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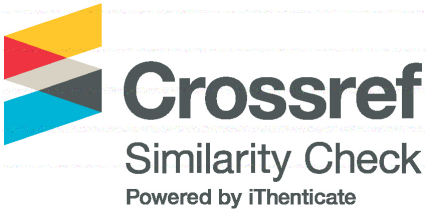


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# THE INFLUENCE OF YEAST EXTRACT AND JASMONIC ACID ON PHENOLIC ACIDS CONTENT OF *IN VITRO* HAIRY ROOT CULTURES OF *ORTHOSIPHON ARISTATUS*

***Iryna Smetanska, Oksana Tonkha, Tetjana Patyka, Dase Hunaefi, Diaa Mamdouh, Mykola Patyka, Anatolyi Bukin, Mikhailo Mushtruk, Natalia Slobodyanyuk, Alina Omelian***

## ABSTRACT
Phenolic acids represent a big group of plant secondary metabolites that can be used as food additives, nutraceuticals, and pharmaceuticals. Obtaining phenolic acids from the plant *in vitro* cultures provide an attractive alternative to produce high-value plant-derived products. The impact of yeast extract and jasmonic acid on the induction of defense responses and consequently the production of phenolic acids in *vitro* hairy root cultures of *O. aristatus* have been investigated. Treatment of *O. aristatus* cultures with jasmonic acid caused accumulation of 12.98 mg.g$^{-1}$ DW of phenolic acids, elicitation with yeast extract resulted in the highest amount of phenolic acids, particularly in 17.99 mg.g$^{-1}$ DW as compared to 4.03 mg.g$^{-1}$ DW for the non-treated cultures. Individual phenolic acids showed a different response to elicitation. Particularly rosmarinic acid content on the control plot reached 2.89 mg.g$^{-1}$ DW, while after the treatment with jasmonic acid is increased to 10.84 mg.g$^{-1}$ DW and after yeast application, it was 14.31 mg.g$^{-1}$ DW. Also, caffeic acid content increased until 0.75 and 2.01 mg.g$^{-1}$ DW after application of jasmonic acid and yeast extract, while at the control plot its concentration was 0.58 mg.g$^{-1}$ DW. Application of yeast extract influenced synthesis of phenolic acids *in vitro* cultures of *O. aristatus* stronger as jasmonic acid treatment.

**Keywords:** elicitors; yeast extract and jasmonic acid; phenolic acids; *in vitro* hairy root culture; *Orthosiphon aristatus*

## INTRODUCTION
Due to the increased demand of consumers to obtain food, enriched with natural health-promoting components, the integrating of phenolic acids into the food matrix is an important task of food producers.

Phenolic acids are industrially relevant compounds, finding use and application in the food, pharmaceutical, cosmetic, and chemical industries. They are used as the raw material for the production of vanillin and preservatives, as a crosslinking agent for the preparation of food gels and edible films. It is expected that the market size of phenolic acids will grow exponentially by 2025, driven by increasing consumer inclination towards the health benefits of the products. For example, the natural ferulic acid market size was over USD 35 million in 2018, and the industry expects consumption at over 750 tons by 2025 **(Valanciene et al., 2020)**.

Phenolic acids are responsible for the adaptations of plants to environmental stresses because they are involved in various defensive and protective mechanisms against microorganisms, insects, and herbivores **(Vamanu and Nita, 2013)**. When infected by the pathogenic microorganism, plants respond with rapid activation of various spatially and temporally regulated defense reactions. These responses include oxidative cross-linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, and incrustation of cell wall proteins with phenolics, particularly phenolic acids **(Kim et al., 2001; Murthy et al., 2014)**.

Phenolic acids are synthesised from the amino acid phenylalanine in the shikimate pathway **(Ogata et al., 2004)**. In terms of conjugation with other metabolites in the plant, phenolic acids can be classified as free and bound phenolic acids **(Yang et al., 2008)**.

The application of phenolic acids, particularly chlorogenic, caffeic, and rosmarinic acid, influences the aroma and taste of food products **(Hunaefi et al., 2013)**. Thus, the characteristic aromas of cocoa and coffee, consisting of the mixture of chlorogenic and rosmarinic acids, have been produced by cell cultures of *Tlaeobroma cacao* and *Coffea arabica*, respectively **(Bais et al., 2002; Palianytsia et al., 2014)**. Rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxy phenyl lactic acid**,** becomes one of the most essential phenolic substances as an ingredient of functional food and food supplements due to

its antioxidant activities **(Khadeer et al., 2011; Shetty, 2001)**.

Numerous plants are characterized by a high content of phenolic acids, among them *Orthosiphon aristatus*. This plant belongs to the *Lamiaceae* family and is important in Indonesian folk medicine **(Hunaefi and Smetanska, 2013)**. This plant has been reported to have high phenolic compounds in which rosmarinic acid is the most important and abundant one **(Mushtruk et al., 2020a)**. **Kumar and Gupta (2008)** indicated, that *O. aristatus* can be used for the development of functional foods, especially due to the high content of rosmarinic acid. However, mass cultivation of tropical *O. aristatus* is considered to be relatively difficult due to specific cultivation conditions.

Plant *in vitro* cultures can be used as a source of valuable secondary metabolites, which can further applied as food additives, nutraceuticals, and pharmaceuticals. The synthesis of phytochemicals by *in vitro* cultures in contrast to these in plants is not dependent on environmental conditions and quality fluctuations. In many cases, the chemical synthesis of metabolites is not possible or economically infeasible **(Smetanska, 2018)**.

Plant cell cultures have been proposed for the production of phenolic acids **(Petersen and Simmonds, 2003)**. *Lithospermum erythrorhizon* cell culture synthesized rosmarinic acid, which was not presented in plant tissues **(Szabo, Thelen and Petersen, 1999)**. It has been reported that cell cultures of *Coffea arabica* as well as of *Theobromo cacao* synthesized chlorogenic acid **(Kurata et al., 1998; Rao and Ravishankar, 2002)**.

However, in many cases *in vitro* hairy root cultures can be preferred as comparing to *in vitro* callus or cell suspension cultures for mass production of the targeted compound as they are more genetically stable and can possess a higher rate of biomass accumulation in comparison to cell cultures **(Cai, Kastell and Smetanska, 2014)**. *In vitro,* hairy root cultures of *Coleus blumei* were reported to accumulate rosmarinic acid **(Bauer, Kiseljak and Jelaska, 2009)**.

Plant *in vitro* cultures shows physiological and morphological responses to microbial, physical, or chemical factors, known as elicitors **(Kovalyshyna et al., 2020b)**. Elicitation is one of the most efficient strategies that lead to the intensification of the synthesis of many secondary metabolites in the plant *in vitro* cultures **(Georgiev, Eibl, and Zhong, 2013)**. Elicitors play an important role in the modulation of biosynthetic pathways and can be used as effective tools to stimulate the synthesis of plant secondary metabolites **(Kurata et al., 1998)**. Since phenolic acids protect plants from environmental changes, the way to induce their synthesis is to apply unfavorable factors for example to simulate pathogen or herbivores attack, osmotic or irradiation stress, etc. Biotic stress can be caused by biotic elicitors as yeast extracts, particularly cell wall fragments of *Saccharomyces cerevisiae* as well as jasmonic acid, which can be formed by the action of the plant on microbial cell walls, also methyl jasmonate, salicylic acid, and ethephon **(Hunaefi and Smetanska, 2013; Schreiner et al., 2011)**.

From the experimental test of elicitors and precursors on the synthesis of phenolic acid, jasmonic acid rapidly influenced the synthesis pathways of phenolic acids. This is most likely because jasmonic acid is reported to function as a signal transducer in plant cell cultures whereby it activates specific genes responsible for defense mechanisms resulting in higher production of almost all secondary metabolites within a short time-lapse. Jasmonic acid and its precursor, linolenic acid, are involved in the whole complex cascade between signal chain reactions of the elicitor-receptor complex and gene activation.

**Ogata et al. (2004)** reported that yeast extracts 5 $g.L^{-1}$ as an elicitor induced an increase of rosmarinic acid content in *Lithospermum erythrorhizon* cell suspension cultures of 4 fold compared to the group of control. Similarly, **Kim et al. (2001)** reported that the stimulation of rosmarinic acid biosynthesis in *Agastache rugosa* O. Kuntze in response to the addition of yeast extract could elevate the rosmarinic acid content up to 5.7-fold compared to non-elicited suspension cells. A more dramatic increase 7 fold of control in rosmarinic acid accumulation had reported in the same plant *O. aristatus* using a cell suspension culture system by **Sumaryono et al. (1991)**. **Hunaefi and Smetanska (2013)** reported that among several treatments with stress substances, elicitation with yeast extract resulted in the highest amount of total phenolic compounds, particularly in 324.5 $mg.g^{-1}$ DW (dry weight), in shoot cultures of *O. aristatus* as compared to 151.2 $mg.g^{-1}$ DW for the non-treated cultures. The content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after the addition of yeast extract: a maximum was reached in 24 h **(Petersen and Simmonds, 2003)**. When the plant cells were treated with yeast extract on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times.

It has been reported that jasmonic acid and its methyl ester influenced the phenylpropanoid pathway by activating the enzyme phenylalanine ammonia-lyase in cell suspension cultures of soybean (*Glycine max*) **(Gueven and Knorr, 2011)**. Similar results were obtained in studies where jasmonic acid was used as an enhancer for the synthesis of phenolic compounds, particularly anthocyanins with a maximum production of phenolics on the 2nd day after application **(Curtin, Zhang, and Franco, 2003)**.

Therefore, in our research, we intended to prove the use of *in vitro* cultures *O. aristatus* as sustainable sources of phenolic acids through the cultivation of them under the influence of biotic elicitors as yeast extract and jasmonic acid.

## Scientific hypothesis

The hypothesis of research work hinged on the assumptions that the synthesis of phenolic acids in *vitro* cultures could be positively influenced by the application of biotic elicitors, particularly jasmonic acid and yeast extract. As an object of the study *in vitro* hairy root culture of *Orthosiphon aristatus* has been selected due to the high content of valuable phenolic acids. According to obtained results the increase of the content of phenolic acids under the influence of elicitors, especially yeast extract, has been approved.

## MATERIAL AND METHODOLOGY

### Establishment and cultivation of *in vitro* hairy root cultures of *O. aristatus*

*O. aristatus* hairy root culture was established according to **Gabr et al. (2018)**. Seeds of *O. aristatus* were treaded with 70% ethanol for 20 – 30 seconds and then transferred

into 10% sodium hypochlorite solution for 5 min. Afterward, they were washed three times in sterilized bidestilled water and sewn on MS (Murashigi and Skoog) medium into 250 mL Erlenmeyer flasks. After 4 weeks sterilized plants were cut into 1 – 1.5 cm pieces (±100 mg fresh weight.$L^{-1}$) and placed in 250 mL Erlenmeyer flasks containing 50 mL liquid MS medium and *A. rhizogenes* suspension for 15 min and then transformed to MS medium. After 24 hours 500 mg.$L^{-1}$ cefotaxime have been added to eliminate *A. rhizogenes*.

*In vitro* hairy root culture of *O. aristatus* was transferred to a new medium every four weeks and maintained at 25 ±2 °C on a rotary shaker at 100 rpm. The best line of *in vitro* culture was the third generation from the first establishment. This line has been used for represented research.

## Treatment with elicitors

Liquid MS media were prepared as follows: 1) MS medium (Control); 2) MS medium and 100 μM jasmonic acid; 3). MS medium and 5 g.$L^{-1}$ yeast extract.

## Extraction of phenolic acids and identification by HPLC

20 mg of the powdered samples were extracted for 15 minutes with 750 μL of 70% methanol (VWR, Germany) in bi-distilled water in an ultrasonic water bath on ice. Afterward, the samples were centrifuged for 5 minutes at 13000 $min^{-1}$ in Eppendorf centrifuge. The supernatants were collected and the pellets were re-extracted twice with 500 μL 70% methanol in bi-distilled. 40 μL of cinnamic acid (VWR, Germany) diluted in 70% methanol in bi-distilled was used as an internal standard. The collected supernatants from each sample were concentrated in a rotary evaporator (SPD 111V Speed Vac. Concentrator, Thermo Scientific, USA; CVC 3000V, Vacuubrand GmbH, Wertheim, Germany) at 25 °C under vacuum to remove the solvent completely. The residues were dissolved in 1 mL HPLC MilliQ water. The samples were filtered through 0.22 μM cellulose acetate filters (Corning® Costar® Spin-X® Plastic Centrifuge Tube Filters, Sigma-Aldrich Chemie GmBH, Steinheim, Germany). Analyzing of phenolic acids was performed on HPLC (UltiMate SR-3000, Dionex, Idstein, Germany), equipped with LPG-3400SD pump, WPS-3000SL automated sample injector, AcclaimPA C16-column (3 μm, 2.1 x 150 mm, Dionex) and DAD-3000 diode array detector (Dionex) and software Chromeleon 6.8. The column was operated at a temperature of 35 °C. The mobile phase consisted of 0.1% phosphoric acid (VWR, Germany) in HPLC water (eluent A) and 40% acetonitrile (VWR, Germany) in HPLC water (v/v, eluent B). A multistep gradient was used for all separations with an initial injection volume of 40 μL and a flow rate of 0.4 mL.$min^{-1}$. The multistep gradient was used as follows: 0 – 1 min: 0.5% B; 1 – 10 min: 0.5 – 40% B; 10 – 12 min: 40% B; 12 – 18 min: 40 – 80% B; 18 – 20 min: 80% B; 20 – 24 min: 80 – 99% B; 24 – 30 min: 99 – 100% B; 30 – 34 min: 100 – 0.5% B; 34 – 39 min: 0.5% B. Diode array detection was used for the identification of the compounds. Retention times and UV/visible absorption spectra of the peaks were compared with those of the authentic standards. The phenolic acid quantity was calculated from HPLC peak areas. The standard curves were obtained by plotting the peak area ratios (Y-axis) of standard compounds to the internal standard versus the concentrations of standard compound (X-axis). According to these standard curves, the linear regressions (Y = mX + b) and correlation coefficients as well as corrected with response factors were computed.

The concentration of phenolic acids was calculated using the following equation (1):

$$phenolic\ acid\ concentration\left[\mu mol\ g^{-1}DW\right]=\frac{AEPh\cdot RFPh\cdot cST}{AESt}\cdot\frac{V\,\mathrm{Pr}}{VInj}\cdot\frac{1}{PE} \quad (1)$$

Where:

AEPh – Peak area phenolic acid; RFPh – Response factor phenolic acid; cSt – Concentration internal standard (nmol); AESt – Peak area internal standard; VP – Volume after extraction (μL); VInj – Injection volume (μL); PE – Sample weight (mg).

## Statistical Analysis

Experimental results concerning this study are reported as means ± standard deviation (*SD*). The data were subjected to analysis of variance (ANOVA) and the level of statistical significance was set at $p$-values <0.05 using SPSS version 17.0 (SPSS Inc. Chicago, IL, USA). Correlation analyses between the parameters were done using the "ProcCorr" procedure in SAS for Windows (SAS Institute, Cary, NC, USA).

## RESULTS AND DISCUSSION

The analysis of the growth dynamic of hairy roots of *O. aristatus* have shown, that the biomass increased from 3.1 g FW (fresh weight) $flack^{-1}$ on the 1st day of the experiment to 8.2 g FW $flack^{-1}$ on the 7th day, 11.4 g FW $flack^{-1}$ on the 10th day up to 150 g FW $flack^{-1}$ on the 14th day (Figure 1).

Application of jasmonic acid caused a decrease in fresh weight accumulation by 78% on the 14th day of the experiment as compared to the control plot, but treatment with yeast extract led to a 23% increase of the biomass toward the control.

To improve the production of phenolic compounds in *vitro* hairy root cultures, supplementation of elicitation with yeast extract and jasmonic acid has been used **(Cai et al., 2014)**. However, previously there was no report about the influence of these elicitors on the synthesis of phenolic acids *in vitro* hairy root cultures of *O. aristatus*.

The analysis of total phenolic acid in *vitro* cultures of *O. aristatus* have shown that during the cultivation time of 14 days hairy roots of untreated hairy roots accumulated 1.35 mg.$g^{-1}$ DW of the substances at the beginning and 4,03 mg.$g^{-1}$ DW by the end of the experiment (Figure 2). Treated with elicitors cultures answered during the first 24 hours after application, however, jasmonic acid caused a 3-times decrease in phenolic acid content as compared to the untreated hairy roots. In contrast, yeast extract application caused stimulation of the synthesis of phenolic acids by almost 2.5-times as compared to the control. The positive effect of the treatment with jasmonic acid has been observed between the 3rd and 7th days of the experiment when the content of phenolic acids increased from 1.11 to 9.30 mg.$g^{-1}$ DW. Yeast extract application influenced

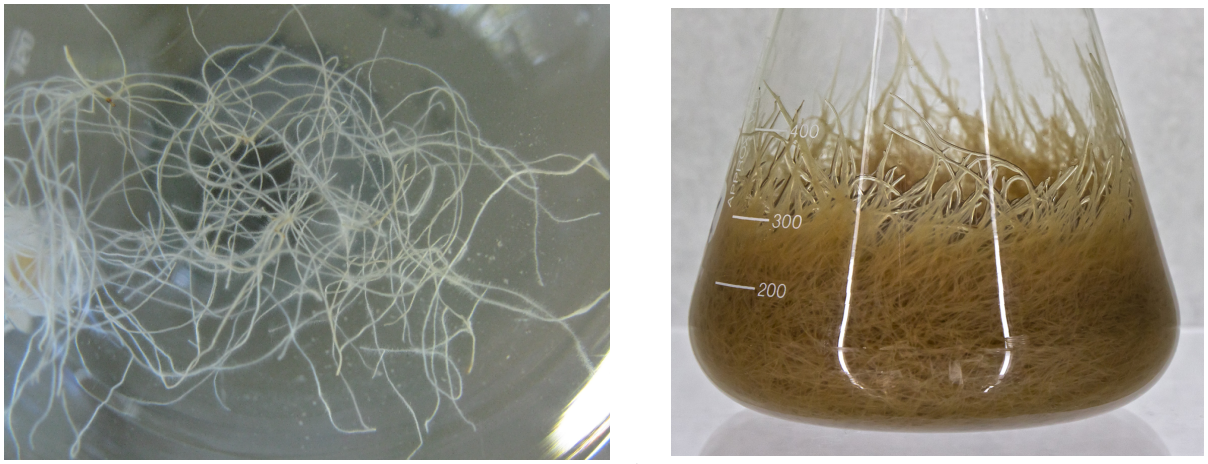



**Figure 1** Hairy root culture of *O. aristatus* on the 1st and 14th day of experiment.

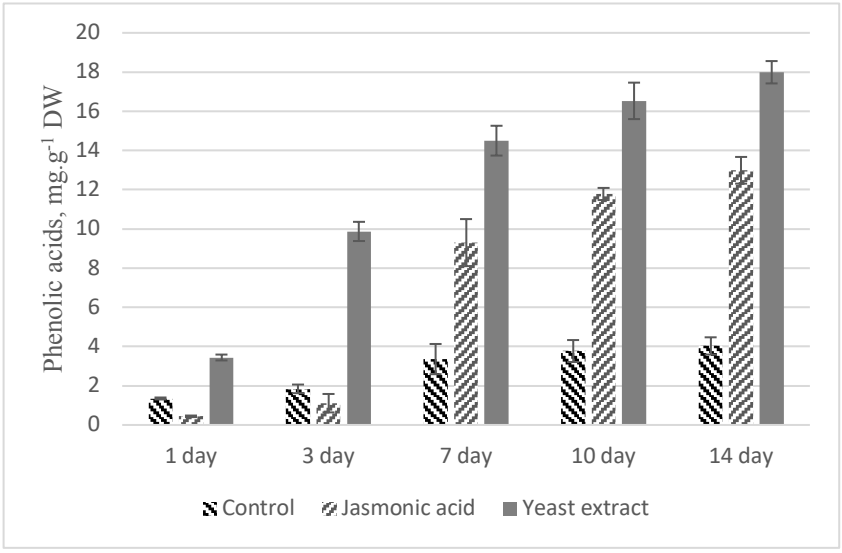



**Figure 2** Impact of elicitors on the content of phenolic acids in *in vitro* hairy root cultures of *O. aristatus.*

positively the process of phenolic acid synthesis during the first 7 days after application, afterwards, the intensity of increment of phenolic acid accumulation became slower.

By the end of the experiment, both elicitors caused the rising of total phenolic acid, particularly jasmonic acid 3.2-times, and yeast extract 4.5-times more in comparison to the control plot.

Authors of scientific works **(Yazaki, 2017; Izuishi et al., 2020; Sheiko et al., 2019)** reported that yeast extracts added in a quantity of 5 $g.L^{-1}$ into the cultivation media of *Lithospermum erythrorhizon* cell suspension cultures induced an increase in phenolic acid content by 4-folds as compared to the control. Authors of scientific works **(Guo et al., 2020; Kovalyshyna et al., 2020a)** reported that the stimulation of rosmarinic acid biosynthesis in *Agastache rugosa* O. Kuntze in response to the application of yeast extract elevated phenolic acid content up to 5.7-folds in comparison to non-elicited suspension cell cultures. A more dramatic increase in phenolic acid accumulation, particularly 7-fold of control, had been reported in the cell suspension culture of *O. aristatus* after yeast extract treatment **(Sumaryono et al., 1991; Kasem, 2018)**. The content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after the addition of yeast extract **(Kumar and Gupta, 2008)**. When the plant cells were treated with yeast extract on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times.

Treatment with yeast extract stimulated synthesis of anthraquinones in cell cultures of *Morinda citrifolia* **(Doernenburg and Knorr, 1997)**, rosmarinic acid in *Coleus blumei* **(Swamy et al., 2018)**, flavonoids in *Glycyrrhiza uralensis* **(Zhang et al., 2009)**, isoflavonoids in *Pueraria candollei* **(Udomsuk et al., 2011)**.

Increasing of isoflavonoid biosynthesis by 5 – 6 times was observed by jasmonic acid addition to soy plant callus suspension culture **(Gueven and Knorr, 2011; Palamarchuk et al., 2019)**.

Analyzing individual phenolic acids in *vitro* hairy root culture of *O. aristatus* has shown the presence of vanillic, chlorogenic, caffeic, p-coumaric, sinapic, and rosmarinic acids (Figure 3). Rosmarinic acid was the major one, followed by caffeic and vanillic acids. Rosmarinic acid content on the control plot reached 2.89 $mg.g^{-1}$ DW, while after the treatment with jasmonic acid it increased to 10.84 $mg.g^{-1}$ DW and after yeast application, it was 14.31 $mg.g^{-1}$ DW (Table 1).

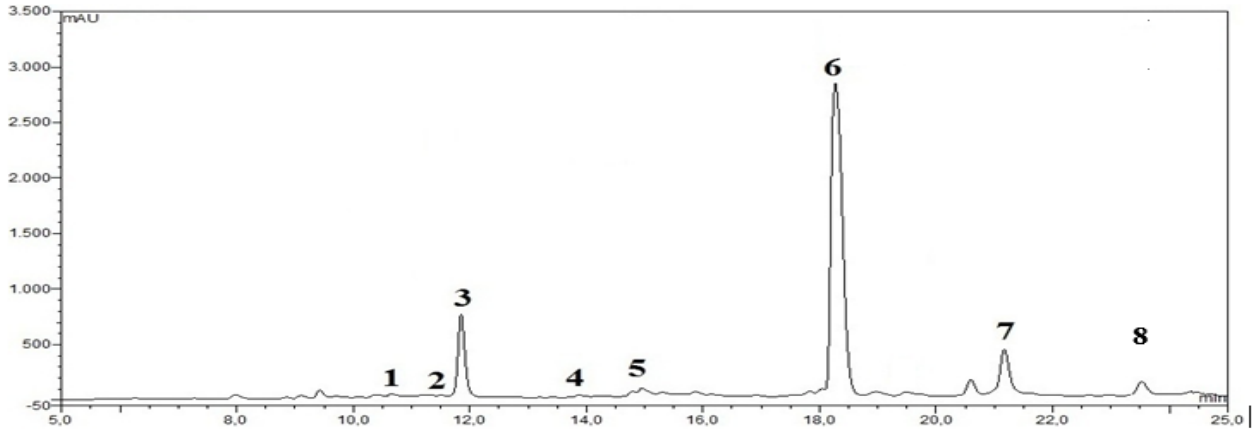
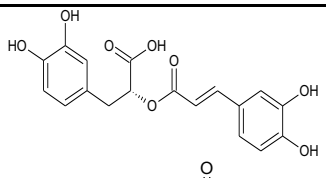
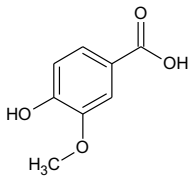
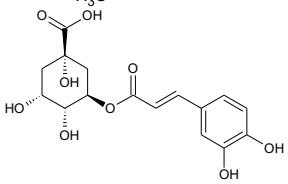
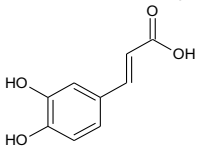
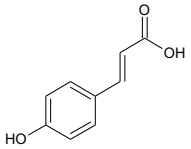
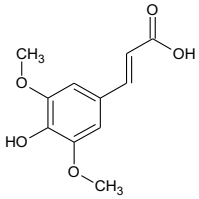



**Figure 3** HPLC chromatogram of *in vitro* culture of *O. aristatus.* Note: 1 – vanillic acid; 2 – chlorogenic acid; 3 – caffeic acid; 4 – p-coumaric acid; 5 – sinapic acid; 6 – rosmarinic acid; 7 – cinnamic acid.

**Table 1** The individual response of phenolic acids of *in vitro* culture of *O. aristatus* subjected to different treatments.

| Phenolic acids | Chemical structure | Control, mg.g$^{-1}$ DW | Jasmonic acid | | Yeast extract | |
|---|---|---|---|---|---|---|
| | | | mg.g$^{-1}$ DW | Fold of control | mg.g$^{-1}$ DW | Fold of control |
| rosmarinic acid |  | 2.89 ± 0.36 | 10.84 ± 0.95 | 3.75 | 14.31 ± 0.78 | 4.95 |
| vanillic acid |  | 0.31 ± 0.03 | 0.87 ± 0.11 | 2.80 | 1.15 ± 0.14 | 3.72 |
| chlorogenic acid |  | 0.07 ± 0.01 | 0.08 ± 0.01 | 1.10 | 0.13 ± 0.02 | 1.90 |
| caffeic acid |  | 0.58 ± 0.02 | 0.75 ± 0.08 | 1.30 | 2,01 ± 0.13 | 3.46 |
| *p*-coumaric acid |  | 0.04 ± 0.01 | 0.10 ± 0.02 | 2.40 | 0.14 ± 0.02 | 3.50 |
| sinapic acid |  | 0.14 ± 0.02 | 0.35 ± 0.04 | 2.5 | 0.25 ± 0.02 | 1.82 |

The basal level of vanillic acid (control) was 0.031 mg.g$^{-1}$ DW, by elicitation with jasmonic acid its content was found higher by 2.8-fold while in the yeast treated culture vanillic acid content was 3.72-fold of the level of control.

The highest concentrations of caffeic and coumaric acids produced by *in vitro* culture of *O. aristatus* were elicited with yeast extract, which was 3.46 and 3.50-fold higher compared to the control level.

The results corresponded with previous investigators using elicitors for producing phenolic acids, particularly rosmarinic acid, in different plants. Elicitors had proven to induced significantly rosmarinic acid production in sprout

cultures of *O. aristatus* **(Hunaefi et al., 2018; Chua et al., 2018)** and cell cultures of *Lithospermum erythrorhizon* **(Ogata et al., 2004; Peng et al., 2019)**. Since in plants, phenolic acids are supposed to act as a preformed constitutively accumulated defense compound and therefore most of the successful cases reports in enhancement rosmarinic acid were by elicitors treatments **(Debnath et al., 2011; Mushtruk et al., 2020b)**.

Induction of rosmarinic acid accumulation in *O. aristatus* cell culture by yeast extract was not limited to cells in the lag phase but could also be provoked when cells were in the growth or stationary phase **(Castro et al., 2017)**. Authors of scientific works **(Bober et al., 2020; Zheplinska et al., 2019; Schulenburg et al., 2016)** reported that phenolic acid accumulation by precursors supplementation had reached a maximum at day 15 on *in vitro* culture of *Oregano*.

Overall, among all treatment yeast extract and jasmonic acid elicitation resulted in higher accumulation for phenolic acids as compared to the control. Phenolic acids were more responsive to yeast extract as jasmonic acid treatment.

These observations show how, in a single system, different phenolic acids of secondary metabolite products can show distinct differences in their responses

## CONCLUSION

The aim of the represented research work was to establish the hairy root culture of *O. aristatus* and to develop the technology of its cultivation for obtaining valuable phenolic acids. The analysis of the growth dynamic of hairy roots of *O. aristatus* has shown, that during the cultivation on MS medium the biomass increased during 14 days of cultivation from 3.1 g until 15.0 g FW flack$^{-1}$ on the 14$^{th}$ day. Application of jasmonic acid caused a decrease in fresh weight accumulation by 78% on the 14$^{th}$ day of the experiment as compared to the control plot, but treatment with yeast extract led to a 23% increase of the biomass toward the control.

The analysis of total phenolic acid in *vitro* cultures of *O. aristatus* has shown that during the cultivation time of 14 days hairy roots of untreated hairy roots accumulated 1.35 mg.g$^{-1}$ DW of the substances at the beginning and 4.03 mg.g$^{-1}$ DW by the end of the experiment. Treated with elicitors cultures answered during the first 24 hours after application, however, jasmonic acid caused a 3-times decrease in phenolic acid content and yeast extract application caused stimulation of the synthesis by 2.5-times as compared to the control. The positive effect of the treatment with jasmonic acid has been observed between the 3$^{rd}$ and 7$^{th}$ days of the experiment, but of the yeast extract application during the first 7 days after application. By the end of the experiment, both elicitors caused the rising of total phenolic acid, particularly jasmonic acid 3.2-times, and yeast extract 4.5-times more in comparison to the control plot.

Among all phenolic acids in the hairy root cultures, rosmarinic acid was the major one, followed by caffeic and vanillic acids. Its content on the control plot reached 2.89 mg.g$^{-1}$ DW, while after the treatment with jasmonic acid is increased to 10.84 mg.g$^{-1}$ DW and after yeast application, it was 14.31 mg.g$^{-1}$ DW.

It is possible to conclude, that the hairy root culture of *O. aristatus* can be used for obtaining phenolic acids. The technology of *in vitro* cultivation can be optimized through the application of elicitors as jasmonic acid and yeast extract. However, the stimulation of the synthesis of phenolic acids after the application of yeast extract is more promising.

For further investigation of the proper use of elicitors, it would be advisable to investigate the application of elicitors during different stages of culture growth.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE SUSTAINABILITY OF RICE FARMING PRACTICES IN TIDAL SWAMPLANDS OF SOUTH SUMATRA INDONESIA

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## ABSTRACT
Tidal swamplands are considered the national food security platform in Indonesia. Residues from the excessive chemical input used in the rice production affecting the environment, farmers' health, and the safety of the product. Similarly, one can expect that excessive chemical use in tidal swamplands can also threaten rice production system sustainability. This study aimed to assess and identify factors influencing the sustainability of rice farming practices in tidal swamplands of South Sumatra, Indonesia. A survey was carried out to obtain information through direct interviews with 150 farmers in Muara Telang, South Sumatra, Indonesia. The Indonesia rice check was employed as indicators to assess the sustainability of rice farming practices in tidal swamplands. A percentage index was to categorize the sustainability status. A regression analysis with the Tobit model was applied to identify factors influencing the sustainability of rice farming practices. The result showed that the average sustainability index was 25.53%. It indicated rice farming practices in tidal swamplands tend to be unsustainable. The significant factors influencing the sustainability of rice farming practices were farmer's education and household size. A policy recommendation is proposed to enhance the implementation of sustainable agriculture practices by the rice farmers in tidal swamplands.

**Keywords:** index; rice check; rice farm; sustainable agriculture; tidal swampland

## INTRODUCTION
Suboptimal lands have an essential role in the food security of Indonesia. One of the suboptimal land types in Indonesia is tidal swamplands. Tidal swamplands are located close to the sea or river such that water availability in tidal swamplands for rice cultivation depends on the tides. The difference between irrigated rice fields and tidal swamplands is water management. There are some primary, secondary, and tertiary canals to the rice field that has sufficient water availability **(Widjaja-Adi, Ratmini, and Swastika, 1997)**. Tidal swamplands are available in some regions of Indonesia such as Sumatra, Kalimantan, Papua, and Sulawesi only. The biggest area of tidal swamplands in Sumatra is in South Sumatra Province. Tidal swamplands were a government reclamation project in the 1970s. The project involved migrants from Java Island to Sumatra Island. The reclaimed area is cultivated for rice farming. The total area of tidal swamplands in South Sumatra is 266,674 hectares and 161,917 hectares are in Banyuasin Regency **(Statistical Center Bureau of Banyuasin Regency, 2018)**. It indicates that a potential exists for food security. Therefore, tidal swamplands are recognized as the food barn of South Sumatra. However, some problems such as pyrite or $FeS_2$, peat, soil acidity, salinity, and others threaten the productivity of tidal swamplands **(Wildayana and Armanto, 2018)**. Furthermore, the farmers in tidal swamplands still cultivate rice using chemicals such as pesticides, insecticides, and fungicides excessively, and intensively **(Roche, 1994; Zahri et al., 2018)**. It was caused by the label of chemicals stating the chemicals will not reduce rice production. The most used pesticide by the farmers is pesticide containing high toxicity **(Amir et al., 2012)**. Some factors influencing the excess chemical use are behavior, perception, and lack of knowledge **(Jallow et al., 2017)**. The problems can be threats to the ecology of tidal swamplands.

Many studies from some countries such as Australia **(Cohen, 2007)**, Kenya **(Tsimbiri et al., 2015)**, and Indonesia **(Mariyono, Kuntariningsih and Kompas, 2018)** stated that ecology degradation and decreased farmers' health occurred because of the chemical use such as pesticide, fertilizer, and others. The state is supported by a phenomenon in which farmers still use chemicals in higher doses than recommended **(Chauhan and Singhal, 2006)**. The impacts of excess chemical uses in the long-term are environmental degradation, $CO_2$ emission, health problem, externality, and others **(Yuan et al., 2017; Zeng et al., 2017)**. Therefore, preventive action should be taken.

In terms of food safety, excess chemical use affects rice quality **(Hong-xing et al., 2017)**. Many chemical residues

are found in rice **(Añasco et al., 2010)**. So the food safety of rice is still in doubt. The case was caused by sustainable agriculture practices that have not been implemented properly. Therefore, it is also important to note that agricultural practices or agriculture production systems must be eco-friendly **(Mishra, 2013)**. So that the rice produced by farmers obtains a worthy price and good quality according to food safety criteria. Sustainable agriculture practices can improve yield and farmers' income. The recent studies investigated socio-economic factors influencing sustainable agriculture practices such as age, household size, education, farm size, and others **(Prokopy et al., 2008; Tey et al., 2014; Dessart, Barreiro-Hurlé and Bavel, 2019)**.

Based on the problems above, a study regarding the sustainability of rice farming practices in tidal swamplands is a concern to monitor the tidal swamplands' environment. One of the tools to assess the sustainability of rice farming practices is a rice check. The rice check first appeared in 1986 by the Department of Agriculture New South Wales, Australia. The goal is to improve the quantity and quality of rice production and as a recommendation and learning platform for farmers. The rice check helped the farmers to figure out when the crops must be provided fertilizer, chemicals, and others so that the chemicals do not damage the environment and agricultural resources in Australia. Furthermore, The Australia rice check includes the allowed pesticides, appropriate application methods of chemicals, and the proper doses accord to the recommendation. The document educated the Australian farmers not to use the chemical excessively. The Australia rice check is targeted to achieve rice production 6 to 8 $t.ha^{-1}$. **Singh, Brennan and Lacy (2007)** explained that the Australia rice check changed Australian farmers' behavior and agriculture practices. The Australian farmers also got the benefit through increasing rice production.

In Malaysia, the Malaysian rice check was introduced in 2002 by the Department of Agriculture Malaysia. The farmers were expected to pay attention to their rice farm. The chemical uses were regulated on the document as well. Furthermore, the sustainability indicator of rice farming practices in Vietnam was issued by The Ministry of Agriculture and Rural Development Vietnam in 2008. The document was well known as Vietnam Good Agricultural Practice (Viet GAP). The indicators emphasized chemical use, post-harvest process, and marketing of rice.

According to **Tilman et al. (2002)**, the sustainability of agricultural production practices needs to be assessed for food security and safety strategy. Thus, this study aimed to assess and identify factors influencing the sustainability of rice farming practices in tidal swamplands. Furthermore, research on the sustainability assessment of rice farming practices in tidal swamplands does not exist yet. So this study is necessary to be carried out.

### Scientific hypothesis

This study had two hypotheses:

1. The rice farming practices are sustainable in tidal swamplands.
2. The socio-economic factors such as age, education, household size, farm size, and farming experience influence the sustainability of rice farming practices.

## MATERIAL AND METHODOLOGY

### Study area

This study was conducted in Muara Telang, South Sumatra, Indonesia (Figure 1). The location was considered because it is the biggest area of tidal swampland's agriculture for rice production. The total area of Muara Telang is 341.57 $km^2$. The location is also a production center of tidal swampland rice in South Sumatra Province. Muara Telang District is a tidal swamp area with an altitude of 0.5 m to 2.25 m above sea level. The average monthly temperature is 27 °C. The relative humidity is 87%. The average annual rainfall is approximately 2,400 mm. The region has a topography with a land slope of less than 3%. It is very potential for the development area of food crops, particularly rice.

### Data Collection

The primary data were collected through face-to-face interviews with the farmers. The 150 farmers were chosen by a simple random sampling technique since the area is similarly affected by tidal water. Some questions regarding the farmers' socioeconomic situation were addressed, i.e., age, education, household size, farm size, and farming experience. This study also covered several agricultural input use information such as seed, fertilizer (nitrogen, phosphor, and potassium), and chemical (herbicide, pesticide, and fungicide).



**Figure 1** Tidal swamplands in Muara Telang, South Sumatra, Indonesia.

**Table 1** Sustainability assessment worksheet for rice farming practices.

| Indicators of Rice Farming Practices | Amount or Frequency | Max Score | Min Score |
|---|---|---|---|
| **Seedling (Rice check 1,2,3)** | | | |
| Planting time | Not Following = 0, Oct-Dec = 1 | 1 | 0 |
| Variety | Not Following = 0, Mekongga, Ciherang, Inpari 30 Ciherang Sub 1 = 1 | 1 | 0 |
| Amount of seeds (80 kg.ha$^{-1}$) | <80 kg.ha$^{-1}$ = 0, 80 kg.ha$^{-1}$ = 1, >80 kg.ha$^{-1}$ = -1 | 1 | -1 |
| **Land preparation (Rice check 4,6)** | | | |
| Depth (20 – 40 cm) | Not Following = 0, within 20 – 40 cm = 1 | 1 | 0 |
| Planting distance (20 × 20cm) | Not Following = 0, within 20 × 20 cm = 1 | 1 | 0 |
| **Fertilizer (Rice check 7)** | | | |
| Timing<br>1$^{st}$ application (15 – 20 days after planting) | Not Following = 0, within 15 – 20 days = 1 | 1 | 0 |
| 2$^{nd}$ application (35 – 40 days after planting) | Not Following = 0, within 35 – 40 days =1 | 1 | 0 |
| 3$^{rd}$ application (50 – 55 days after planting) | Not Following = 0, within 50 – 55 days =1 | 1 | 0 |
| Amount of N fertilizer (200 kg.ha$^{-1}$) | <200 kg.ha$^{-1}$ = 0, 200 kg.ha$^{-1}$ = 1, >200 kg.ha$^{-1}$ = -1 | 1 | -1 |
| Amount of P fertilizer (75 kg.ha$^{-1}$) | <75 kg.ha$^{-1}$ = 0, 75 kg.ha$^{-1}$= 1, >75 kg.ha$^{-1}$ = -1 | 1 | -1 |
| Amount of K fertilizer (50 kg.ha$^{-1}$) | <50 kg.ha$^{-1}$ = 0, 50 kg.ha$^{-1}$ = 1, >50 kg.ha$^{-1}$ = -1 | 1 | -1 |
| Organic fertilizer (Livestock dung, etc.) | No = 0, Used =1 | 1 | 0 |
| **Weed Control (Rice Check 9)** | | | |
| Frequency (herbicide use) | 2 times = 0, 0 – 1 = 1, over 3 times = -1 | 1 | -1 |
| Amount of herbicide (5 L.ha$^{-1}$) | <5 L.ha$^{-1}$ = 0, 5 L.ha$^{-1}$ = 1, >5 L.ha$^{-1}$ = -1 | 1 | -1 |
| Organic herbicide | No = 0, Used = 1 | 1 | 0 |
| Pulling up weeds by hands | No = 0, Yes =1 | 1 | 0 |
| **Pest Control (Rice Check 10)** | | | |
| Frequency (insecticide use) | 2 times = 0, 0 – 1 time= 1, over 3 times = -1 | 1 | -1 |
| Amount of insecticide (5 L.ha$^{-1}$) | <5 L.ha$^{-1}$ = 0, 5 L.ha$^{-1}$ = 1, >5 L.ha$^{-1}$ = -1 | 1 | -1 |
| Organic insecticide | No = 0, Used = 1 | 1 | 0 |
| Disease Control (Rice Check 10) | | | |
| Frequency (fungicide use) | 2 times = 0, 0 – 1 time= 1, over 3 times = -1 | 1 | -1 |
| Amount of fungicide (5 L.ha$^{-1}$) | <5 L.ha$^{-1}$ = 0, 5 L.ha$^{-1}$= 1, >5 L.ha$^{-1}$ = -1 | 1 | -1 |
| Organic fungicide | No = 0, Used = 1 | 1 | 0 |
| **Water Management (Rice Check 8,12,13)** | | | |
| Following irrigate and drainage schedule | Yes = 0, No = 1 | 1 | 0 |
| Observing depth of water | Yes = 0, No = 1 | 1 | 0 |
| **Total Score** | | 24 | -10 |

**Table 2** Sustainability index of rice farming practices.

| Sustainability index value (%) | Category |
|---|---|
| >70.0 | Sustainable |
| 60.1 – 70.0 | Somewhat sustainable |
| 50.1 – 60.0 | Intermediate sustainable |
| 40.1 – 50.0 | Possibly quite unsustainable |
| 20.0 – 40.0 | Possibly unsustainable |
| <20.0 | Possibly very unsustainable |

The Indonesia rice check was employed as indicators to assess the sustainability of rice farming practices in tidal swamplands. The rice check is a guideline document of sustainable rice farming practices. There were 24 rice farming practices used as indicators. The practices and indicators have been modified to be suitable for tidal swamplands. The sustainability assessment worksheet for rice farming practices was provided as a questionnaire in Table 1. The Indonesia rice check was presented by The Ministry of Agriculture Indonesia in 2017. It referred to

the Australia rice check. It emphasized farmers to achieve optimal rice production. The document is a result of the agreement among researchers, farmers, and agricultural extension officers based on 3 aspects of sustainability which are social, economic, and ecology. By adopting the rice check, The Indonesia Government expected the farmers had adopted the best technology to achieve optimal and sustainable rice production **(The Ministry of Agriculture, 2017)**.

## Data Analysis

The sustainability indexing of rice farming practices referred to **Taylor et al. (1993)**. The farmers applying practices based on the sustainability assessment worksheet or questionnaire would be given a score of 1. However, the farmers who do not apply practices based on the questionnaire would be given a score of 0 or negative. The sustainability index value of rice farming practices was built on a range of 0 to 100%. It was created to obtain tangible results and facilitate the comparison of numerical scales among the rice farmers.

Then, the values were categorized according to the sustainability index. The six categories for the sustainability index of rice farming practices are in Table 2.

Furthermore, regression analysis with the Tobit model was applied to identify the factors influencing the sustainability of rice farming practices. The Tobit model was applied because the model can estimate and accommodate bias on censored data. The data structure of the sustainability index of rice farming practices or dependent variable (Y) is known as censored data because there were some values of zero (0) on observation data or index. The independent variables (X) used were the socio-economic characteristics of the farmer. **Igbokwe (2000)** reported that the socio-economic characteristics of farmers influenced rice farming practices. Therefore, the independent variables used in this study were age ($X_1$), education ($X_2$), household size ($X_3$), farm size ($X_4$), and farming experience ($X_5$). A regression equation with the Tobit model created in this study was:

$$Y_i^* = \alpha + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + u_i$$
$$Y_i = Y^* \text{ if } Y^* > 0$$
$$Y_i = 0 \text{ if } Y^* \leq 0 \quad (1)$$

Where:

$Y_i^*$ denotes the sustainability index of rice farming practices; $\alpha$ is the intercept of the model; $\beta_1 \ldots \beta_n$ (n = 1, 2, 3...) are estimated parameters; $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ represent age, education, household size, farm size, and farming experience respectively; $u_i$ indicates an error term.

## Statistical analysis

Two statistical analyses were used in this study. The descriptive statistics were performed in Microsoft Excel 2010. Furthermore, the data analysis for the parametric statistics which was regression analysis with the Tobit model was performed in STATA 15.1. The *p*-values used for this study were $p$ <1%; 5% and 10%.

# RESULTS AND DISCUSSION

## Socio-economic characteristics and input uses of rice farmers in tidal swamplands

The socio-economic characteristics of rice farmers in Muara Telang can be seen in Table 3. The majority of farmers are still in productive age. Therefore, they are still able to cultivate rice and work on rice farms. The farmers' education in tidal swamplands is still low. The average farmers' formal education is to 7.72 years. It indicates that the farmers got an education in primary school only. The average household size is two or three people. A farmer's household usually consists of the farmer, his wife, and one child or two children (unmarried). However, some farmers have more than two children and the minority of them live alone since his wife passed away and his children married and moved to the city. Furthermore, the average farmers' farm size is 4.72 ha. The farmers got a grant which was 2 ha of rice farms from the government. However, some of them sold rice farms. Moreover, some of them have 8 ha or more. Consequently, some farmers have smaller rice farms. The average farming experience of farmers is over 20 years. Farming is the main job in Muara Telang. The farmers cultivated rice and worked in the rice farm or wetland before becoming migrants in the reclamation project of tidal swamplands in the 1970s.

This study found that excess agricultural input uses tidal swamplands (Table 4). The implication of the excess agricultural input uses is inefficient rice production in tidal swamplands **(Purba et al., 2020)**. The average seed use was 85.82 kg.ha$^{-1}$. The case was occurred due to the cultivation system in tidal swamplands. The cultivation system in tidal swamplands is direct seed spreading. It is well-known as *sonor*. It is carried out without seedling. The practice is also followed by burning the land for land clearing. It is one of the cases that trigger unsustainability **(Wildayana, Armanto and Junedi, 2017)**. The impact of the practice was no regulated depth and planting distance. Besides, some farmers still used the local variety with limited technology. It made the sustainability score of rice farming practices low.

**Table 3** Socio-economic characteristics of rice farmers.

| Variables | Mean | Std. Deviation |
|---|---|---|
| Age (year) | 44.74 | 11.45 |
| Education (year) | 7.72 | 3.25 |
| Household size (individual) | 2.56 | 1.21 |
| Farm size (ha) | 4.72 | 3.93 |
| Farming experience (year) | 24.25 | 11.34 |

**Table 4** Input use of rice farmers.

| Input | Mean | Std. Deviation |
|---|---|---|
| Seed (kg.ha$^{-1}$) | 85.82 | 20.94 |
| Nitrogen fertilizer (kg.ha$^{-1}$) | 239.09 | 120.05 |
| Phospor fertilizer (kg.ha$^{-1}$) | 149.20 | 76.39 |
| Potassium fertilizer (kg.ha$^{-1}$) | 132.95 | 73.28 |
| Herbicide (L.ha$^{-1}$) | 6.54 | 3.18 |
| Insecticide (L.ha$^{-1}$) | 5.34 | 3.27 |
| Fungicide (L.ha$^{-1}$) | 5.64 | 2.77 |

**Table 5** Result of rice farming practices sustainability assessment.

| Measure | Value (%) |
|---|---|
| Mean | 25.53 |
| Median | 26.14 |
| Mode | 27.27 |
| Min – Max | 0.00 – 72.73 |

This study found that the fertilizer and chemical uses in tidal swamplands were still high. Based on the government recommendation, the fertilizer uses for nitrogen, phosphor, and potassium should be 200 kg.ha$^{-1}$, 75 kg.ha$^{-1}$ and 50 kg.ha$^{-1}$ respectively **(The Ministry of Agriculture, 2017)**. The case is in line with **Han and Zhao (2009)** that the farmers in China also use a higher amount of fertilizer than is recommended. The chemical uses for pest and disease control such as herbicide, insecticide, and fungicide are also higher than the recommendation. The recommendation of chemical uses is 5 L.ha$^{-1}$ for herbicide, insecticide, and fungicide respectively. **Abhilash and Singh (2009)** reported that chemical uses in India were higher than the recommendation. The chemical uses would be possible to increase and would threaten the sustainability of tidal swamplands.

## Assessment of rice farming practices sustainability in tidal swamplands

The average index of rice farming practices sustainability in tidal swamplands was 25.53%. It means rice farming practices were in the category of possibly unsustainable. It occurred because some farmers indeed carried out sustainable agriculture practice but only the easy practices such as pulling up weeds by hands, the timing of fertilizer application, and others. The farmers disregarded the important practices in sustainable agriculture like the amount of fertilizer and chemicals **(Mishra et al., 2018)**. The maximum index was 72.73. Despite there was a farmer in the category of sustainable, no farmer has a perfect performance in sustainable agriculture practices with a score of 100% (Table 5).

Furthermore, over 50% of the farmers were in the category of possibly unsustainable. Moreover, 47 rice farmers (31.33%) were in the worst category, namely possibly very unsustainable. Then, 23 rice farmers (15.33%) had a sustainability index in the category of possibly quite unsustainable. There was one farmer in the category of intermediate sustainable, somewhat sustainable, and sustainable (Figure 2). The farmer in the sustainable category can be a role model for farmer-to-farmer learning so that the other farmers would adopt and apply sustainable agriculture practices. Farmer-to-farmer learning can improve social capital, income, and technology adoption **(Taweekul et al., 2010)**. The agricultural extension officers also are needed to enhance farmers' knowledge related to sustainable farming practices. The agricultural extension role is expected can improve the adoption of sustainable agriculture practices for the farmers **(Hosseini, Mohammadi and Mirdamadi, 2011; Anang, Bäckman and Sipiläinen, 2020)**.

Most of the farmers were in the possibly unsustainable category. The finding is supported by some studies in Malaysia. The studies explained that rice farming practices were possibly unsustainable in granary areas of Malaysia **(Mohamed et al., 2016a)** and Kelantan **(Terano et al., 2015)**. The majority of farmers were unsustainable for rice farming practices with a score of less than 40%. A study by **Roy, Chan and Rainis (2014)** showed that more than 50% of the rice farming were unsustainable in Bangladesh. The main cause of the case is excessive and intensively chemical use.

## Factors influencing the sustainability of rice farming practices in tidal swamplands

According to **Veall and Zimmermann (1996)**, if the value of Pseudo $R^2$ is adequate ( >50%), the Tobit model is fit. The value of Pseudo $R^2$ in this study was 69%. The result of the regression analysis with the Tobit model is provided in Table 6. The intercepts of the model were negative. It indicated the sustainability of rice farming practices in tidal swamplands is unsustainable. The result was supported by the previous finding revealing the sustainability of rice farming practices in tidal swamplands is possibly unsustainable with the score index range of 20 to 40%. Age had a positive value. However, it does not significantly influence the sustainability of rice farming practices in tidal swamplands. **Mohamed et al. (2016b)** also declared that age is not a determinant factor in the sustainability of rice farming practices in Malaysia.

Education was positive and significantly affected the sustainability of rice farming practices in tidal swamplands. It occurred since some farmers began to aware of the environmental issue and sustainability **(Francis and Porter, 2011)**.

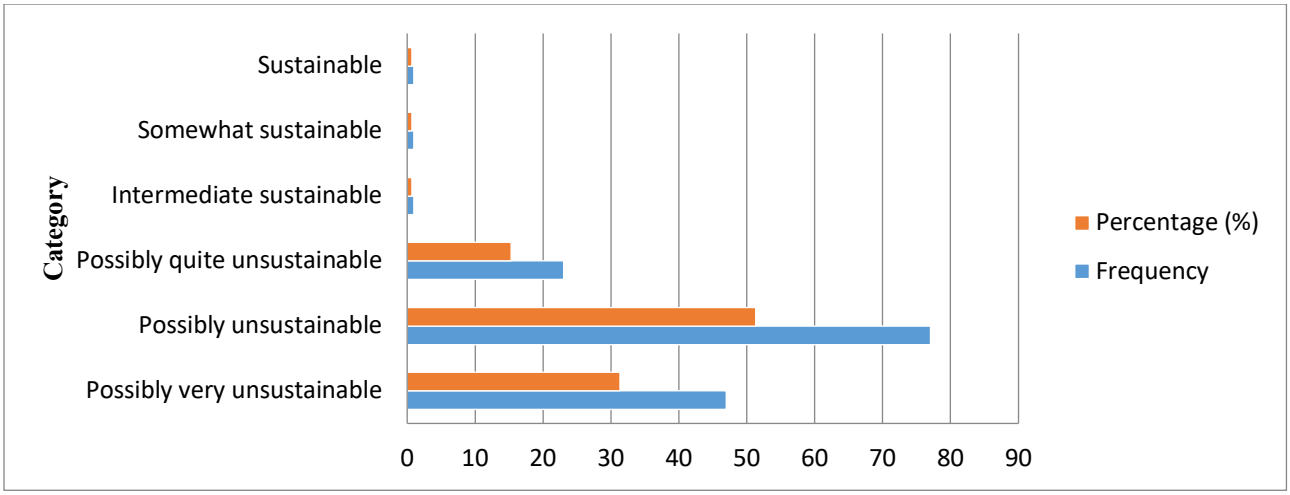


**Figure 2** Categorization of the farmer sustainability index.

**Table 6** Result of regression analysis with Tobit model.

| Variables | Coefficient | Std. Error | t-stat | *p* |
|---|---|---|---|---|
| Constant | -5.14 | 1.87 | -2.75 | 0.01** |
| Age | 0.09 | 0.60 | 0.15 | 0.39 |
| Education | 1.70 | 0.20 | 8.50 | 0.00*** |
| Household size | 4.59 | 0.58 | 7.91 | 0.00*** |
| Farm size | 0.05 | 0.19 | 0.26 | 0.385 |
| Farming experience | -0.28 | 0.15 | -1.87 | 0.070* |
| Pseudo $R^2$ | | | 0.69 | |
| *p* >Chi-square | | | 0.00 | |

Note: ***= Significant at $p$ <0.01; **= Significant at $p$ <0.05; *= Significant at $p$ <0.10.

The farmers got information from television or social media regarding sustainable agriculture. Currently, the role of electronic media is important to build the capacity of farmers **(Zeweld et al., 2017)**.

It is such an informal education that can improve the farmers' knowledge regarding sustainable agriculture. Education significantly influenced sustainable agriculture practices in Nigeria **(Omoare and Oyediran, 2020)**, Vietnam **(Thanh and Yapwattanaphun, 2015),** and the USA **(D'Souza, Cyphers and Phipps, 1993)**.

Furthermore, household size had a significant value on the sustainability of rice farming practices in tidal swamplands. The household size significantly influenced sustainable agriculture practices in the Philippines **(Mariano, Villano and Fleming, 2012)** and Ethiopia **(Kassie et al., 2009)**. The household size in agriculture is related to labor. Labor availability is important in sustainable agriculture **(Teklewold, Kassie and Shiferaw, 2013)**. The rice farmers in tidal swamplands employed family labor on the rice farm. The case occurred because sustainable agriculture required more labor than conventional agriculture **(Rigby and Cáceres, 2001)**. The most of inputs used for rice farms with sustainable farming practices must be made by own. Economically, the farmers can save some money to pay hired laborers if the farmers employed the family laborers. It is a reason why household size influences the sustainability of rice farming practices. However, the current case that occurred in the tidal swampland is the labor forces move to an urban area. The labor forces seek a job in the city as construction laborers or others. The decreasing force of labor in agriculture would threaten rural development **(Peng, Tang and Zou, 2009)**.

The other finding obtained that farm size did not influence the sustainability of rice farming practices in tidal swamplands. The tidal swamplands owned by farmers are fragmented. Therefore, the farmers are tough to manage and maintain their rice farms sustainably. The other reason is if a farmer carried out sustainable agriculture practices but the surrounding farmers did not; the surrounding farmers' chemical will pollute the rice farm with sustainable agriculture practices. This finding is also in line with **Terano et al. (2015)** and **Mohamed et al. (2016b)** that farm size did not affect the sustainability of rice farming practices significantly in Malaysia.

The farming experience negatively influences the sustainability of rice farming practices in tidal swamplands. The farming experience had a negative value on the sustainable agriculture practices of farmers in Bangladesh **(Ghosh and Hasan, 2013)** and Nigeria **(Oyewole and Sennuga, 2020)**. The experienced farmers were not willing to change their rice farming practices. They thought that sustainable rice farming practices are difficult and spend much of their time. On the other hand, they could buy the agricultural input without making it by themselves. It is required a way to change their paradigm to achieve sustainable agriculture. The ways are through training, field school, empowerment program, or others **(Berg et al., 2020)**.

## CONCLUSION

This study concluded that rice farming practices in tidal swamplands were unsustainable. The majority of farmers had an average sustainability index of 25.53%. It indicated that rice farming practices in tidal swamplands were possibly unsustainable. The factors affecting the sustainability of rice farming practices in tidal swamplands were education and household size. In terms of policy recommendations, farmer-to-farmer learning and extension are considered to encourage and educate the farmers to implement sustainable agriculture practices. Also, an empowerment program for the young generation in tidal swamplands must be considered to prevent labor movement from the agriculture sector in the rural area to the industry sector in the urban area.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE INFLUENCE OF CAVITATION EFFECTS ON THE PURIFICATION PROCESSES OF BEET SUGAR PRODUCTION JUICES

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## ABSTRACT
In the juices of sugar beet, the viscosity of the produced viscosity is determined. They contain sugars and non-sugary compounds. If they are in the form of associated or complex compounds, then when their state changes. Well under the action of external factors or at their removal from a solution it is obligatory. Its rheological properties will also change. Therefore, with the help of determining the viscosity, it is possible to conclude the complex processes that take place in juices under the action of the effects of vapor condensation cavitation, namely: the force between Leculiary bonds, the size of molecules, and the length of chemical bonds, etc. The paper presents studies of the influence of vapor-condensation cavitation effects on the change of such rheological properties of cell and diffusion juice as viscosity and surface tension. The viscosity of the steam-treated juice is affected by complex transformational changes that occur with the associated compounds under the effects of vapor-condensation cavitation, which leads to their destruction and this leads to a decrease in their molecular weight and changes in concentration. Studies have shown that with increasing steam consumption for juice processing in the range of 0 – 1.5% by weight of juice the upper tension increases. Such legitimacy is also an indirect confirmation of the processes of destruction of the association. important compounds of diffusion juice under the influence of the effects of steam condensation cavitation.

**Keywords:** diffuse juice; associates; cavitation effects; hydrodynamic cavitation; surface tension

## INTRODUCTION
Diffusion juice is a complex solution of sucrose and various organic and inorganic compounds. According to the hypothesis of L. D. Bobrovnik, most of them are contained in the form of associated and complex compounds of varying degrees of stability, and the processes occurring during the lime-carbon dioxide purification of diffusion juice are largely processes of transformation of complexes. Sucrose also takes part in it, which as a result passes to a free state **(Alves et al., 2017)**.

To destroy and remove some non-sugars from the solution by coagulation, precipitation, or adsorption, it is necessary to spend thermal energy or energy of chemical reaction, which occurs according to the typical technological scheme of juice purification in the conditions of preliminary and main defecation **(Sheiko et al., 2019)**.

Thermal energy at all stages of purification is supplied to the juice mainly through recuperative heat exchange equipment **(Sukhenko et al., 2019)**. However, as practice shows, heating the juice through the heat exchange surface is not able to cause radical changes in hydrated substances of the most common associates in the juice system, or in real complex compounds formed by metals and other, mostly organic compounds that are always present in the juice **(Zheplinska et al., 2019)**. This led to the fact that the radical destruction of natural associates in the known methods of purification is realized through the use of the energy of a chemical reaction, i.e. reagent treatment of the juice **(Zheplinska et al., 2020)**.

But this method is quite long and requires a large cost of chemical reagent. At the same time, as the experiment proves, it is possible to destroy the associated structures in another way, namely by introducing water vapor into the juice. This is especially evident in the method proposed by L. D. Bobrovnik in Cuba for the purification of raw cane juice "mesclado", i.e. in production, where the cost of purification is 0.03% by weight of raw materials (while in sugar production they are 3.0%). During the application of the method, there was an increase in the purity of the juice by more than 2 units and the sedimentation rate of the coagulated particles of colloidal dispersion substances **(Palamarchuk et al., 2019)**.

Positive results were obtained in the case of treatment of diffusion juice with water vapor with the simultaneous introduction of lime milk. The use of this method allowed not only to increase the purity of the juice and reduce its

color but also to reduce the cost of lime milk for cleaning **(Bhatia et al., 2016)**.

Hydrodynamic cavitation is used in the technological processes of food production relatively recently. But the available experience of such use allows to state that from all known influences hydromechanical is the most effective **(Somaratne et al., 2019)**. Industrial studies of the use of hydrodynamic cavitation to intensify the process of pre-defecation have shown that these conditions also significantly reduces the duration of the process and improves the conditions for further processes of purification of diffusion juice **(Matyashchuk et al., 1988)**. However, the authors note that a positive effect is observed only during the formation of cavitation bubbles of size Ro = 200300 μM when there are soft cavitation regimes.

## Scientific hypothesis

There are no data on the effect of steam condensation cavitation on the treated medium in the literature. It is possible that by analogy with hydrodynamic cavitation, the energy released due to the collapse of steam bubbles is sufficient to destroy some of the complex and associated compounds. If so, the constituent elements released under these conditions will be ion carriers and will be able to participate in reactions with the calcium ion. Indirect evidence of such destruction may be a change in the viscosity and surface tension of the juice.

If under the action of cavitation, the coagulation spatial structure of non-sugars is formed, which dramatically increases the strength of the system, the viscosity of the juice will increase and, conversely, in the case of destruction of associates and chains of macromolecular compounds, the mobility of the system will increase. The latter also occurs with partial complete coagulation of some compounds, such as proteins, the excretion of which should also reduce the viscosity.

## MATERIAL AND METHODOLOGY

These works mainly cover the final technological result but do not consider the physical nature of the effects that occur during hydrodynamic or vapor-condensation cavitation treatment, and the mechanisms of their manifestation on the physico-chemical transformations of diffusion juice compounds, which necessarily occur. This is the subject of our research.

The direct objects of research were cellular and diffusion juices of beet sugar production. Cell juice was obtained by pressing beetroot chips on the mechanical press (Figure 1).

Diffusion juice was obtained by performing the process of extraction from beetroot chips using water, that is, extraction in the system of solid body-fluid was carried out. The study was performed on a laboratory installation of Figure 2.

Hydrodynamic cavitation is the local discontinuities of flow continuity caused by a decrease in pressure with the formation of cavities (bubbles, caverns) filled with steam, gas, or a mixture thereof, which subsequently slam (collapse) in a pulsed mode, creating a hydro in slam. The main active factor of hydrodynamic cavitation is the intense impulses created during the slam of a large number of small bubbles. Powerful micro-pulses of liquid compression with gas evolution, ionization, and rearrangement of the liquid structure, create favorable conditions for the intensification of hydromechanical, thermal, mass transfer, and chemical processes. Similar to such cavitation, phenomena occur when a jet of water vapor is injected through a nozzle into a turbulent liquid stream with less than steam pressure and a lower temperature. The bubbles of superheated vapor relative to the liquid are crushed, and then quickly condense and slam, creating microhydroshocks with similar to hydrodynamic cavitation effects – this is steam condensation cavitation. The scheme of the laboratory installation is presented in Figure 3.

Cell juice was obtained in the laboratory using a mechanical press by squeezing. Diffusion juice was also obtained in the laboratory by extraction in a screw diffusion apparatus. The extraction temperature was 75 ºC during the whole process until the dry matter content in the diffusion juice reached 14.2%. This equipment for the production of cell and diffusion juices are presented in Figure 2 and Figure 3, respectively. After squeezing the cell juice, the dry matter content of 22.8 units was also determined. The dry matter content was determined using a precision refractometer with an error of 0.01%. After obtaining the juices, they were immediately filtered through a filter with a pressure of 0.2 MPa. The filtration time was not determined because this was not the purpose of the study. It was necessary to obtain pure solutions without suspended particles so that they do not interfere with the determination of viscosity and surface tension at the temperature of the juices. Steam condensation kiting was performed on the apparatus (Figure 3) with the following parameters: vapor pressure of 0.2 MPa and an increase in juice temperature within 4 – 8 ºC. All studies were performed with beets of the same quality for 5 days, i.e. five repetitions, which showed a deviation of less than 1.5% in statistical processing.

Physical phenomena that cause a strong destructive or intensifying action are united by a general pattern: they occur in liquid media during a sharp change in external pressure and are accompanied by intense growth or flattening of the formed bubbles, if any, contained in the liquid. A distinctive feature of these phenomena is the Spatio-temporal localization of energy, which allows at a relatively low level of energy to form directed pulses of high power **(Mushtruk et al., 2020)**.

The dynamic viscosity was determined using a Hepler 9 viscometer and a rotary viscometer REOTEST.

Surface tension was measured using the Rebinder method, as well as a stalagmometer. Rebinder's method is to measure the pressure required to form and separate the gas bubble in the liquid from the surface of the capillary. The scheme of the device and the analysis was performed with the difference that the differential pressure gauge was not placed vertically but at an angle of 45, which reduced the effect of hydrostatic pressure on the accuracy of measurements.

The method of measuring surface tension using a stalagmometer is described in the following scientific papers **(Mysels, 1990)**.

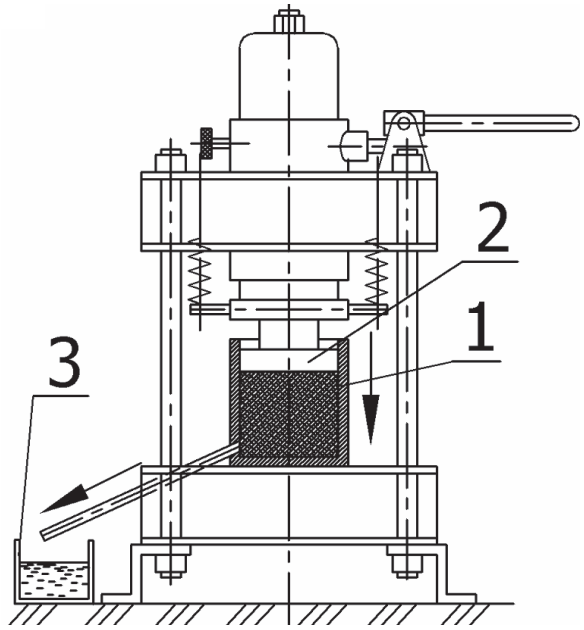


**Figure 1** Mechanical press for obtaining cell juice from sugar beet. Note: 1 – cylindrical sleeve; 2 – the piston; 3 – capacity for collecting the juice.




**Figure 2** Diffusion apparatus MT-131 for obtaining diffusion juice.

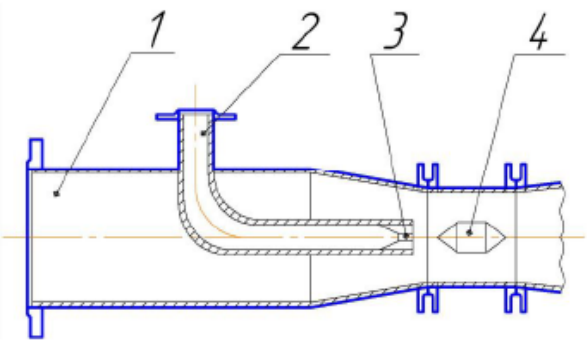


**Figure 3** Steam condensation cavitation device. Note: 1 – l ime milk pipeline; 2 – a branch pipe for steam supply; 3 – nozzle; 4 – distribution insert.

**Statistical 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

Diffusion and cell juices of sugar beets were filtered, treated with steam at a constant potential of 0.2 MPa, and a temperature difference of 4 – 8 ºC. The authors of scientific works **(Buniowska et al., 2017; Verma et al., 2018; Shmyrin, Kanyugina and Kuznetsov, 2017)**, process the diffusion juice at a constant potential in the range from 0.4 to 0.7 MPa. The authors of scientific works **(Nadeem et al., 2018; Avalos-Llano, Molina and Sgroppo, 2020; Rahimi et al., 2020)**, process the diffusion juice at a constant potential in the range from 0.4 to 1.7 MPa.Using a Hepler viscometer, the determination was performed at a constant temperature of 20 ºC for the duration of the fall of the ball, the constant k of which was 0.01035 PA·cm$^3$.g$^{-1}$, the density of the ball material – 2.399 g.cm$^{-3}$. The value of the dynamic viscosity was determined by the formula (1):

$$\eta = \tau \times (d_1 - d_2) \times k, \qquad (1)$$

where:

η – dynamic viscosity, Pa · s;

τ – duration of falling of the ball, s;

$d_1$, $d_2$ – respectively, the density of the material of the ball and the liquid, $\frac{kg}{m^3}$;

k is the constant of the ball, $\frac{Pa \cdot m}{g}$

The results obtained are given in Table 1.

As can be seen, with increasing steam consumption, which corresponds to the more intense effect of vapor condensation cavitation on the juice, the viscosity of the latter decreases for both diffusion and cell juices. The viscosity of cell juice is always higher than that of diffusion juice, which is explained not only by the higher dry matter content but also by the preservation of the structure of associates and macromolecular compounds, which is partially destroyed in diffusion juice by thermal disaggregation. The authors of the following scientific papers **(Dalfré Filho, Assis and Genovez, 2015; Luo et al., 2019; Dhar et al., 2015)** claim that with increasing steam consumption, the viscosity of diffusion juices increases.

Similar dependencies were obtained in the case of using a viscometer REOTEST. The authors of scientific works **(Guo et al., 2018; Ozerov and Sapronov, 1985; Jiang, 2015; Almohammed et al., 2015)** used the viscosimeter of other brands and performed a chromatographic analysis of the obtained samples.

It is possible to deny the results of research, citing the fact that during the treatment of juices steamed their dilution with steam condensation. That's the way it is, but to prove that when steam is treated with steam, the decrease in viscosity occurs not only due to the dilution of the juice with condensate, we conducted studies on the effect of dilution on the viscosity of diffusion juice. The data are presented in Figure 4, which shows that when diluting the juice with distilled water to dry matter content, which is similar when treating the same juice with steam, the viscosity of the juice is greater than 6% of the viscosity of the diffusion juice treated with steam. The authors of the following scientific works **(Johnson, Zhou, and Wangersky, 1986; Lee et al., 2012; Lebovka et al., 2007)** conducted similar studies and found that the viscosity of the juice ranged from 10 – 15%.

That is, the viscosity of the steam-treated juice is affected not only by dilution but also by the complex transformational changes that occur with the associated compounds under the effects of vapor-condensation cavitation. In subsequent scientific works **(Katariya, Arya and Pandit, 2020; Kim et al., 2016; Kozelová et al., 2011; Krasulya et al., 2016)** such studies were not conducted.

Namely, they break down, which causes a decrease in their molecular weight and a change in concentration, because it is known that the greater the mass of macromolecular compounds, the greater the viscosity of their solution, as long chains of molecules interfere with fluid flow. The authors of scientific works **(Dehghannya et al., 2018; Moser et al., 2017; Zhu et al., 2016; Zdziennicka et al., 2017)** claim that the decrease in molecular weight and change in concentration change depending on the change in low molecular weight compounds.

This follows from the Staudengir equation, which relates viscosity to molecular weight by the formula (2):

$$\eta - \eta_0 / \eta_0 = kMC, \qquad (2)$$

where:

M – average molecular weight of macromolecular compounds;

C – concentration of the solution.

Also, spatial transformations of associates are possible.

Under the action of the effects of steam condensation cavitation, energy is released, which, in our opinion, causes the destruction of natural associates and increases the number of polar molecules of non-sugars of diffusion juice in the solution and thus increases the surface tension.

Therefore, the following studies were determined by us to determine the amount of surface tension of diffusion juice depending on the amount of steam entered into the juice, using the Rebinder method and using a squagometer.

The results of experiments to determine the dependence of the surface tension of the diffusion juice by the Rebinder method from the amount of steam in % to the mass of the juice that went to its treatment, are presented in Table 2.

To verify the results, we used the method of determining the surface tension using a stalagmometer. The results obtained are also presented in Table 2. As can be seen, under the influence of the effects of steam condensation cavitation, the surface tension of diffusion juice, determined by two methods, gives close in absolute value results, which show that with increasing steam consumption for juice

**Table 1** Correlation of juice viscosity on steam consumption for their processing.

| Type of juice | Viscosity, Pa s at steam consumption, % by weight of juice. Diffusion juice (dry matter content 14.2 %) | | | | |
|---|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 1.4 | 2.0 |
| Diffusion juice (dry matter content 14.2 %) | 1.92 | 1,86 | 1.80 | 1.77 | 1.74 |
| Cell juice (dry matter content 22.8%) | 3.04 | 2.87 | 2.78 | 2.70 | 2.66 |

**Table 2** Influence of PC cavitation effects on the value of surface tension of diffusion juice.

| Values/method | Surface tension, N/m at steam consumption, % by weight of juice | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 1.0 | 1.5 |
| Rebinder | 0.705 | 0.710 | 0.715 | 0.720 |
| Stalagmometer | 0.710 | 0.713 | 0.718 | 0.722 |

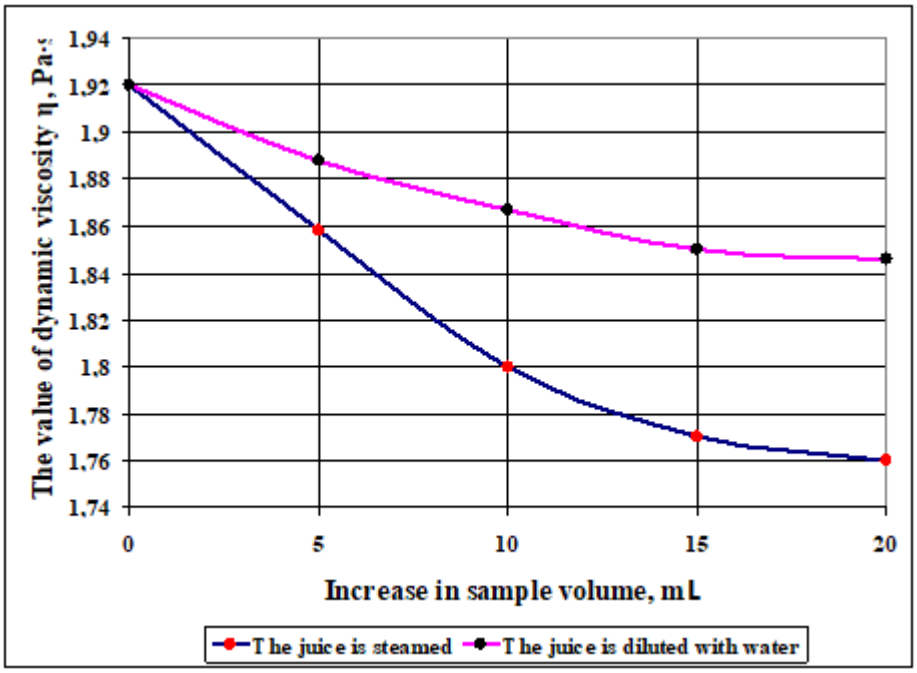


**Figure 4** Change in viscosity of diffusion juice when treated with steam or diluted with water.

treatment within 0 – 1.5% by weight of juice surface tension increases.

The authors of **(Noguiera Felix et al., 2019; Sasikumar, Chutia and Deka, 2019; Cervantes-Elizarrarás et al., 2017)** conducted studies that show that with increasing steam consumption for juice treatment within 0 – 3.5% by weight of juice, the surface tension decreases. This pattern is also an indirect confirmation of the processes of destruction of the associated compounds of diffusion juice under the influence of the effects of steam condensation cavitation. However, because the difference between the final and initial values of the surface tension is within the error of the methods used, to explain the processes that occur by changing the surface tension is not entirely correct. Although in general, the constant increase in the value of surface tension confirms the hypothesis of partially destructive nature of cavitation of high molecular weight and associated compounds of diffusion juice.

Thus, it was found that the viscosity of cell and diffusion juices under the action of vapor-condensation cavitation decreases, and the surface tension increases, which indicates the destruction or change of spatial orientation of the associated and complex compounds of diffusion juice.

## CONCLUSION

Thus, the application of the effects of steam condensation cavitation is a promising area of intensification of technological processes in sugar production. In many cases, cavitation processes are still insufficiently studied and quantitative relationships have not been established to calculate the effects obtained, but the implementation of proven solutions can help increase production efficiency. The paper presents studies of the influence of vapor-condensation cavitation effects on the change of rheological properties of cell and diffusion juice – viscosity and surface tension.

The viscosity of the steam-treated juices was affected by complex transformational changes that occur with the associated compounds under the effects of vapor-condensation cavitation, leading to their destruction and a decrease in their molecular weight and changes in concentration.

Studies have shown that with increasing steam consumption for juice treatment in the range of 0 – 1.5% by weight of the juice, the surface tension increases and the value of the dynamic viscosity coefficient decreases. This pattern is also an indirect confirmation of the processes of destruction of the associated compounds of diffusion juice under the influence of the effects of steam condensation cavitation.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE QUALITY CHARACTERISTICS OF SAUSAGE PREPARED FROM DIFFERENT RATIOS OF FISH AND DUCK MEAT

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## ABSTRACT
The article aims to determine the effect of combining duck meat (*Anas platyrhynchos*) and freshwater fish (*Hypophthalmichthys molitrix*) on the quality (nutritional value, functional and technological properties, sensory attributes) of meat-containing semi-smoked sausages. The optimal version of the recipe with the highest quality indicators of semi-smoked meat sausages with duck meat and freshwater aquaculture was determined. It was found that the protein content of meat-containing, semi-smoked sausages with different ratios of duck and fish meat was 17.90% – 21.34% higher than that of the analog. A sample containing 50% duck and 30% fish has an ideal protein: fat ratio of 1:1. The following high functional properties of model minced experimental semi-smoked sausages were established: up to 72.75% moisture, water binding capacity of up to 71.47%, and water holding capacity of up to 60.60%. A comparative analysis of the rheological properties of minced meat showed that increasing the proportion of duck meat in minced meat modelling systems improves the strength of the system and increases shear stress. The addition of fish raw materials improves the elasticity of meat-containing systems. The optimal ratio of duck and fish is 50:30 for plastic and easily minced mince system. Combining duck meat with raw fish increases the stability of the emulsion to 74.8%. Analysis of the sensory parameters of the experimental sausages confirmed that the best ratio of duck to freshwater fish is 50:30.

**Keywords:** fish; duck; combination; smoked; sausage

## INTRODUCTION
Semi-smoked sausage is a popular meat product made from different types of raw materials: pork, beef, chicken, etc. **(Sălăgean et al., 2012; Drachuk et al., 2018)**.

The production of meat-containing semi-smoked sausages, in which the mass fraction of raw meat is not more than 60%, is topical today in Ukraine. Due to the requirements for meat-containing sausages, meat ingredients are more widely used in recipes in combination with protein fillers **(Strashynskiy et al., 2016)**.

Duck meat is valued by consumers for its dense muscular structure, which determines its high functional and technological properties, pronounced taste, and nutritional value **(Qiao et al., 2017; Bozhko et al., 2020)**. Duck meat is in great demand both in Ukraine and around the world **(Biswas, Banerjee and Bhattacharyya, 2019)**. Thus, products made from duck meat have high-quality characteristics and consumer attractiveness.

On the other hand, it is possible to introduce into the recipe of meat-containing semi-smoked sausages muscle tissue of food raw materials from freshwater aquaculture as a source of high-quality protein with appropriate functional properties **(Tischenko, Bozhko and Pasichnyi, 2016; Lago et al., 2017; Nawaz et al., 2019)**.

Given the fact that duck and fish meat differ from traditional types of meat in structure and physicochemical properties, it is important to select recipe components rationally to ensure the quality characteristics of the new range of semi-smoked meat-containing first- and second-grade sausages using these raw materials **(Bozhko et al., 2020; Ramadhan, Huda and Ahmad, 2014)**.

The authors have proven the effectiveness of the combination of duck meat and freshwater aquaculture meat **(Bozhko et al., 2018a; Bozhko, Pasichniy and Bordunova, 2016)**. Replacing pork with duck meat in boiled sausage recipes allows the product to be balanced in terms of protein and fat and increases the functional and technological performance and cooking yield. Through the use of fish meat in sausage technology as a structural component of the minced meat system, its water-binding capacity can be increased. The content of salt-soluble myofibrillar proteins with high hygroscopicity is between 75% and 80% of total freshwater fish protein. This causes high juiciness and cooking yield of products from fish raw materials.

Meat-containing semi-finished products with the meat of different types of freshwater aquaculture have been developed **(Bozhko et al., 2018b)**. It is determined that

when combining Peking duck and freshwater fish (*Carassius gibelio*) as part of frozen chopped meat-containing semi-finished products, it is possible to produce high-quality products.

The analysis of functional-technological and organoleptic indicators confirmed an increase in water binding capacity to 81.54%, in the water holding capacity to 76.47%, in emulsion capacity to 98.0%, and in emulsion stability to 69.49%. The developed semi-finished products of the combined composition had high organoleptic characteristics and were safe in terms of microbiological parameters **(Bozhko et al., 2018b)**.

Thus, prospects for the development of new products require further study of the combination of waterfowl and freshwater aquaculture as the ingredients in meat-containing semi-smoked sausages.

### Scientific hypothesis

This project was carried out to determine the effect of a duck meat (*Anas platyrhynchos*) and freshwater fish (*Hypophthalmichthys molitrix*) combination on the quality (physicochemical and sensory properties) of meat-containing semi-smoked sausages. We are expecting to determine the optimal recipe with the highest quality for semi-smoked sausages containing duck meat and freshwater aquaculture due to the optimal ratio.

## MATERIAL AND METHODOLOGY

### Semi smoked sausage preparation

Three different ratios of duck *(Anas platyrhynchos)* and fish (*Hypophthalmichthys molitrix)* were used in this research, where sample 1 = 30:50, sample 2 = 40:40, and sample 3 = 50:30. Other used ingredients were pork fat – 10%, soya bean protein isolate – 4%, hemp protein – 6%, salt 2.3 – 2.8%, black pepper 0.1%, sodium nitrite – sample 1 – 0.003%, 2 – 0.004%, 3 – 0.005%, nutmeg – 0.05% and fresh garlic – 0.2%. The control (analog) was semi-smoked meat containing sausage with the following proportions of ingredients: duck 40%, pork fat 15%, pork heart 20%, soya bean protein isolate 5%, dry whey 5%, fibre 5%, and chicken skin 10%.

Meat, fish, pork heart, and chicken skin were mixed into a mixer cutter (Philipps, Germany). Soya bean protein isolate was hydrated in a 4:1 water: isolate ratio for 15 min. Minced ingredients were mixed for 8 min. The forcemeat was stuffed into the casings. The sausages were settled at a temperature of 4 – 8 °C for 2 hours, then dried in an oven at a temperature of 90 ±10 °C for 60 minutes. Sausages were cooked for 40 minutes at a temperature of 80 ±5 °C, cooled and smoked at a temperature of 43 ±7 °C for 5 hours, and dried at a temperature of 10 °C for 2 days.

#### Raw Protein Measurement

Protein measurements were performed using the Kjeldahl method **(ISO 937, 1978)**. 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 0.01N sulfuric acid.

#### Fat Measurement

Total fat was measured by the Soxhlet method **(ISO 1443, 1973)**. 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.

#### Ash Measurement

Ash content was determined by ashing samples overnight at 520 °C in a muffle furnace (Germany).

#### Definition of the energy value

Energy value was calculated by the Atwater general factor system. The average values of energy are expressed as the number of calories per 1 gram of the macronutrient. The Atwater general factor system includes energy values of 4 kcal per gram ($kcal.g^{-1}$) (17 $kJ.g^{-1}$) for protein, 4 $kcal.g^{-1}$ for carbohydrates, and 9 $kcal.g^{-1}$ (37 $kJ.g^{-1}$) for fat **(FAO, 2003)**.

#### 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, 1997)**. 5 g of the sample was placed in a container, dried for 1 hour at 150 °C.

#### Methods of measuring functional indicators

WBC (water binding capacity) of minced meat was determined by the pressing method **(Pasichnyi, 2013)**. $WBC_a$ is the water-binding capacity to the moisture of the sample; $WBC_m$ is the water-binding capacity to the mass of the sample. 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)**.

#### 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 statistical analysis data were 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 deviation of the mean (SDM). The smallest acceptable difference for probes from the one sample was pointed at 5%. Probes with more differences were not considered. We selected the value of reliability as $p < 0.05$.

## RESULTS AND DISCUSSION

The data obtained for the nutritional value of semi-smoked sausages are presented in Table 1.

It was found that the sausages had a high protein content, which ranged from 20.47 to 19.89 g.100g$^{-1}$ of product, which was 17.90% – 21.34% higher compared with the analog (control). This was due to the inclusion of protein ingredients such as duck meat, silver carp, soy isolate, and hemp protein in the recipe. Due to the use of high-protein components in the technology, it was possible to reduce the mass fraction of fat in sausages. Thus, in the analog, the fat content was 31.03 g.100g$^{-1}$, which is on average 75% higher than in the experimental samples. The fat content in the combined sausages ranged from 14.42 to 20.98 g.100g$^{-1}$, which significantly affected the energy value of products. The caloric content of the experimental samples decreased relative to the control by 51% on average. According to physiological norms, the ratio of protein and fat in the human diet should be 1:1 **(Trumbo et al., 2002**). According to the present research, a similar content of protein and fat was obtained in a sample of 50% duck and 30% fish. The set values meet the requirements for commercial smoked sausages.

The problem of protein deficiency in diets remains unresolved **(WHO, 2004)**. This is especially true for countries with low economic development **(Eshetie, 2017)**. After all, the diets of their inhabitants mainly consist of vegetables and cereals. Adequate protein intake is crucial for health and development **(Elmadfa and Meyer, 2017; Santarpia, Contaldo, and Pasanisi, 2017; Brandhorst and Longo, 2019)**. As a rule, animal protein, including that of fish, is of higher quality for human consumption due to its amino acid composition and good digestibility **(Cieślik et al., 2017; Khalili Tilami and Sampels, 2017)**. Meat-containing semi-smoked sausages with duck and freshwater fish will supplement the human diet with a high-quality, easily digestible protein.

Proteins play a major role in the formation of functional and technological properties of forcemeat systems **(Strashynskiy et al., 2016; Kang et al., 2016)**. The introduction of freshwater aquaculture proteins into the meat-containing system and their combination with duck muscle tissue and vegetable proteins allow model forcemeat systems with a high level of hydration to be obtained. Duck meat proteins have high functional properties, in particular the ability to bind and retain water in minced meat systems **(Adzitey, 2012; Ismail et al., 2010)**.

The results of the study of functional and technological properties of model minced meat systems with different ratios of duck and freshwater fish are presented in Table 2.

Analysis of the data in Table 2 shows that the amount of moisture in the experimental samples ranges from 71.00% to 72.75%. The high moisture content of minced meat is due to the high moisture-binding capacity of the systems. The results of physicochemical properties are within the normal range of previously reported experimental smoked sausages **(Bozhko et al., 2018c)**.

The WHC of minced meat systems is 58.44% – 60.60%, which is 5.05% – 8.93% higher than in the control. Due to the balancing of the ingredient composition, the pH value of the experimental samples decreased by 14.70% – 16.54%, which helped to shift the pH from the isoelectric point of muscle and fish proteins and increase their ability to attach water molecules. Due to the processes of hydration, the $WBC_a$ of experimental samples was in the range of 69.60% – 71.47%, which confirms the high ability of model meat and fish systems to retain moisture in the structure of the forcemeat **(Fennema, 1990)**.

In the technology of sausage production, an important and basic operation of the technological process is the preparation of minced meat with a strong structure and the highest water-binding capacity. It is known that the strength of the forcemeat structure is due to the number of proteins that can pass from cellular structures into a continuous phase and form a dispersion medium and affect the structural and mechanical properties of products **(Smith, 2001; Bouton, Harris and Ratcliff, 1981)**.

Analysis of structural and mechanical parameters of the combined minced meat (Table 3) proves that changing the ratio of the same ingredients in the recipe makes it possible to directly regulate the rheological properties, thereby affecting product quality increasing the content of fish raw material to 50% in the formulation of sample 1 reduced the shear stress by 35.5% compared with the control sample and by 11.5% and 18.8%, respectively, according to samples 2 and 3. The forcemeat of all test samples was more elastic in comparison with the control. The results are within the normal range of commercial smoked sausages.

Increasing the proportion of duck meat in minced meat modeling systems enhanced the strength of the system and, accordingly, increased the shear stress.

Also, as the content of dense duck meat increased, the plasticity of the forcemeat decreased. Thus, the minced meat with the highest plasticity had a fish content of 50% and a duck meat content of 30% – 25.9 ±0.38 cm$^2$.g$^{-1}$, while in the sample with a duck content of 50% and 30% fish meat, this indicator was 21.6 ±0.37 cm$^2$.g$^{-1}$. That is, for a plastic and easy-to-form system, the optimal ratio of duck to fish is 50:30.

An important role in the production of a juicy sausage is played by the emulsifying properties of the minced dispersed system **(Tian et al., 2019; Santhi, Kalaikannan and Sureshkumar, 2017)**. The data in Figure 1 shows the effect of the ratio of duck to fish in minced smoked sausages on the emulsifying capacity (EC) and the stability of the emulsion (SE).



A B C

**Figure 1** Samples of semi-smoked meat containing sausages with different ratio of duck and fish. Note: Ratio of duck and fish: A – 30:50; B – 40:40; C – 50:30

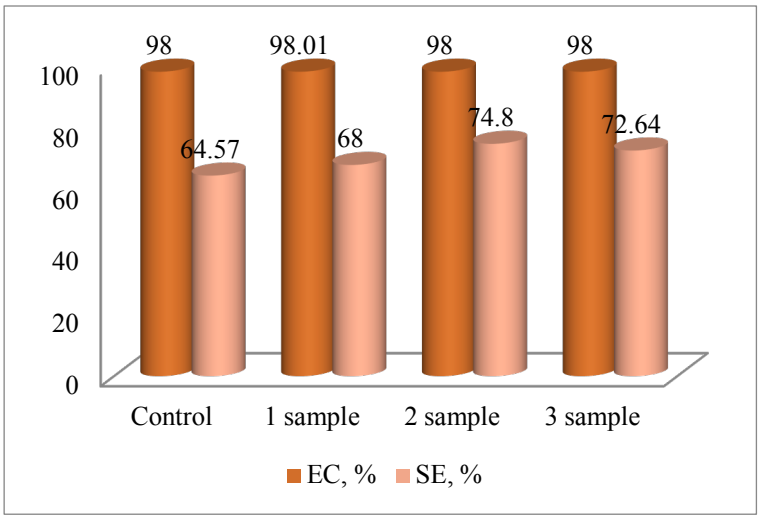


**Figure 2** Analysis of emulsifying properties of forcemeat with different ratios of duck to fish.

**Table 1** Nutritional value of combined minced meat semi-smoked sausages.

| Parameter | Control | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|---|
| Protein, g.100g$^{-1}$ | 16.87 ±0.85 | 20.47 ±0.23 | 20.18 ±0.54 | 19.89 ±0.54 |
| Fat, g.100g$^{-1}$ | 31.03 ±0.13 | 14.42 ±0.09 | 17.70 ±0.09 | 20.98 ±63 |
| Ash, g.100g$^{-1}$ | 0.98 ±0.05 | 1.51 ±0.02 | 1.30 ±0.04 | 1.10 ±0.01 |
| Energy value, kcal | 364 | 212 | 241 | 269 |

**Table 2** Functional and technological properties of combined minced meat of semi-smoked sausages.

| Sample | Moisture,% | WBC $_a$,% | WBC $_m$,% | WHC,% | pH |
|---|---|---|---|---|---|
| Analog (Control) | 70.51 ±0.31 | 69.34 ±0.60 | 98.34 ±0.85 | 55.63 ±0.05 | 7.62 ±0.03 |
| 1 | 72.75 ±2.20 | 71.47 ±1.64 | 97.46 ±2.16 | 58.44 ±0.36 | 6.50 ±0.37 |
| 2 | 71.00 ±3.60 | 69.60 ±1.45 | 97.35 ±2.31 | 60.60 ±0.96 | 6.40 ±0.10 |
| 3 | 71.80 ±0.36 | 70.17 ±2.17 | 98.66 ±3.01 | 60.50 ±0.27 | 6.36 ±0.55 |

**Table 3** Structural and mechanical properties of combined minced meat of semi-smoked sausages.

| Samples | Shear stress, Pa | Plasticity, cm$^2$/g | Effective viscosity, Pa × s |
|---|---|---|---|
| Analog (Control) | 1407 ±23.7 | 20.5 ±0.16 | 817 ±17.3 |
| 1 | 1038 ±19.1 | 25.9 ±0.38 | 579 ±21.9 |
| 2 | 1157 ±20.1 | 23.7 ±0.52 | 691 ±27.5 |
| 3 | 1234 ±21.9 | 21.6 ±0.37 | 684 ±24.1 |

**Table 4** The effect of the duck meat and fish ratio on the organoleptic characteristics of semi-smoked meat containing sausages.

| Sample | Appearance | Colour | Flavor | Texture | Taste | Juiciness | The total score, points |
|---|---|---|---|---|---|---|---|
| Control | 4.7 ±0.05 | 4.7 ±0.05 | 4.8 ±0.08 | 4.6 ±0.03 | 4.5 ±0.04 | 4.5 ±0.01 | 4.6 ±0.12 |
| 1 | 3.1 ±0.03 | 3.3 ±0.06 | 3.1 ±0.05 | 2.8 ±0.04 | 3.1 ±0.02 | 4.0 ±0.03 | 3.2 ±0.11 |
| 2 | 3.6 ±0.04 | 3.5 ±0.03 | 3.6 ±0.02 | 3.5 ±0.01 | 3.4 ±0.02 | 3.8 ±0.05 | 3.6 ±0.09 |
| 3 | 4.7 ±0.04 | 4.6 ±0.04 | 4.5 ±0.01 | 4.3 ±0.02 | 4.6 ±0.03 | 4.7 ±0.02 | 4.5 ±0.11 |

Analysis of the data in Figure 2 shows that the emulsifying properties of minced meat with different ratios of duck meat and freshwater fish meat were almost at the same level. This confirms the data of **Kornyenko and Ghuseva (2018)** regarding the high ability of fish proteins to retain fat in the emulsion state. Water-soluble and salt-soluble fractions of fish muscle proteins promote the formation of a solvate shell on the surface of fat capsules and prevent their adhesion **(Huang et al., 2018; Santana, Huda and Yang, 2015)**.

When studying the stability of the emulsion under the influence of heat treatment, it was found that with the increase in the proportion of duck meat in the sausage recipe, the stability of the emulsion increased. In the sample with a ratio of 50% duck meat to 30% fish meat, the CE was 72.64 ±1.25%, which is 12.5% greater than the control. According to EC and SE indicators, minced fish is close to minced duck meat **(Huda, Putra and Ahmad, 2011)**. The emulsifying ability of minced poultry meat is 75%, and the stability of the emulsion is about 70% **(Strashynskiy et al., 2016; Yancheva, Dromenko and Grinchenko, 2017)**; thus, the combination of this raw material with multicomponent functional mixtures based on plant and animal proteins and food additives, i.e. fillers **(Pasichniy et al., 2015)** will effectively develop meat products with a combined composition of raw materials.

The data for organoleptic quality indicators, namely appearance and color, aroma, texture, taste and juiciness, and overall acceptability of meat-containing semi-smoked sausages with different ratios of duck and freshwater fish, were assessed on a five-point scale by nine semi-qualified experts. The results are shown in Table 4. The evaluation results showed a significant difference in appearance and color, flavor juiciness, texture, and total evaluation between control and experimental samples containing duck and fish meat in all three ratios. The data indicated that the addition of duck and fish had a significant effect on the sensory attributes of sausages.

Freshwater aquaculture is not a traditional ingredient in meat products, one of the reasons being the inherent odor of fish products. Therefore, when combining poultry and fish, it is important to maintain the optimal ratio in terms of organoleptic characteristics.

Among the samples, the highest score on all indicators was given to sample number 3, with a ratio of duck: fish 50:30. Higher color intensities are due to the higher content of duck meat, which has a dark color. A similar effect was observed **(Biswas, Chakraborty and Sarkar, 2006)** in the study of patties with duck and broiler chicken meat. The consistency of the sausages improved with the increase in the proportion of duck meat. When duck meat was combined with the meat of broiler chickens, this effect was not observed. In contrast, the consistency deteriorated, as found by **Huda et al. (2010)**. However, in other studies, no difference was observed **(Kumar et al., 2015)**.

## CONCLUSION

It was found that the protein content of meat-containing semi-smoked sausages with different ratios of duck and fish meat was 17.90% – 21.34% higher than the analog.

A sample containing 50% duck and 30% fish has an ideal protein: fat ratio of 1:1. High properties of model minced experimental semi-smoked sausages were established: moisture content of up to 72.75%, a $WBC_a$ of up to 71.47%, and a water holding capacity of up to 60.60%.

A comparative analysis of the rheological properties of minced meat showed that increasing the proportion of duck meat in minced meat model systems increases the strength of the system and, accordingly, the growth of shear stress. Furthermore, the addition of fish improves the elasticity of experimental forcemeat systems. The optimal ratio of duck and fish meat for a plastic and easy formatted minced meat system is 50:30.

The combination of duck meat with fish raw materials helps to increase the stability of the emulsion to 74.8%. Analysis of the sensory attributes of experimental sausages confirmed that the best ratio of duck meat and freshwater fish in the recipe is 50:30.

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# THE CONTRIBUTION OF PROTEINS, FATS, CARBOHYDRATES, AND ALCOHOL TO THE TOTAL ENERGY VALUE OF THE DIET: A CROSS-SECTIONAL STUDY

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## ABSTRACT
While eating, people receive energy, as well as the necessary nutrients. However, different food products contain different amounts of macro- and micronutrients, so their consumption can vary significantly depending on eating habits. The diets of 105 people, first-year students of the Peoples` Friendship University of Russia from six different regions of the Earth: CIS countries; Latin America; Central and South Africa; North Africa, the Middle East, and Central Asia; Central and South Asia; Iran and Azerbaijan - were analyzed. Nutrition was evaluated by the frequency method. The median calorie intake among all examined was 2756 kcal per day. At the same time, the proportion of proteins from the total calorie content was 15.3%, from fats – 37.5%, from carbohydrates – 47.1%, from alcohol – 0.5%. It was not possible to detect significant differences in the consumption of macronutrients or their contribution to the energy value of the diet between the regions. The exception was ethanol consumption: students coming from Central and South Africa consumed significantly more alcoholic drinks compared to students from Russia, Ukraine, and Moldova. Due to the extremely high level of dispersion within each of the studied groups, it was not possible to detect significant differences in the consumption of macronutrients, depending on the region of origin of the student. That is, intra-group differences turned out to be significantly more than inter-group differences. At the same time, the share of macronutrients in ensuring the total calorie content of the diet in this study turned out to be close to the data obtained in large cities of other countries and significantly differed from the results of the assessment of diets of rural residents. Probably, the student's nutrition is formed largely under the influence of the urban environment and individual taste preferences; ethnic and cultural affiliation plays a much smaller role.

**Keywords:** calorie content; energy substrates; macronutrients; regional features; ethnicity

## INTRODUCTION
During a meal, a person receives energy, as well as the necessary nutrients. Proteins, fats, and carbohydrates are macronutrients. Each of them performs certain functions. However, different foods contain different amounts of them, so their consumption may vary depending on food predilections and habits **(Tutelyan, 2012)**.

Proteins are one of the main components of the diet. During digestion, they break down to amino acids, which are absorbed in the small intestine. In the future, amino acids are involved in the resynthesis of the body's peptides and proteins. Proteins are part of all the basic structures of cells, tissues, and organs, perform enzymatic functions, participate in the transport of substances, are the basis of protein hormones, and carry out intercellular signaling. Amino acids are also involved in the synthesis of protein derivatives - neurotransmitters and hormones **(Galchenko, Morozova and Zaletova, 2017)**. Another function of amino acids is that they act as nitrogen donors in the synthesis of nitrogen-containing compounds of a non-protein nature, such as nucleotides, heme, creatine, etc. Amino acid metabolism usually begins with transamination or deamination reactions, after which either new amino acids or α-keto-acids are formed, or α-keto-acids and an amine residue. Then it goes into the ornithine cycle, which regulates the nitrogen balance of the body. The main product of this cycle is urea. Nitrogen-free amino acid residues are converted into pyruvate, acetyl-CoA, alpha-ketoglutarate, succinyl-CoA, and fumarate, which enter the Krebs cycle, and subsequently into the electron transfer chain, which ends with the synthesis of ATP. With a decrease in the level of glucose in the bloodstream, one of the derivatives of asparagine and aspartic acid, oxaloacetate, will participate in glucose resynthesis - gluconeogenesis **(Severin, 2011a)**.

Fats form the basis of the cell membrane, are the main source of carbon and hydrogen atoms, and perform energy and signal functions **(Rustan and Drevon, 2005)**. Also, lipids act as thermal insulation and are the precursors of bile acids and steroids. Lipid metabolism begins with emulsification by bile acids. After this, the surface area of the fat droplets increases many times, and they undergo

hydrolysis. Hydrolysis products – fatty acids, monoacylglycerols, and diacylglycerols – as well as bile acids, cholesterol, and hydrophobic vitamins, form mixed micelles **(Severin, 2011b)**. They are absorbed into the cells of the small intestine mucosa, where they break down into the main components. Fatty acids can be deposited, form the structural components of cells, and also oxidize to acetyl-CoA and enter the citrate cycle. Ketone bodies, phospholipids, triacylglycerol, eicosanoids, and cholesterol are also formed from fatty acids. Further, cholesterol is involved in the synthesis of bile acids, esters, and steroids **(Wakil, 1962)**.

The main function of carbohydrates is energy supply. They participate in the synthesis of fatty acids, amino acids, nucleotides, which are part of the glycocalyx, which acts as cellular receptors, antigens, and also participates in parietal digestion and transport of substances. Carbohydrates can also be stored as glycogen. The main monomer of carbohydrates in humans is glucose. Its metabolism begins with the formation of glucose-6-phosphate, which subsequently participates in various processes of synthesis and decomposition. The breakdown of glucose-6-phosphate, glycolysis, can occur both in the presence of oxygen and in its absence. Different products form depending on this. So, with a lack of oxygen, pyruvate is converted into lactate, which is transposed into muscle or liver. In the presence of oxygen, pyruvate is converted to acetyl-CoA in the pyruvate dehydrogenase complex. Then acetyl-CoA enters the citrate cycle. The result is the release of energy as ATP and carbon dioxide and water formation. The reverse route from pyruvate to glucose is also possible – gluconeogenesis. It provides maintenance of blood glucose during fasting and physical exertion. Also, glucose-6-phosphate can enter the pentose phosphate pathway, which ends with the formation of hexoses and pentoses. Some pentoses may also be part of nucleotides. Other monosaccharides and heteropolysaccharides, lipids, amino acids, and glycerol can also be formed from glucose (Figure 1) **(Severin, 2011c)**.

Ethanol can be synthesized in the body or ingested with food. Exogenous ethanol is rapidly absorbed in the stomach and small intestine. Ethanol metabolism mainly occurs in the liver and begins in the oral mucosa. Ethanol is subsequently oxidized by the action of alcohol dehydrogenase and acetaldehyde dehydrogenase to acetaldehyde and acetic acid, respectively. Next, acetic acid is converted to acetyl-CoA. It, in turn, is oxidized in the citrate cycle to carbon dioxide, and with its excess is used for the synthesis of fatty acids, fat, and cholesterol. Ethanol as an amphiphilic substance can enhance the permeability of the blood-brain barrier, disrupt the structure and functions of cell membranes, and cause a change in metabolism in almost all organs. So, as a result of ethanol catabolism, the number of NADH increases, which leads to increased lactate formation and slows down gluconeogenesis **(Severin, 2011d)**.

Thus, the metabolism of all macronutrients is closely related. All of them, to one degree or another, are capable of mutual transformations and are predecessors of each other. Synergy concerning basic functions (primarily energy) leads to the fact that the ratio of incoming macronutrients with food can vary significantly, without leading to immediate clinical manifestations of food imbalance. The general scheme of interconversion of macronutrients is presented in Figure 1.

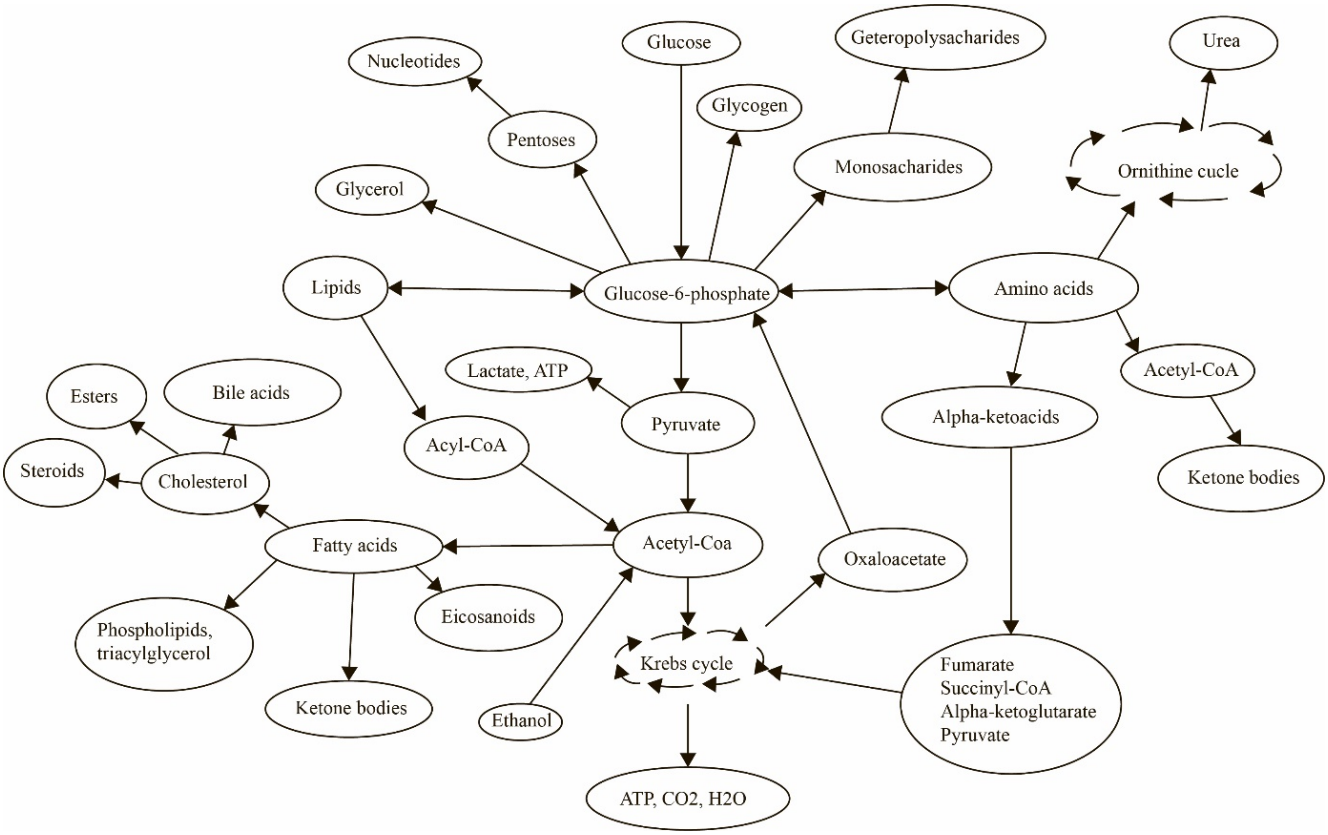


**Figure 1** The general scheme of macronutrient metabolism.

### Scientific hypothesis

The objective of the study was to understand how national and cultural traditions influence the nature of people's nutrition. Besides, even with comparable caloric intake, the contribution of various macronutrients to the total energy value can vary significantly depending on the products that make up the diet.

## MATERIAL AND METHODOLOGY

In this study, 105 people (71 women and 34 men), first-year students of the Peoples' Friendship University of Russia from 33 countries of North and South America, Africa, Europe, and Asia, were examined. The subjects were divided into six groups based on the previous region of residence: 18 people from South, Southeast Asia, Mongolia, and China, 4 people from Latin America, 12 people from North Africa, the Middle East, Central Asia, and Afghanistan, 14 people from South and Equatorial Africa, 4 people from Iran and Azerbaijan, and 54 people from Russia, Ukraine, and Moldova. The age of the subjects was from 20 to 51 years (Me = 23 years).

Evaluation of the actual nutrition was carried out by the method of frequency analysis with Nutrilogic software ("Nutrilogic" LLC, Ryazan, Russia). Food consumption for 3 – 6 months was taken into account. The interview was fully structured. Everyone was asked to recall the features of their diet and the frequency of consumption of main dishes and products. Subjects were suggested to estimate the intake of each group of products separately. The same situation was with the evaluation of food consumption frequency: people had to choose one of the given variants. Photographic measures were used for estimating the amount of food. There were color images of portions of a known weight of different products and dishes included in the Nutrilogic program package. The life-size portions of different weights were presented in photographs on the monitor screen, and the subject determined how much his average portion was comparable with the shown one. Data on the chemical composition of the foods in Nutrilogic is mainly based on the Tutelyan handbook **(Tutelyan, 2012)**.

The fractions of proteins, fats, and carbohydrates in the total calorie intake were calculated based on the fact that the energy value of proteins is 4 kcal.g$^{-1}$, fats – 9 kcal.g$^{-1}$, carbohydrates – 4 kcal.g$^{-1}$ **(Norms of physiological needs, 2009b)**, ethanol – 7 kcal.g$^{-1}$ **(Kolman and Rum, 2000)**. The mass of ethanol consumed was calculated via the formula $m = V * \rho$, where $\rho$ is the density of ethanol equal to 0.79 g.mL$^{-1}$ **(Chemical Encyclopedia, 1999)**.

### Statistical analysis

Statistical data processing was carried out using the SPSS 23 application software package. Analysis of the research results was carried out using descriptive statistics. The sample was not representative; accordingly, the data are presented as medians and quartiles. The differences between the two independent groups were determined by the Mann-Whitney criterion. The differences between all groups were determined by the Kruskal-Wallis test. To determine significance in all tests $p < 0.05$.

## RESULTS AND DISCUSSION

The median caloric value of the diet among all groups was 2756 kcal per day, and the intake of proteins, fats, carbohydrates, and alcohol was 101 g, 109.6 g, 311.8 g, and 2.6 ml, respectively. The values within the groups varied significantly, the difference between the values of 25 and 75 percentiles in most cases was more than

**Table 1** Consumption of macronutrients and energy value of the diet in groups.

| Region | Energy, kcal, median (percentiles 25%; 75%) | Proteins, g median (percentiles 25%; 75%) | Fats, g median (percentiles 25%; 75%) | Carbohydrates, g median (percentiles 25%; 75%) | Alcohol, ml median (percentiles 25%; 75%) |
|---|---|---|---|---|---|
| South and Southeast Asia, Mongolia and China | 2300 (1660; 3907) | 78.4 (61.3; 155.6) | 99.3 (70.8; 159.08) | 287.9 (205.5; 454.9) | 1.7 (0; 14.7) |
| Latin America | 2624 (976; 5852) | 102.7 (37.3; 178.9) | 109.2 (38.4; 270.4) | 307.5 (125.8; 698.2) | 6.5 (0; 11.6) |
| North Africa, Middle East, Central Asia, and Afghanistan | 2722 (1704; 3808) | 116.3 (61.9; 174.1) | 104.2 (75.5; 149.0) | 304.8 (186.2; 435.8) | 6.6 (1.1; 14.9) |
| South and Equatorial Africa | 2746 (1911; 4045) | 108.7 (74.6; 166.1) | 102.2 (79.1; 155.8) | 343.25 (196.7; 446.6) | 15.4 (3.95; 28.5)* |
| Iran and Azerbaijan | 3305 (1634; 5572) | 145.9 (57.9; 249.9) | 137.8 (76.4; 259.1) | 373.25 (164.9; 563.1) | 4.1 (0; 9.3) |
| Russia, Moldova and Ukraine | 2841 (2080; 3769) | 100.7 (76.4; 143.6) | 116.8 (85.8; 166.4) | 349.9 (203.8; 490.4) | 1.3 (0; 8.4)* |
| All regions | 2716 (1970; 3910) | 101.0 (73.6; 153.2) | 109.6 (77.2; 163.4) | 311.8 (206.2; 464.9) | 2.6 (0; 11.5) |

Note: * – $p < 0.05$.

**Table 2** The share of various macronutrients in the total calorie intake.

| Region | Proteins, % | Fats, % | Carbohydrates, % | Alcohol, % |
|---|---|---|---|---|
| South and Southeast Asia, Mongolia and China | 13.9% | 40.1% | 51.3% | 0.4% |
| Latin America | 16.1% | 38.7% | 48.0% | 1.4% |
| North Africa, Middle East, Central Asia and Afghanistan | 17.5% | 35.6% | 45.9% | 1.4% |
| South and Equatorial Africa | 16.2% | 34.6% | 51.3% | 3.1%* |
| Iran and Azerbaijan | 18.1% | 38.7% | 46.3% | 0.7% |
| Russia, Moldova and Ukraine | 14.5% | 38.1% | 51.6% | 0.2%* |
| All regions | 15.3% | 37.5% | 47.1% | 0.5% |

Note: * – $p$ <0.05.

twofold. Such a high dispersion of values did not allow us to identify significant differences in the consumption of macronutrients in multiple comparisons (Kruskal-Wallace test) and even in pairwise comparisons (Mann-Whitney test), despite considerable differences in median indices between regions. The exception was alcohol consumption for the CIS countries and South and Equatorial Africa: the latter consumed much more ethanol. Table 1 shows the daily consumption of macronutrients and calorie intake.

The contribution of proteins to the energy value of the diet in all regions (medians) was 15.3%, of fats – 37.5%, of carbohydrates – 47.1%, of alcohol – 0.5%. Similarly to the absolute indicators of macronutrient consumption, when comparing their shares in the total calorie content, it was not possible to identify significant differences (except for the consumption of the contribution of alcohol between students from Equatorial and South Africa and the CIS countries).

Table 2 presents the distribution of caloric intake by macronutrients among groups of students.

In the Russian Federation, the recommended dietary allowances for macro- and micronutrients, as well as for the energy value of the daily diet are presented in the **Norms of physiological needs (2009a)**. According to the document, for the adult population, protein intake should be 11 – 12% of the total calorie content, fats – 30 – 33%, carbohydrates – 58%. The examined students predominantly consumed more proteins and fats, abundantly contained in fast food, at the expense of a decrease in the proportion of carbohydrates.

Only about a quarter of the examined students consumed the recommended in Russia amounts of macronutrients (proteins – 65 – 117 g/day (m) 58 – 87 g/day (f); fats – 70 – 154 g/day (m) 60 – 102 g/day (f); carbohydrates – 257 – 586 g/day) **(Norms of physiological needs, 2009b)**. This once again emphasizes the degree of intra-group differences.

Similar studies have been conducted in other countries. So, **Matthys et al. (2003)** found that among adolescents in Belgium on average 35.7% of the calorie intake was in fats, 49% – in carbohydrates. In adolescents in Spain, the proportion of protein in the caloric content of the diet was 17.8%, fats – 34%, carbohydrates – 46.4% **(Lopez-Sobaler et al., 2019)**. According to other data, the calorie content of the diets of Spanish children consisted of 16.5% of proteins, 36.5% of fats, 45.4% of carbohydrates **(Madrigal et al., 2020)**. According to **Merkiel and Chalcarz (2016)**, children in Poland received 12% of their energy from protein, 33.4% from fats, and 54.1% from carbohydrates. In the United States, children's diets were 15% protein energy, 31% fat, and 53% carbohydrate **(Martinez et al., 2017)**. At the same time, the diets of North American adults included 80 grams of protein, 77 grams of fats, and 262 grams of carbohydrates daily **(Raatz et al., 2017)**. **Shan et al. (2019)** estimated the energy contribution of macronutrients in a similar population and found that the share of proteins was 16.4%, fats – 33.2%, carbohydrates – 50.5%. When assessing the nutrition of Canadians, the energy contribution of proteins was 16.5%, fats – 31%, carbohydrates – 50.1% **(Garriguet, 2007)**. In a study in South Africa, the contribution to the energy value of adult diets was 11 – 18% of proteins, 17 – 37% of fats, and 47 – 69% of carbohydrates **(Mchiza et al., 2015)**. Similar results were obtained in the UK: the share of proteins was 17%, fats – 36%, carbohydrates – 47% **(Scarborough et al., 2016)**, as well as in Denmark and Finland: the contribution of proteins was 18.4% and 16%, fats – 36.7% and 35%, carbohydrates – 45% and 44.5%, respectively **(Jakobsen et al., 2015; Ahola et al., 2017)**. Similar data were obtained in Australia: pregnant women consumed 19.1% of their energy from protein, 37.6% from fat, and 41.5% from carbohydrates **(Blumfield et al., 2015)**. In a study by **George et al. (2018)** the proportion of fats in the total calorie content of the diet was greater than the proportion of carbohydrates, which was not previously encountered.

In 2018, Chinese authors evaluated the energy value of the diets of people in the countryside and cities of China. In rural areas, the contribution to protein-calorie content was 13%, fats – 32%, carbohydrates – 55%. In large cities, the share of proteins, fats, and carbohydrates in the total calorie intake was already 16%, 37%, and 47%, respectively **(Yu, Lopez-Olmedo and Popkin, 2018)**. The results of this study, obtained in cities, are largely consonant with ours, while the nutrition of people from

villages in China was more consistent with the standards recommended in Russia. In another study by Chinese authors, the energy contribution of proteins to the diet of the elderly was 12.3%, fats – 30.8%, carbohydrates – 56% **(Xu et al., 2015)**, which is close to the data corresponding to eating behavior in the Chinese countryside. On the contrary, the nutrition of older people in Portugal was closer to the eating behavior of urban residents of China: 17.7% and 18.8% of calories consumed were provided by proteins in women and men, respectively, 34.6% and 34% – fats, 49.8% and 46.6% – carbohydrates **(Santos et al., 2015)**. **Bitok et al. (2017)** assessed the consumption of macronutrients in elderly people from different countries. The number of proteins was 65 grams, fats – 84 grams, carbohydrates – 204 grams, which is much less than our results. In Switzerland, by 2016, the consumption of proteins was 83.8 grams for men, 70 grams for women, fats – 83.2 and 70.4 grams, carbohydrates – 244 and 208 grams, respectively, which was also less than the values we obtained **(Marques-Vidal et al., 2015)**.

In the study of **R.B. Levy-Costa et al. (2005)**, it was observed that the nutrition of people in rural areas in Brazil was also more in line with the "Norms of physiological needs" than the results of our study: 12.8% of the calories were proteins, 27.6% were fats, and 59.6% were carbohydrates. An even smaller proportion of fats was in another study in South Africa. According to **Kolahdooz, Spearing, and Sharma (2013)**, the share of proteins in the energy value of the diet was 11 – 13%, fats – 18%, carbohydrates – 67 – 69%. A very low proportion of fat was also noted in a 2013 study in the United States. Approximately 18.2% of the total daily caloric content was ensured by protein intake, 11.4% from saturated fatty acids, and 67.7% from carbohydrates **(Huth et al., 2013)**. These results are similar to those obtained by **Kolahdooz, Spearing and Sharma (2013)**, with the difference that the protein/fat ratio in South Africa was shifted towards the latter.

### Limitations of the study

The efficiency of statistical data processing was decreased as a result of the fact that the quantitative composition of the groups varied greatly. For the same reason, as well as due to unequal gender composition, the groups were not divided by gender.

Another limitation of the study was the nutritional assessment method itself. Since the frequency analysis is based on the patient's subjective memories, the risk of accidental bias is relatively high. But still, in this case, the frequency method was preferable to the diary method, since nutrition was assessed over a rather long period and the transfer of the study from a cross-sectional format into perspective one would have been accompanied by the loss of a large number of subjects (if not all of them). Actually, in our particular case, the diary method was impossible at all, since the subjects' nutrition was assessed for the period before theyr arrival in Russia. It would be unacceptable to evaluate only the Russian group by the diary method within the framework of the methodological postulate of counteracting a systemic bias.

## CONCLUSION

Even though the medians and the values of the 25$^{th}$ and 75th percentiles in the groups were significantly different, the huge variance of the indicators within each group did not reveal significant differences in the absolute consumption or contribution of any of the macronutrients to the total energy value of the diet between the groups. The exception was the difference in alcohol consumption and its share in total calories between students from the CIS countries and South and Equatorial Africa: the latter consumed significantly more ethanol-containing drinks, despite the prevailing stereotypes.

At the same time, we found that the consumption and ratio of macronutrients among residents of big cities of different countries have much greater similarities than between people living in cities and rural areas even within the same state.

The urban environment is likely to be crucial in shaping the diet of first-year students. Also, the results made us conclude that the nature of students' nutrition depends primarily on individual preferences, regardless of which camp the student came to Russia.

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# THE POSSIBILITY OF IMPROVING MEAT QUALITY BY USING PEAS AND FABA BEANS IN FEED FOR BROILER CHICKENS

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## ABSTRACT

The price-to-quality ratio of broiler chicken meat is one of the reasons why consumers use these products in their daily diet. The quality of poultry products such as meat is closely associated with the nutritional value and quality of feed fed to broilers. In composing broiler diets, the main focus is turned on crude protein (CP). Because of the trend for the poultry industry to reduce its dependence on soybean meal as a source of protein, the role of pulses in the supply of protein and energy, which could partly replace the soybean meal in poultry diets, increases. Due to considerable progress in plant breeding, the present research study used peas (*Pisum sativum*) of the var. 'Bruno' and var. 'Pinochio' as well as faba beans (*Vicia faba minora*) of the var. 'Lielplatone' was developed in Latvia. Higher protein diets induce a higher meat protein content, accordingly, feed proteins are hydrolyzed in the digestive system into amino acids (AA) that, after absorption, are used to form proteins and to build different body tissues. The most important AA was twofold lower in peas and faba beans than in soybean meal. Although the amount of AA in feed is not directly proportional to the content of AA in broiler chicken meat, the amount of AA in poultry feed can significantly affect an important meat quality criterion for consumers – the nutritional value of protein. Therefore, the research aimed to identify changes in AA composition and chemical composition – a qualitative property of poultry meat – as a result of adding pulses (peas and beans) to poultry diets. The research results showed that the diets with an addition of faba beans and peas had a positive effect on the level of essential amino acids (EAA) and non-essential amino acids (NEAA) in broiler meat, decreased cholesterol concentration in meat, and increased energy value of meat.

**Keywords:** faba beans; peas; broiler nutrition; meat quality

## INTRODUCTION

In recent decades, poultry consumption tended to increase in most of the world, which could relate to the growing consumer interest and belief that white meat is better and healthier than red meat. According to research studies on consumer buying habits **(Kaygisiz, Bolat and Bulut, 2019; Xazela et al., 2017)**, the price and quality of broiler chicken meat are one of the reasons why consumers use these products in their daily diet. From a nutritional aspect, poultry meat is a valuable source of proteins, vitamins, and minerals, and has a relatively low-fat content. The chemical composition of muscle tissue is an important element of broiler meat quality **(Grashorn, 2005)**.

The set of properties that defines consumer perception of meat quality has expanded to include extrinsic factors, such as animal welfare, the sustainability of production and meat concerning human health, as well as a combination of muscle, fat, and moisture, the nutritional value of meat compositional analysis such as AA **(Purslow, 2017)**. Quality is defined as the usefulness of a good or service for consumption **(Juran and Godfrey, 1998)**; therefore, one can assert that the quality of food plays a great role in food purchases. However, the importance of consumer subjective views regarding product choices should be emphasized as well. The organoleptic characteristics of a food (e.g. color, taste, consistency) is what each consumer perceives individually; therefore, they could be regarded as subjective assessment criteria that depend on the individual perception of each consumer **(Mulvey, 2008)**. Consumers' expanded perception of food quality encompasses more than just organoleptic or other food characteristics. For consumers, the quality of food is the desired characteristics of the food that could justify the value-added of the food: e.g. the type of food production (organic or conventional), the environmental impact of food production, feed components used in production (protein sources, phytonutrients), functional properties of food (a low cholesterol level and fat content, a balanced amount of AA), etc. **(Yamada et al., 2008)**.

Food quality is strongly linked to the functional properties of the food and the health of individuals. From the standpoint of consumer interests, broilers should have not only high slaughter yields and desirable carcass

conformation scores but also good aesthetic, sensory, and nutritional characteristics. This means that food should also contain biologically active principles making beneficial effects on the functions of the human body, contribute to the maintenance of health and reduce the risk factors for various diseases **(Maggini, Pierre and Calder, 2018)**. Under the presence of functional ingredients in various types of food, consumers are allowed to fully balance their diet and have vitamins, minerals, proteins, etc. **(Holban and Grumezescu, 2018)**. **Röhr et al. (2005)** point out that consumers are ready to pay a higher price if it is guaranteed that the producer supplies are safe and quality products. A similar finding was made by **Nagyová et al. (2019)**, indicating that 84% of respondents bought high-quality food and 60% bought it for health reasons.

The nutritional value of protein is determined by essential AA, while the composition, amount, and ratio of desired AA in products vary; therefore, more attention should be paid to the biological value of protein in food, as the spectrum and amount of AA are important for the health of the human body. **Kim, Do and Chung (2017)** point out that broiler meat is an excellent source of protein not only because of the quantity of the protein (20%) but also because of the quality of it – more than 40% of AA are essential ones.

It should be stressed that the quality of poultry products such as meat is closely associated with the nutritional value and quality of feed fed to broilers. Nutrition can have a crucial effect on the chemical composition of broiler meat. Broilers have high dietary protein requirements, due to the importance of protein as a major constituent of the biologically active compounds in the body, assistance of proteins in the synthesis of body tissues, and growth of the body. Furthermore, proteins are forming enzymes and hormones, which play important roles in the physiology of any living organism **(Beski, Swick and Iji, 2015)**. The enrichment of poultry meat with substances beneficial to human health has been studied for several decades **(Grashorn, 2005; Swaggerty et al., 2020; Selle et al., 2020)**. Each of the nutrients added to poultry feed causes a specific effect: they are biological and chemical substances that, when added to feed rations for broilers, are transformed by metabolic processes in the birds body and contribute to the digestion of feed, the growth, and development of the organism while at the same time producing higher quality products and achieving higher production efficiency. Positive effects are achieved both by improving food conversion and increasing the vitality of birds, stimulating their immune system, regulating the microflora of their digestive system, etc., as a result of which the quality of meat is enhanced.

In composing broiler diets, the main focus is turned on crude protein (CP) because the protein is the critical constituent of poultry diets together with the other nutrients **(Cheeke, 2005)**. One of the factors that can have a highly variable effect on the chemical composition and quality of poultry meat includes different protein and energy values of formulated feed rations. Higher protein diets induce a higher meat protein content, while reducing the fat content of the muscles. Feed proteins are hydrolyzed in the digestive system into AA that, after absorption, are used to form proteins and to build different body tissues **(Beski, Swick and Iji, 2015)**.

The level of protein in broiler feed on poultry farms in Latvia is mainly provided by imported feed materials containing CP – soybean meal (SBM) and sunflower products, cornflour, etc. –, which are mostly imported feed protein sources. **Regulation (EC) No 178/2002** lays down rules on the production, labeling, traceability, and control of feed that have been developed at the EU level, which are incorporated into the Member States' legislation. However, imported protein crops produced outside the EU are not subject to the same environmental, health, and GMO regulatory restrictions as EU-produced products, thereby affecting the quality of livestock products as well. In the world market, however, the prices of protein-rich feedstuffs continue rising **(Indexmundi, 2017)**, thereby significantly affecting the total price of feed and, consequently, the cost of livestock products. There is a trend for the poultry industry to reduce its dependence on soybean meal as a source of protein and replace it with sustainable alternatives. Consequently, interest in using grain legumes such as faba beans (*Vicia faba*) and peas (*Pisum sativum*) as alternatives to conventional protein sources has been increasing **(Nalle, Ravindran and Ravindran, 2010; Stagnari et al., 2017)**. Beans and peas play an increasingly important role in animal feed owing to their high-quality protein. Pulses can provide a diet rich in protein and AA. Research studies done in Latvia have revealed that the total amount of AA in domestically grown peas varies from 202.4 $g.kg^{-1}$ to 248.9 $g.kg^{-1}$, while in beans from 235.7 $g.kg^{-1}$ to 267.9 $g.kg^{-1}$; the amount of essential AA in peas was in the range of 67.6 – 74.2 $g.kg^{-1}$, while in beans in the range of 79.3 – 86.9 $g.kg^{-1}$ **(EUROLEGUME, 2018)**.

The amount of AA in feed is not directly proportional to the content of AA in broiler chicken meat because of a certain AA conversion rate from feed to meat in the bird organism. Nevertheless, the amount/level of AA in poultry feed can significantly affect a meat quality criterion – the nutritional value of protein – and can enhance the level of AA **(Temesgen, Retta and Tesfaye, 2018)**. Therefore, the research aimed to identify changes in AA composition – a qualitative property of poultry meat – as a result of adding pulses (peas and beans) to poultry diets.

### Scientific hypothesis

The quality properties (amino acids and chemical composition) of meat that are important for consumers could be enhanced by including pulses (peas and faba beans) in broiler diets.

## MATERIAL AND METHODOLOGY

### Management and birds

A feeding experiment was done on cross Ross 308 broiler chickens (n = 240). The stocking density complied with the rules of **Council Directive 2007/43/EC**; the max live weight density was 33 kg per $m^2$ or 12 chicks per $m^2$. The broilers were placed on the floor and sawdust was used for their bedding; the broilers were provided free access to feed and water. In the first week, the temperature in the poultry building was set at 31 °C; from the second week, the temperature was gradually reduced to 28 °C, and from the fourth week onwards, the temperature was set at 22 °C. The fattening period was 42 days and divided into three stages: from 1 – 10 days (starter), 11 – 26 days (grower) and

27 – 42 days (finisher), following the standards for the cross Ross 308 **(Aviagen, 2018)**. Additions of faba beans and peas were included in the feed of experimental groups only at the grower and finisher stages. On day 10, the broiler chickens (n = 240) having a similar body weight (BW) 276.00 ±23.2 g standard deviation (SD) were divided into 8 groups, and the experiment had two replications per treatment for each group (n = 30).

## Dietary treatments

During the starter period, all the groups of broiler chickens were fed the same diet: wheat – 635.80 $g.kg^{-1}$; soybean meal (SBM) – 255.60 $g.kg^{-1}$; sunflower – 6.00 $g.kg^{-1}$; rapeseed cake – 13.00 $g.kg^{-1}$; technological, nutritional, physiological and zootechnical additives 89.60 $g.kg^{-1}$. The starter feed contained 24.93% of dry matter (DM) CP, 3.27% of DM crude fat, 2.16% of DM crude fiber, 5.70% of DM crude ash, 0.88% of DM calcium (Ca), 0.68% of DM phosphorus (P), 2980 $kcal.kg^{-1}$ metabolizable energy (ME), – 1.00% of DM lysine (Lys), 0.40% of DM methionine (Met) and 0.31% of DM cysteine (Cys).

From the 11$^{th}$ day of the experiment, the diets were as follows: for the control group (C) – the basal diet (control, without peas and faba beans); for the seven experimental groups (group PB200 to group PB100 + FBL150) – the basal diet with SBM was partially replaced with different amounts of peas and faba beans. The peas 'Bruno' (PB) developed by the Institute of Agricultural Resources and Economics as well as the peas 'Pinochio' (PP) and the faba beans 'Lielplatone' (FBL) grown in Latvia were tested in the feeding experiment (Table 1). Crude protein contents in the pulses (dry matter) were as follows: PB – 300.00 ±23.62 $g.kg^{-1}$, PP – 284.26 ±61.54 $g.kg^{-1}$, FBL – 330.71 ±41.11 $g.kg^{-1}$, while in SBM it was 587.95 $g.kg^{-1}$. The pea and bean varieties studied contained equivalent amounts of crude fat, crude fiber, crude ash, calcium, and phosphorus. As is known from the results of the previous researches, the amount of pea and faba beans in broiler diets is limited due to the tannins they contain, which are anti-nutritive compounds and affect the nutritional flavor as well as the digestive system of the birds, thus limiting the number of peas and beans the amount of feed **(Crépon, 2006)**. This indicates that peas and beans cannot completely replace SBM in poultry feed not only because of antinutritional factors but also of their lower crude protein contents.

The feed mixtures for broilers were granulated appropriate for the grower and finisher stage 2.5 – 4.0 mm diameter, 4.0 – 8.0 mm in length, reaching a temperature of up to 84 °C during the granulation process. The granulation process did not significantly affect the chemical composition of peas and beans, even though the proportion of soluble protein increased by 0.61% and that of undegradable intake protein decreased by 5.97% in the total crude protein content of peas during the granulation process, compared with the non-granulated peas. Such a change in crude protein components may contribute to the digestibility and absorption of crude protein of peas in the bird organism.

The AA contents of peas, faba beans and SBM are reported in Table 2. The Lys content in peas was in the range of 1.40 – 1.50%, 1.71% in faba beans, and 2.80% in SBM, while the amount of Met in SBM was threefold higher than that in the peas and beans (Table 2). The amounts of other essential AA in peas and beans were also about twofold lower than that in SBM. This means that the amounts of AA most important for metabolism in the broiler organism were significantly lower in peas and faba beans than in SBM.

The EAA content of PB and PP was in the range of 8.70 – 9.38%, and 11.99% in FBL. SBM had an EAA content of 20.52%. This means that SBM had a higher EAA content than peas and faba beans by 11.14 – 11.82% and 8.53%, respectively. The NEAA content in pea protein was in the range of 8.16 – 9.62%, in faba bean protein – 11.57%, but 21.62% in SBM protein. This means that the NEAA content of SBM protein was 11.98 – 13.44% higher than that of pea protein and 10.03% higher than that of faba bean protein (Table 2).

The grower stage feed contained 23.63 – 25.64% of DM CP, while the finisher stage feed had a CP content of 22.01 – 24.48% of DM. At the grower stage, the 8$^{th}$ group was fed a lower CP content diet (0.8% lower than that for the C group) that contained a mixture of 150 $g.kg^{-1}$ FBL and 100 $g.kg^{-1}$ PB. However, at the finisher stage, such a mixture of 150 $g.kg^{-1}$ FBL and 100 $g.kg^{-1}$ PB in the diet had a lower CP content (by 2.47%), compared with the diet for the C group; however, the CP content in the diets for the experimental groups, compared with that for the C group, was not significantly different ($p$ >0.05) at both feeding stages (Table 3). Using SBM, PB, PP, and FBL, for the experimental groups, a ME of 750 – 1196 $kcal.kg^{-1}$ was provided at the grower stage and 664 – 1158 $kcal.kg^{-1}$ at the finisher stage, exceeding the ME provided for the C group by 94 – 494 $kcal.kg^{-1}$, which might be explained by a 4 – 5 times higher ST content of peas and faba bean, compared with that of SBM. Such a difference in the CP content could not negatively affect the live weight of broilers at the selling age, compared with the C group. The contents of crude fat, crude ash, fiber, Ca and P in the broiler diets were equal for all the groups.

## Chemical analysis

A test of the feed, peas, and faba beans were performed to calculate a precise feed ratio for the relevant period. During the experiment, feed tests for all the groups were performed on the 1$^{st}$ day, 10$^{th}$ day, and 27$^{th}$ day. At the final stage of the experiment (on day 42 of rearing), 10 birds from each group were slaughtered and dissected.

The biochemical analyses of feed and broiler breast samples were carried out at the Latvia University of Life Sciences and Technologies (LLU) Research Laboratory of Biotechnology **(LATAK, 1999)**. 100 g of each experimental diet was collected and sent for analysis.

Dry matter, crude protein, and ash were analyzed in the diets according to the method AOAC **(Herlich, 1990)**. The samples of meat were analyzed for DM **(ISO 6496, 1999)**, CP **(ISO 5983-2, 2009)**, crude fat **(ISO 6492, 1999)**, crude ash **(ISO 5984, 2002/COR 1:2005)**, P **(ISO 6491, 1998)**, yet the cholesterol level was determined using the method described by **Sperry and Webb (1950)**. AA contents in feed and meat were determined by FTIR spectroscopy.

A chemical score ($C_S$) for broiler breast meat was calculated for each AA using the following formula:

$$C_s = A_x/A_s \quad (1)$$

where $A_x$ is the content of an AA in the investigated protein (%) and as is the content of an AA in the standard protein (the same whole egg protein) **(Alfaig et al., 2014)**.

An AA score was determined by comparing the essential amino acid (EAA) contents of the samples to the AA contents suggested for humans **(Straková et al., 2009)**. The AA content recommended for children aged 2 – 5 years is used to calculate an AA score of the samples (Table 7) **(WHO, 1985; Straková et al., 2009)**. The essential AA index (EAAI) is a geometrical average of CS values **(Písaříková, Kráčmar and Herzig, 2005; Straková et al., 2009)**.

A biological value ($B_y$) for the experimental food materials was calculated from the EAAI using Oser's method **(Oser, 1959)** as follows:

$$B_y = 1.09 \times (EAAI) - 11.7 \quad (2)$$

## Statistical analysis

Statistical analyses were performed with SPSS for Windows version 20.0 statistical software package. The dietary energy and other variables were compared statistically by ANOVA. Statistical significance was calculated using a t-test. Differences between the control and experimental groups were considered significant at $p \leq 0.05$. Data were reported as mean ± SD.

## RESULTS AND DISCUSSION

Any broiler diet has to have such EAA as Lys and Met. The Met content of peas was in the range of 0.20 – 0.22%, while that of faba beans was 0.21%; the Met content of SBM was higher – 0.61%. However, no significant difference in Met content between the feed mixtures for the experimental and control groups was identified (Table 4).

The AA content (g.kg$^{-1}$ DM) in the experimental diets varied. The Cys content was significantly higher in diets PB200, PP200, but significantly lower in diets PB300 and PB100 + FBL100 at the grower stage. The Cys contents were similar for all the experimental groups at the finisher stage. The Lys content was significantly higher in diets PP300 and PB100 + FBL150 at both feeding stages. The analysis of AA composition (% of DM) in the C and experimental diets is presented in Table 4.

The highest values in the EAA group among the finisher feeds were recorded for Arg (0.96% of DM) and Leu (1.01% of DM), but the lowest value – Cys (0.3% of DM). In the non-essential AA group, Gln was found to be the most abundant (3.26% of DM), but Tyr had the lowest value (0.43% of DM). They were followed by His in diets PP200 (0.34% of DM) and PB300 (0.32% of DM), with the lowest values recorded for diets FBL100 and PB100 + FBL100 (0.29% of DM) (Table 4).

In total, the significantly higher average EAA levels (6.04, 6.44 and 6.28% of DM) and TAA amounts (12.8, 13.15 and 13.02% of DM) were recorded in experimental finisher stage diets PP200, PB300 and PB100 + FBL150, respectively, but the lowest – in the basal diet (Table 4).

## Biochemical composition of broiler meat

The effect of various protein diets on meat quality parameters is shown in Table 5. The CP content was significantly ($p < 0.05$) higher in breast meat of broilers fed diets FBL100 and PB100 + FBL100 (21.34 and 21.4% respectively), compared with the C group.

Increased fat content was found in broilers from the C group (1.85% of DM) and diet PB300 (1.84% of DM), but a significant decrease was observed in the broilers from groups PP300 and PB100 + FBL100. No significant difference in the crude ash content was recorded among the treatments ($p > 0.05$).

There was a significant reduction ($p < 0.05$) in the cholesterol concentration of broiler breast meat from the broilers fed diet PP200 supplemented with 200 g.kg$^{-1}$ PP (110.5 mg.100g$^{-1}$) and FBL100 – with 100 g.kg$^{-1}$ FBL (110.6 mg.100g$^{-1}$). In general, cholesterol concentration in meat decreased in all the experimental groups (except PB300) in comparison with the C group (Table 5).

**Table 1** Feed ingredient composition of the basal and experimental diets.

| Items | C | PB200 | PP200 | PB300 | PP300 | FBL100 | PB100 + FBL100 | PB100 + FBL150 |
|---|---|---|---|---|---|---|---|---|
| **Grower stage (11th – 26th day)** | | | | | | | | |
| SBM, g | 227 | 122 | 124 | 70 | 73 | 164 | 112 | 80 |
| PB, g | - | 200 | - | 300 | - | - | 100 | 100 |
| PP, g | - | - | 200 | - | 300 | - | - | - |
| FBL, g | - | - | - | - | - | 100 | 100 | 150 |
| Sunflower, g | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 |
| Rapeseed cake, g | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 |
| Wheat, g | 649 | 649 | 649 | 649 | 649 | 649 | 649 | 649 |
| **Finisher stage (27th – 42nd day)** | | | | | | | | |
| SBM, g | 201 | 106 | 108 | 58 | 61 | 144 | 96 | 67 |
| PB, g | - | 200 | - | 300 | - | - | 100 | 100 |
| PP, g | - | - | 200 | - | 300 | - | - | - |
| FBL, g | - | - | - | - | - | 100 | 100 | 150 |
| Sunflower, g | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Rapeseed cake, g | 200 | 200 | 200 | 200 | 200 | 200 | 200 | 200 |
| Wheat, g | 698 | 698 | 698 | 698 | 698 | 698 | 698 | 698 |

Note: C – Control group; from PB 200 to PB100 + FBL 150 the experimental broiler groups; SBM – soybean meal, PB – peas 'Bruno', PP – peas 'Pinochio', FBL – faba beans 'Lielplatone`.

**Table 2** Amino acid contents of peas, faba beans and soybean meal (% DM).

| Items | PB | PP | FBL | SBM |
|---|---|---|---|---|
| Met | 0.22 ±0.01 | 0.20 ±0.01 | 0.21 ±0.23 | 0.61 ±0.20 |
| Cys | 0.30 ±0.03 | 0.28 ±0.04 | 0.33 ±0.22 | 0.68 ±0.19 |
| Lys | 1.50 ±0.16 | 1.40 ±0.22 | 1.71 ±0.74 | 2.80 ±0.64 |
| Thr | 0.77 ±0.12 | 0.76 ±0.15 | 0.97 ±0.56 | 1.82 ±0.50 |
| Arg | 1.78 ±0.48 | 1.66 ±0.62 | 2.54 ±0.84 | 3.34 ±0.78 |
| Ile | 0.85 ±0.18 | 0.80 ±0.24 | 1.14 ±0.67 | 2.10 ±0.14 |
| Leu | 1.47 ±0.39 | 1.35 ±0.49 | 2.05 ±1.07 | 3.45 ±0.86 |
| Val | 0.95 ±0.17 | 0.90 ±0.22 | 1.21 ±0.68 | 2.21 ±0.61 |
| His | 0.50 ±0.15 | 0.40 ±0.21 | 0.69 ±0.41 | 1.20 ±0.11 |
| Phe | 1.04 ±0.10 | 0.95 ±0.13 | 1.14 ±0.74 | 2.32 ±0.14 |
| Total EAA | 9.38 ±1.74 | 8.70 ±2.33 | 11.99 ±0.61 | 20.52 ±2.54 |
| Tyr | 0.64 ±0.14 | 0.56 ±0.20 | 0.84 ±0.55 | 1.63 ±0.49 |
| Gly | 0.90 ±0.18 | 0.83 ±0.24 | 1.17 ±0.09 | 2.00 ±0.54 |
| Ser | 1.01 ±0.13 | 0.97 ±0.18 | 1.22 ±0.07 | 2.37 ±0.66 |
| Ala | 0.92 ±0.12 | 0.88 ±0.16 | 1.10 ±0.6 | 2.06 ±0.45 |
| Asp | 2.40 ±0.25 | 2.36 ±0.33 | 2.82 ±0.21 | 5.27 ±1.39 |
| Glu | 3.75 ±0.94 | 2.56 ±1.32 | 4.42 ±0.92 | 8.29 ±0.41 |
| Total NEAA | 9.62 ±1.71 | 8.16 ±2.41 | 11.57 ±1.03 | 21.62 ±1.08 |

Note: SBM – soybean meal, PB – peas 'Bruno', PP – peas 'Pinochio', FBL – faba beans 'Lielplatone', % DM – percentage of dry matter. Data are presented as means ±SD – standard deviation.

**Table 3** Composition of feeds used in the broiler feeding experiment at the grower and finisher stages.

| Items | C (control) | PB200 | PP200 | PB300 | PP300 | FBL100 | PB100 + FBL100 | PB100 + FBL150 |
|---|---|---|---|---|---|---|---|---|
| **Grower stage** | | | | | | | | |
| CP, % DM | 24.43 ±0.33 | 25.64 ±0.28 | 24.87 ±0.31 | 24.59 ±0.33 | 25.09 ±0.59 | 25.37 ±0.71 | 24.05 ±0.25 | 23.63 ±0.48 |
| DM, % | 87.93 ±0.28 | 87.94 ±0.42 | 87.84 ±0.31 | 87.79 ±0.25 | 87.41 ±0.27 | 88.27 ±0.28 | 88.05 ±0.42 | 87.48 ±0.25 |
| CFa, % DM | 3.62 ±0.30 | 3.20 ±0.26 | 3.22 ±0.24 | 2.83 ±0.29 | 3.41 ±0.32 | 3.43 ±0.20 | 3.16 ±0.38 | 3.80 ±0.20 |
| CAsh, % DM | 6.01 ±0.33 | 5.89 ±0.21 | 5.45 ±0.33 | 5.02 ±0.41 | 5.34 ±0.33 | 5.70 ±0.28 | 5.72 ±0.42 | 5.86 ±0.34 |
| CFb, % DM | 3.19 ±0.06 | 4.37 ±0.59 | 3.34 ±0.06 | 4.04 ±0.05 | 3.47 ±0.11 | 4.89 ±0.06 | 4.10 ±0.27 | 3.95 ±0.13 |
| Ca, % DM | 1.04 ±0.01 | 0.99 ±0.06 | 0.88 ±0.01 | 0.75 ±0.01 | 0.79 ±0.08 | 0.92 ±0.03 | 0.89 ±0.01 | 0.92 ±0.01 |
| P, % DM | 0.68 ±0.02 | 0.68 ±0.02 | 0.68 ±0.03 | 0.62 ±0.03 | 0.66 ±0.03 | 0.70 ±0.02 | 0.69 ±0.03 | 0.67 ±0.01 |
| ME, kcal.kg$^{-1}$* | 750 ±78 | 1048 ±61$^{S}$ | 1045 ±24$^{S}$ | 1196 ±48$^{S}$ | 1193 ±86$^{S}$ | 844 ±14 | 992 ±32 | 1040 ±53$^{S}$ |
| **Finisher stage** | | | | | | | | |
| CP, % DM | 22.01 ±0.22 | 22.15 ±0.45 | 22.11 ±0.51 | 22.91 ±0.07 | 23.02 ±0.16 | 22.46 ±0.37 | 23.29 ±0.17 | 24.48 ±0.56 |
| DM, % | 88.14 ±0.15 | 87.82 ±0.14 | 87.87 ±0.37 | 87.78 ±0.56 | 88.07 ±0.14 | 88.17 ±0.46 | 87.91 ±0.16 | 87.87 ±0.15 |
| CFa, % DM | 4.91 ±0.45 | 5.03 ±0.14 | 4.71 ±0.45 | 3.89 ±0.48 | 4.21 ±0.56 | 4.25 ±0.40 | 3.93 ±0.51 | 4.08 ±0.36 |
| CAsh, % DM | 6.40 ±1.08 | 5.61 ±1.50 | 5.62 ±1.37 | 5.29 ±0.41 | 5.58 ±1.25 | 8.58 ±0.89 | 5.76 ±0.43 | 5.45 ±0.40 |
| CFb, % DM | 4.23 ±0.07 | 4.48 ±0.16 | 4.63 ±0.34 | 4.09 ±0.22 | 4.46 ±0.31 | 5.02 ±0.05 | 5.73 ±0.11 | 5.87 ±0.17 |
| Ca, % DM | 1.08 ±0.01 | 0.86 ±0.03 | 0.85 ±0.01 | 0.74 ±0.02 | 0.86 ±0.04 | 1.09 ±0.02 | 0.93 ±0.01 | 0.81 ±0.04 |
| P, % DM | 0.74 ±0.042 | 0.62 ±0.015 | 0.66 ±0.036 | 0.60 ±0.05 | 0.68 ±0.029 | 0.68 ±0.030 | 0.65 ±0.055 | 0.66 ±0.050 |
| ME, kcal.kg$^{-1}$* | 664 ±16 | 993 ±21$^{S}$ | 990 ±29$^{S}$ | 1158 ±19$^{S}$ | 1153 ±33$^{S}$ | 777 ±22 | 941 ±18$^{S}$ | 998 ±31$^{S}$ |

Note: % DM – percentage of dry matter, CP – crude protein, CFa – crude fat, CFb – crude fiber, CAsh – crude ash, Ca – calcium, P – phosphorus, ME* - metabolizable energy, intake from SBM, beans and peas. Data are presented as means ± SD – standard deviation. Means with superscript letter $^{S}$ are significantly different at $p$ <0.05 in comparison with the control group. The numbers of each columns that do not have letter have a meaningful difference ($p$ >0.05).

The meat energy value contents were higher in the breast of broilers fed PB100 + FBL100 diets supplemented with 100 g.kg$^{-1}$ FBL with 100 g.kg$^{-1}$ PB (107.84 kcal.100g$^{-1}$), but the lowest – in the C group – from the dietary treatment with SBM (95.26 kcal.100g$^{-1}$). No significant difference ($p$ >0.05) in meat energy value was recorded among the treatments.

**Amino acid contents in breast muscle**

The effects of dietary treatments on the AA contents of broiler breast meat from the eight experimental groups are shown in Table 6.

The results indicated that in most cases, the average level of total AA (TAA) was increased in meat from the broilers fed diets PB100 + FBL100 and PB100 + FBL150. The Met content was significantly higher in muscle from diet PB100

+ FBL150, while Cys – in broiler meat from groups PB200, PB100 + FBL100, and PB100 + FBL150. Compared with the C group diet, diets PB300 and FBL100 significantly decreased ($p$ <0.05) the TAA, whereas diet PB100 + FBL150 increased ($p$ <0.05) the TAA content.

The highest values in the EAA group were recorded for Arg (3.14 g.kg$^{-1}$ DM) and Leu (3.81 g.kg$^{-1}$ DM), but the lowest value – Cys (0.58 g.kg$^{-1}$ DM). In the NEAA group, Gln was found to be the most abundant (7.54 g.kg$^{-1}$ DM), but Tyr had the lowest value – (1.4 g.kg$^{-1}$ DM). In total, the highest average EAA level (23.15 g.kg$^{-1}$ DM) was recorded in experimental diet PB100 + FBL150 (with 150 g.kg$^{-1}$ FBL with 100 g.kg$^{-1}$ PB), but the lowest (20.13 g.kg$^{-1}$ DM) – in diet PB200. On average, diets PP300, PB100 + FBL100 and PB100 + FBL150 increased the total EAA content in breast muscle, but the TAA level was increased by only diets PB100 + FBL100 and PB100 + FBL150 (41.94 and 42.59 g.kg$^{-1}$ DM, respectively).

## The chemical score, amino acid score, EAA index, and biological value

The calculated indicators of breast meat samples are set out in Table 7. The chemical score, the AA score, the EAA index and the biological value of all the tested AA were significantly different between the control and the treatment groups.

A comparison of the protein-supplemented diet groups revealed that the chemical and AA scores were increased in the meat of groups PB100 + FBL100 and PB100 + FBL150 (supplemented with faba beans and peas), but were decreased in the breast meat of broilers fed diets PP200 and PB300 (supplemented with 200 g.kg$^{-1}$ PP and 300 g.kg$^{-1}$ PB, respectively).

Regarding the EAAI and biological value, there were significant increases between the control (C) group and groups PB100 + FBL100 and PB100 + FBL150, respectively.

The results of the use of faba beans in the poultry diet have been contradictory **(Crépon, 2006)**. Faba beans have a quite high level of protein (about 26%), but the presence of anti-nutritional constituents, such as vicine and convicine, and the low level of Sulphur AA content reduce their nutritional value in poultry meat.

Some studies showed adverse effects on poultry performance or growth performance similar to control diets at various inclusion levels **(Dal Bosco et al., 2013)**. Some results indicated that replacing SBM with alternative protein sources had a positive effect on the dietetic value of poultry meat, whereas other data indicate something opposite **(Milczarek, Osek and Pachnik, 2016)**. Parameters used to determine protein quality included chemical score, AA score, essential AA index (EAAI), and biological value **(Aronal, Huda and Ahmad, 2012)**. Proteins with a high content of essential AA are the most important components of poultry meat **(Straková et al., 2002)**.

As is known, birds, especially broiler chicks, have an intense growth rate and high genetic potential for productivity. Therefore, broilers need to feed with a high (20 – 25%) crude protein level. Before the start of the feeding test, the chemical composition of peas, beans, and imported soybeans are grown in Latvia was determined. Regarding chemical composition of a pea, faba bean and soybean used in the trial, our results are in agreement with published data of other authors **(Koivunen et al., 2016)** who reported on significantly lower content of crude protein, crude fat, and ash in faba beans and peas in comparison with soybean meal. However, our results showed a significantly higher content of starch and crude fiber in peas and faba beans. This agrees with the results of **Koivunen et al. (2016)**. Unfortunately, pea starch is less susceptible to enzyme hydrolysis than starch in any of the cereal grains **(Laudadio and Tufarelli, 2010)**. In contrast to the current study, in the study of **Laudadio and Tufarelli (2010),** the content of crude fiber in soybean was higher as in peas. Faba beans are protein and energy-rich legume seeds well adapted to grow in various climatic zones of Europe and widely used for feed and food **(Grosjean et al., 2000)**.

AA composition is key to the evaluation of a dietary protein source. In the present research study, feeding pulses led to a significantly higher level of EAA, NEAA, and TAA in the diets. Among the analyzed finisher stage samples, basal feed with an addition of 200 g.kg$^{-1}$ PP or 300 g.kg$^{-1}$ PB or 150 g.kg$^{-1}$ FBL with 100 g.kg$^{-1}$ PB had a higher content in almost all the groups of AA; however, Sulphur AA (Met and Cys) were the exception in most cases.

AA, termed functional AA, includes Ala, Arg, Asp, Gln, Gly, Pro, and Ser and are important not only for intestinal integrity, nutrient transport, and metabolism but also in immune response **(Dai, Wu and Zhu, 2011; Ren et al., 2013)**. Some authors emphasize the importance of EAA (Lys and Met) in broiler feed. The productivity of broilers, to a great extent, is determined by the EAA content of feedstuffs. This AA is not synthesized in the broiler organism; therefore, the acids must be consumed with feed in a certain quantity and at a certain ratio **(Beski, Swick and Iji, 2015)**. Lys and Met are among the most limiting AA, which is important for the growth of the organism and in the balance of nitrogen.

Met is classified as the first insufficient AA in the organism, as it is available in a limited amount in plant proteins. Met is required to provide the building blocks for immune cells and tissues, therefore, it is an EAA for animals, poultry, including broiler, in particular. Met is required for the growth of feathers and the synthesis of proteins **(Bunchasak, 2009)**.

The second most important EAA needed for the growth of broilers is Lys, which contributes to the growth of chest muscle tissues as well as the growth pace of broilers **(Kidd et al., 2004)**. Deficiency in Lys causes blood circulation problems, a lower level of hemoglobin as well as leads to smaller muscles and bone deterioration **(Burnham, 2005)** and, consequently, the death rate of broilers increases and an increase in the weight of their muscles is lower, which can cause considerable economic losses.

The research showed that soybean meal had a higher EAA content than peas and faba beans by 11.14 – 11.82% and 8.53%, respectively (Table 3). It has to be taken into consideration that the SBM had such anti-nutritive factors as protease inhibitors – trypsin, chymotrypsin, pectin, goitrogens, and other substances that could affect the digestibility of protein present in the feed, metabolic nitrogen retention, and excretion **(Yasothai, 2016)**.

**Table 4** Amino acid contents (% of DM) in broiler feeds.

| Items | C | PB200 | PP200 | PB300 | PP300 | FBL100 | PB100 + FBL100 | PB100+ FBL150 |
|---|---|---|---|---|---|---|---|---|
| **Grower stage** | | | | | | | | |
| Met | 0.49 ±0.018 | 0.5 ±0.019 | 0.48 ±0.017 | 0.45 ±0.018 | 0.47 ±0.019 | 0.48 ±0.023 | 0.49 ±0.028 | 0.45 ±0.0.017 |
| Cys | 0.33 ±0.015 | 0.35 ±0.016$^{S}$ | 0.35 ±0.015$^{S}$ | 0.31 ±0.011$^{S}$ | 0.33 ±0.010 | 0.33 ±0.021 | 0.31 ±0.014$^{S}$ | 0.33 ±0.018 |
| Lys | 0.97 ±0.059 | 1.00 ±0.061 | 1.10 ±0.067 | 0.99 ±0.057 | 1.06 ±0.066$^{S}$ | 0.92 ±0.012 | 0.96 ±0.057 | 1.04 ±0.066$^{S}$ |
| Thr | 0.67 ±0.031 | 0.72 ±0.029 | 0.73 ±0.032 | 0.69 ±0.034 | 0.68 ±0.038 | 0.74 ±0.061 | 0.74 ±0.049 | 0.67 ±0.026 |
| Arg | 0.98 ±0.073 | 1.13 ±0.055$^{S}$ | 1.12 ±0.060 | 1.09 ±0.067 | 1.19 ±0.043 | 1.04 ±0.040 | 1.13 ±0.041$^{S}$ | 1.20 ±0.050$^{S}$ |
| Ile | 0.60 ±0.037 | 0.69 ±0.024 | 0.69 ±0.026 | 0.66 ±0.029 | 0.72 ±0.030$^{S}$ | 0.65 ±0.081 | 0.70 ±0.014 | 0.68 ±0.026 |
| Leu | 1.02 ±0.051 | 1.14 ±0.035 | 1.14 ±0.038 | 1.08 ±0.042 | 1.16 ±0.039 | 1.10 ±0.025 | 1.18 ±0.049$^{S}$ | 1.11 ±0.037 |
| Val | 0.74 ±0.034 | 0.81 ±0.023 | 0.81 ±0.025 | 0.79 ±0.028 | 0.84 ±0.026 | 0.79 ±0.044 | 0.85 ±0.021 | 0.82 ±0.025 |
| His | 0.35 ±0.012 | 0.37 ±0.011 | 0.36 ±0.012 | 0.36 ±0.013 | 0.37 ±0.014 | 0.35 ±0.032 | 0.38 ±0.009 | 0.38 ±0.010 |
| Phe | 0.63 ±0.029 | 0.71 ±0.016 | 0.70 ±0.026 | 0.68 ±0.019 | 0.71 ±0.017 | 0.72 ±0.021 | 0.71 ±0.021 | 0.68 ±0.014 |
| Total EAA | 6.78 ±0.258 | 7.42 ±0.173$^{S}$ | 7.48 ±0.187$^{S}$ | 7.10 ±0.194 | 7.53 ±0.289$^{S}$ | 7.12 ±0.171 | 7.45 ±0.064$^{S}$ | 7.36 ±0.190$^{S}$ |
| Tyr | 0.53 ±0.020 | 0.58 ±0.031 | 0.55 ±0.018 | 0.55 ±0.019 | 0.56 ±0.022 | 0.53 ±0.032 | 0.57 ±0.007 | 0.58 ±0.018 |
| Gly | 0.64 ±0.019 | 0.68 ±0.013 | 0.68 ±0.014 | 0.66 ±0.016 | 0.69 ±0.013 | 0.67 ±0.005 | 0.71 ±0.014 | 0.68 ±0.014 |
| Ser | 0.75 ±0.027 | 0.81 ±0.021 | 0.81 ±0.023 | 0.78 ±0.025 | 0.78 ±0.041 | 0.83 ±0.025 | 0.82 ±0.028 | 0.78 ±0.021 |
| Ala | 0.63 ±0.021 | 0.68 ±0.013 | 0.69 ±0.015 | 0.66 ±0.015 | 0.69 ±0.014 | 0.66 ±0.015 | 0.69 ±0.002 | 0.68 ±0.015 |
| Asp | 1.08 ±0.099 | 1.36 ±0.053 | 1.32 ±0.055 | 1.28 ±0.061 | 1.38 ±0.067$^{S}$ | 1.23 ±0.042 | 1.35 ±0.000 | 1.35 ±0.056 |
| Glu | 3.46 ±0.124 | 3.56 ±0.132 | 3.65 ±0.143$^{S}$ | 3.43 ±0.143 | 3.38 ±0.160 | 3.62 ±0.166 | 3.67 ±0.219$^{S}$ | 3.36 ±0.121 |
| Total NEAA | 7.09 ±0.225 | 7.67 ±0.168 | 7.70 ±0.168 | 7.36 ±0.169 | 7.48 ±0.162 | 7.54 ±0.190 | 7.81 ±0.262 | 7.43 ±0.161 |
| TAA | 13.87 ±0.504 | 15.09 ±0.335$^{S}$ | 15.18 ±0.435$^{S}$ | 14.46 ±0.462 | 15.01 ±0.310$^{S}$ | 14.66 ±0.421 | 15.25 ±0.325$^{S}$ | 14.79 ±0.411 |
| **Finisher stage** | | | | | | | | |
| Met | 0.39 ±0.016 | 0.37 ±0.011 | 0.41 ±0.018 | 0.37 ±0.004 | 0.37 ±0.005 | 0.37 ±0.009 | 0.36 ±0.007 | 0.37 ±0.018 |
| Cys | 0.26 ±0.014 | 0.27 ±0.10 | 0.30 ±0.005 | 0.29 ±0.011 | 0.29 ±0.010 | 0.29 ±0.005 | 0.29 ±0.004 | 0.30 ±0.015 |
| Lys | 0.72 ±0.067 | 0.81 ±0.051 | 0.91 ±0.052 | 0.94 ±0.052$^{S}$ | 0.84 ±0.022$^{S}$ | 0.82 ±0.026 | 0.81 ±0.035 | 0.86 ±0.056$^{S}$ |
| Thr | 0.56 ±0.024 | 0.58 ±0.022 | 0.63 ±0.023 | 0.57 ±0.019 | 0.59 ±0.017 | 0.59 ±0.021 | 0.58 ±0.028 | 0.62 ±0.021 |
| Arg | 0.79 ±0.065 | 0.84 ±0.056 | 0.92 ±0.054 | 0.96 ±0.060$^{S}$ | 0.94 ±0.061 | 0.85 ±0.067 | 0.84 ±0.085 | 0.96 ±0.055$^{S}$ |
| Ile | 0.54 ±0.033 | 0.55 ±0.031 | 0.63 ±0.030 | 0.62 ±0.025 | 0.56 ±0.015 | 0.58 ±0.015 | 0.56 ±0.021 | 0.59 ±0.034 |
| Leu | 0.43 ±0.047 | 0.49 ±0.223 | 0.50 ±0.020 | 1.01 ±0.054$^{S}$ | 0.89 ±0.044$^{S}$ | 0.91 ±0.046$^{S}$ | 0.91 ±0.057$^{S}$ | 0.99 ±0.019$^{S}$ |
| Val | 0.62 ±0.035 | 0.67 ±0.031 | 0.72 ±0.031 | 0.71 ±0.029 | 0.64 ±0.015 | 0.66 ±0.015 | 0.64 ±0.021 | 0.67 ±0.037 |
| His | 0.31 ±0.017 | 0.31 ±0.018 | 0.34 ±0.012 | 0.32 ±0.012 | 0.30 ±0.006 | 0.29 ±0.016 | 0.29 ±0.007 | 0.30 ±0.029 |
| Phe | 0.56 ±0.038 | 0.59 ±0.034 | 0.68 ±0.008$^{S}$ | 0.65 ±0.023 | 0.61 ±0.013 | 0.59 ±0.025 | 0.60 ±0.014 | 0.62 ±0.002 |
| Total EAA | 5.18 ±0.409 | 5.48 ±0.304 | 6.04 ±0.213$^{S}$ | 6.4 ±0.236$^{S}$ | 6.03 ±0.174 | 5.95 ±0.214 | 5.88 ±0.283 | 6.28 ±0.309$^{S}$ |
| Tyr | 0.43 ±0.021 | 0.49 ±0.008 | 0.50 ±0.019 | 0.49 ±0.005 | 0.48 ±0.005 | 0.49 ±0.006 | 0.48 ±0.011 | 0.48 ±0.008 |
| Gly | 0.56 ±0.015 | 0.57 ±0.013 | 0.59 ±0.009 | 0.60 ±0.013 | 0.59 ±0.013 | 0.57 ±0.015 | 0.58 ±0.014 | 0.60 ±0.012 |
| Ser | 0.67 ±0.017 | 0.66 ±0.018 | 0.68 ±0.051 | 0.67 ±0.021 | 0.65 ±0.024 | 0.66 ±0.026 | 0.65 ±0.035 | 0.70 ±0.012 |
| Ala | 0.57 ±0.019 | 0.59 ±0.019 | 0.61 ±0.047 | 0.62 ±0.020 | 0.58 ±0.019 | 0.59 ±0.021 | 0.58 ±0.028 | 0.62 ±0.016 |
| Asp | 1.03 ±0.079 | 1.15 ±0.053 | 1.24 ±0.048 | 1.27 ±0.055$^{S}$ | 1.21 ±0.051 | 1.16 ±0.061 | 1.15 ±0.078 | 1.26 ±0.051$^{S}$ |
| Glu | 3.26 ±0.080 | 3.10 ±0.042 | 3.14 ±0.059 | 3.06 ±0.030$^{S}$ | 3.04 ±0.033$^{S}$ | 3.05 ±0.040$^{S}$ | 3.00 ±0.057$^{S}$ | 3.08 ±0.049 |
| Total NEAA | 6.52 ±0.119 | 6.56 ±0.124 | 6.76 ±0.134 | 6.71 ±0.128 | 6.55 ±0.127 | 6.52 ±0.155 | 6.44 ±0.212 | 6.74 ±0.121 |
| TAA | 11.70 ±0.455 | 12.04 ±0.361 | 12.8 ±0.301$^{S}$ | 13.15 ±0.231$^{S}$ | 12.58 ±0.301 | 12.47 ±0.563 | 12.32 ±0.795 | 13.02 ±0.234$^{S}$ |

Note: Data are presented as means ± SD -standard deviation. Means with superscript letter $^{S}$ are significantly different at $p$ <0.05 in comparison with the control group. The numbers of each columns that do not have letter have a meaningful difference ($p$ >0.05).

Our results demonstrated the CP content in the diets for the experimental groups, compared with that for the C group, was not significantly different ($p$ >0.05), yet the overall contents of EAA in groups PP200, PP300, PB100 + FBL150 and TAA in group PP200 were significantly higher than those in the C group at both the grower and the finisher stages. It means that the diet with peas and faba beans contained higher-quality CP than that for the C group (SBM).

From the nutritional aspect, poultry meat is an important source of proteins, vitamins, and minerals, and at the same time has relatively low-fat content, therefore the chemical composition of muscle tissue is an important criterion of broiler meat quality. The biochemical parameters of broiler meat observed in the research indicated that the inclusion of faba beans and peas (100 – 200 g.kg$^{-1}$ of the basal diet) in the feed as higher protein diets can induce a higher meat protein content and, at the same time, reduce the fat content of muscles owing to a decrease in the protein-calorie ratio **(Bogosavljevic-Boskovic et al., 2010)**.

Although authors **Dal Bosco et al. (2013)** reported on a negative effect of faba bean diets due to a lower level of some EAA (Met, Cys, Thr, and Trp) and the presence of vicine, convicine, and tannins as anti-nutritional factors,

**Table 5** Effect of a dietary protein source on the biochemical composition of broiler meat.

| Items | C | PB200 | PP200 | PB300 | PP300 | FBL100 | PB100 + FBL100 | PB100 + FBL150 |
|---|---|---|---|---|---|---|---|---|
| DM, % | 22.33 ±0.74 | 24.47 ±0.84 | 23.26 ±1.61 | 23.41 ±0.68 | 23.33 ±1.29 | 22.46 ±0.45 | 24.04 ±1.11 | 24.05 ±0.42 |
| CP, % DM | 20.32 ±1.28 | 21.23 ±0.95 | 20.09 ±0.07 | 20.71 ±0.39 | 21.05 ±0.47 | 21.34 ±0.73$^{S}$ | 21.41 ±0.93$^{S}$ | 20.76 ±0.34 |
| CFa, % DM | 1.85 ±0.08 | 1.76 ±1.01 | 1.78 ±1.05 | 1.84 ±0.70 | 1.51 ±0.55 | 1.55 ±1.68 | 1.53 ±0.70 | 1.71 ±0.81 |
| CAsh, % DM | 1.38 ±0.02 | 1.58 ±0.09 | 1.41 ±0.10 | 1.40 ±0.06 | 1.51 ±0.03 | 1.51 ±0.13 | 1.55 ±0.06 | 1.52 ±0.01 |
| Ca, % DM | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.01 | 0.02 ±0.01 | 0.01 ±0.01 | 0.01 ±0.01 |
| P, % DM | 0.21 ±0.01 | 0.12 ±0.01 | 0.20 ±0.02 | 0.22 ±0.01 | 0.21 ±0.01 | 0.21 ±0.01 | 0.22 ±0.01 | 0.22 ±0.01 |
| Cholesterol, mg.100g$^{-1}$ | 128.1 ±8.70 | 118.2 ±23.11 | 110.5 ±7.56$^{S}$ | 137.5 ±9.20 | 118.7 ±18.13 | 110.6 ±9.95$^{S}$ | 118.6 ±6.96 | 122.6 ±41.77 |
| Meat energy value, kcal.100g$^{-1}$ | 95.26 | 104.11 | 99.31 | 104.13 | 102.31 | 100.50 | 107.84$^{S}$ | 106.05 |

Note: % DM – percentage of dry matter, CP – Crude protein, CFa – Crude fat, CAsh – Crude ash, Ca – Calcium, P – phosphorus. Data are presented as means ±SD – standard deviation. Means with superscript letter $^{S}$ are significantly different at $p$ <0.05 in comparison with the control group. The numbers of each columns that do not have letter have a meaningful difference ($p$ >0.05).

**Table 6** Amino acid contents in breast muscle of broilers at 42 days of age, g.kg$^{-1}$.

| Items | C | PB200 | PP200 | PB300 | PP300 | FBL100 | PB100 + FBL100 | PB100+ FBL150 |
|---|---|---|---|---|---|---|---|---|
| Met | 1.38 ±0.02 | 1.37 ±0.01 | 1.23 ±0.02 | 1.21 ±0.09$^{S}$ | 1.32 ±0.05 | 1.27 ±0.03 | 1.37 ±0.12 | 1.49 ±0.01$^{S}$ |
| Cys | 0.61 ±0.03 | 0.64 ±0.01$^{S}$ | 0.62 ±0.01 | 0.61 ±0.01 | 0.62 ±0.01 | 0.58 ±0.00$^{S}$ | 0.65 ±0.02$^{S}$ | 0.64 ±0.01$^{S}$ |
| Lys | 4.06 ±0.13 | 3.03 ±0.95$^{S}$ | 3.80 ±0.18 | 3.77 ±0.21 | 3.91 ±0.10 | 3.75 ±0.13 | 4.01 ±0.16 | 4.03 ±0.17 |
| Thr | 1.99 ±0.11 | 2.04 ±0.04 | 1.87 ±0.07$^{S}$ | 1.86 ±0.11$^{S}$ | 1.96 ±0.04 | 1.86 ±0.06$^{S}$ | 1.96 ±0.11 | 2.02 ±0.06 |
| Arg | 2.99 ±0.05 | 3.14 ±0.01$^{S}$ | 2.95 ±0.02 | 2.81 ±0.13 | 2.88 ±0.05 | 2.73 ±0.10$^{S}$ | 2.98 ±0.14 | 3.07 ±0.03$^{S}$ |
| Ile | 2.38 ±0.08 | 2.45 ±0.04$^{S}$ | 2.09 ±0.08$^{S}$ | 2.19 ±0.11 | 2.29 ±0.06 | 2.18 ±0.04 | 2.33 ±0.10 | 2.38 ±0.02 |
| Leu | 2.63 ±0.84 | 1.47 ±0.07$^{S}$ | 3.52 ±0.16 | 3.52 ±0.16 | 3.58 ±0.12$^{S}$ | 3.50 ±0.07 | 3.71 ±0.17$^{S}$ | 3.81 ±0.07$^{S}$ |
| Val | 2.37 ±0.09 | 2.44 ±0.01 | 2.17 ±0.10 | 2.20 ±0.10 | 2.30 ±0.05 | 2.18 ±0.01 | 2.39 ±0.09 | 2.34 ±0.01 |
| His | 1.43 ±0.04 | 1.50 ±0.02 | 1.40 ±0.08 | 1.39 ±0.03 | 1.54 ±0.01$^{S}$ | 1.34 ±0.06 | 1.50 ±0.07 | 1.47 ±0.02 |
| Phe | 1.99 ±0.04 | 2.04 ±0.06 | 1.87 ±0.03 | 1.76 ±0.06 | 1.85 ±0.03 | 1.74 ±0.08$^{S}$ | 1.85 ±0.08 | 1.90 ±0.06 |
| Total EAA | 21.84 | 20.13 | 21.54 | 21.31 | 22.25$^{S}$ | 21.13 | 22.77$^{S}$ | 23.15$^{S}$ |
| Tyr | 1.59 ±0.04 | 1.63 ±0.10 | 1.50 ±0.07 | 1.45 ±0.04 | 1.47 ±0.06 | 1.40 ±0.09$^{S}$ | 1.47 ±0.09 | 1.55 ±0.02 |
| Gly | 2.22 ±0.06 | 2.17 ±0.03 | 2.58 ±0.36$^{S}$ | 2.00 ±0.07 | 2.23 ±0.05 | 1.95 ±0.05 | 2.27 ±0.05 | 2.12 ±0.03 |
| Ser | 1.71 ±0.07 | 1.78 ±0.01 | 1.60 ±0.09 | 1.63 ±0.14 | 1.67 ±0.01 | 1.58 ±0.07 | 1.67 ±0.11 | 1.77 ±0.03 |
| Ala | 2.48 ±0.14 | 2.57 ±0.06 | 2.49 ±0.08 | 2.31 ±0.24$^{S}$ | 2.46 ±0.10 | 2.34 ±0.14 | 2.54 ±0.17 | 2.47 ±0.08 |
| Asp | 4.05 ±0.14 | 4.20 ±0.09 | 3.81 ±0.09 | 3.69 ±0.30 | 3.94 ±0.10 | 3.76 ±0.21 | 3.98 ±0.36 | 4.09 ±0.27 |
| Gln | 7.22 ±0.15 | 7.54 ±0.10$^{S}$ | 6.84 ±0.07$^{S}$ | 6.78 ±0.06$^{S}$ | 6.94 ±0.14 | 6.84 ±0.09$^{S}$ | 7.25 ±0.09 | 7.46 ±0.03$^{S}$ |
| Total NEAA | 19.27 | 19.89$^{S}$ | 18.83 | 17.85$^{S}$ | 18.71 | 17.88$^{S}$ | 19.17 | 19.44 |
| TAA | 41.11 | 40.02 | 40.37 | 39.16$^{S}$ | 40.96 | 39.01$^{S}$ | 41.94$^{S}$ | 42.59$^{S}$ |

Note: Data are presented as means ±SD – standard deviation.

which reduce energy and protein digestibility by forming tannin-protein complexes, our findings demonstrated that addition to diets of faba bean protein alone or in a mix with peas could elevate the content of dry matter in broiler meat **(Dal Bosco et al., 2013)**.

We found a similar situation regarding the content of CP in dry matter. The highest content was shown by the diets containing 100 g.kg$^{-1}$ FBL with 100 g.kg$^{-1}$ PB (PB100 + FBL100). Also, the crude ash content of dry matter increased statistically significantly ($p$ <0.05) in the muscle of broilers within all the experimental groups, especially in the group fed basal feed with 200 g.kg$^{-1}$ PB of the total diet.

Our results are not in agreement with the data published by other authors **Milczarek, Osek and Pachnik (2016)** who reported that the introduction of different varieties of faba beans in starter/grower feed for broilers did not affect the content of basic meat components – dry matter, crude ash, and crude protein.

Even though our results demonstrated also an increase in crude fat content within all the experimental groups in comparison with the C group of broilers, **Milczarek, Osek and Pachnik (2016)** reported that the use of faba beans in the diet of broilers modified the muscle fatty acid profile in a health-enhancing direction.

Concerning Ca, our research demonstrated that the addition of faba beans and peas in various amounts did not affect statistically significantly the level of Ca in broiler muscle meat. We founded the same situation regarding the level of P. Therefore, we would suggest that diets with faba beans and peas do not harm key minerals in broiler meat.

An important indicator of meat quality is the content of cholesterol. As regards this parameter, our experiment showed a statistically significant decrease in all the experimental diets, except for the group of broilers fed 300

**Table 7** Calculated nutritional indicators of broiler chicken muscle tissue.

| Items | Chemical score of AA (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | C | PB200 | PP200 | PB300 | PP300 | FBL100 | PB100 + FBL100 | PB100+ FBL150 |
| Met | 33.66 ±2.19 | 33.41 ±1.84 | 30.0 ±1.19 | 29.51 ±1.67 | 32.20 ±2.30 | 30.98 ±2.68 | 33.41 ±2.04 | 36.34 ±1.73 |
| Cys | 25.42 ±0.59 | 26.67 ±0.53 | 25.83 ±0.79 | 25.42 ±1.58 | 25.83 ±1.59 | 24.17 ±1.93 | 27.97 ±0.64 | 26.67 ±1.18 |
| Phe | 31.59 ±1.99 | 32.38 ±1.85 | 29.68 ±1.02 | 27.94 ±0.93 | 29.37 ±1.07 | 27.62 ±1.30 | 29.37 ±1.39 | 30.16 ±1.75 |
| His | 68.1 ±2.37 | 71.43 ±3.53 | 66.67 ±4.08 | 66.19 ±4.44 | 73.33 ±4.12 | 63.81 ±4.05 | 71.43 ±2.44 | 70.0 ±3.44 |
| Ile | 29.75 ±2.08 | 30.63 ±1.92 | 26.13 ±1.02 | 27.38 ±0.93 | 28.63 ±1.06 | 27.25 ±1.30 | 29.13 ±2.38 | 29.75 ±1.58 |
| Leu | 28.59 ±1.37 | 15.98 ±1.80 | 38.26 ±0.38 | 38.26 ±1.03 | 38.91 ±1.49 | 38.04 ±1.72 | 40.33 ±2.38 | 41.41 ±1.12 |
| Val | 32.47 ±1.79 | 33.42 ±1.66 | 29.73 ±0.82 | 30.14 ±1.33 | 31.51 ±1.23 | 29.86 ±1.50 | 32.74 ±1.92 | 32.05 ±1.52 |
| Lys | 56.39 ±6.15 | 42.08 ±5.60 | 52.78 ±0.99 | 52.36 ±1.70 | 54.31 ±1.78 | 52.08 ±2.17 | 55.69 ±7.44 | 55.97 ±4.78 |
| Thr | 40.61 ±1.82 | 41.63 ±1.73 | 38.16 ±0.98 | 37.96 ±1.18 | 40.0 ±1.35 | 37.96 ±1.68 | 40.0 ±1.78 | 41.22 ±1.46 |
| Met | 55.2 ±3.60 | 54.8 ±3.02 | 49.2 ±1.94 | 48.4 ±2.74 | 52.8 ±3.77 | 50.8 ±4.41 | 54.8 ±3.35 | 59.6 ±2.84 |
| Cys | 24.4 ±0.57 | 25.6 ±0.50 | 24.8 ±0.76 | 24.4 ±1.15 | 24.8 ±1.24 | 23.2 ±1.51 | 26.0 ±0.61 | 25.6 ±0.91 |
| His | 75.26 ±2.63 | 78.95 ±3.90 | 73.68 ±4.51 | 73.16 ±4.90 | 81.05 ±4.55 | 70.53 ±4.48 | 78.95 ±2.70 | 77.37 ±3.80 |
| Ile | 85 ±5.94 | 87.5 ±5.48 | 74.64 ±2.92 | 78.21 ±2.63 | 81.79 ±3.04 | 77.86 ±3.71 | 83.21 ±6.82 | 85.0 ±4.50 |
| Leu | 39.85 ±14.7 | 22.27 ±0.53 | 53.33 ±0.52 | 53.33 ±1.43 | 54.24 ±2.08 | 53.03 ±2.40 | 56.21 ±1.91 | 57.73 ±1.31 |
| Val | 67.71 ±3.46 | 69.71 ±3.23 | 62.9 ±1.54 | 62.86 ±2.77 | 65.71 ±2.56 | 62.29 ±3.13 | 68.29 ±3.50 | 66.86 ±3.01 |
| Lys | 70.0 ±7.64 | 52.24 ±6.95 | 65.52 ±1.23 | 65.0 ±2.12 | 67.41 ±2.20 | 64.66 ±2.69 | 69.14 ±9.24 | 69.48 ±5.93 |
| Thr | 58.53 ±2.62 | 60.0 ±2.49 | 55.0 ±1.43 | 54.71 ±6.14 | 57.65 ±5.55 | 54.71 ±6.55 | 67.65 ±2.57 | 59.41 ±4.61 |
| EAAI (%) | 393.28 ±7.32 | 376.7 ±10.07 | 383.33 ±10.71 | 379.06 ±15.18 | 399.08 ±16.33 | 374.43 ±19.97 | 405.74 ±8.35$^{S}$ | 411.55 ±12.08$^{S}$ |
| Biological value | 416.98 ±7.99 | 398.9 ±10.98 | 406.13 ±11.67 | 401.47 ±17.80 | 423.29 ±17.80 | 396.42 ±21.77$^{S}$ | 430.56 ±9.10$^{S}$ | 436.89 ±13.17$^{S}$ |

Note: Data are presented as means ± SD -standard deviation.

g.kg$^{-1}$ PP of the total diet. This could be explained by the highest content of CP from peas (21.05% of DM) in this experimental PP300 group in comparison with the other experimental diets. Our results are in agreement with those of researchers **Kiczorowska, Samolińska and Andrejko (2016)** who also reported on the hypocholesterolemic effect of pea seeds (*Pisum sativum L.*).

An important criterion of meat quality is the energy value of meat that was higher in all the experimental groups (from 4.25% in group PP200 to 13.20% in group PB100 + FBL100) than in the C group. Our results are not in agreement with the data of authors **Dal Bosco et al. (2013)** who reported that the introduction of faba beans in starter and grower feeds for broiler chickens did not affect the meat energy value. This probably could be explained by the high value of the dry matter in broiler meat as a result of our experiment. But, the inclusion of 200 – 300 g.kg$^{-1}$ peas of the basal diet in the experimental broiler feed demonstrated a lower energy value than that for the PB100 + FBL150 group fed 150 g.kg$^{-1}$ FBL with 100 g.kg$^{-1}$ PB, which could be explained by an imbalance between energy intake and energy expenditure **(Bogosavljevic-Boskovic et al., 2010)**.

AA are absorbed at the intestinal level and have effects on the physiology of the intestine, and AA might also affect muscle composition and meat quality. Macroorganisms cannot synthesize EAA or indispensable AA and therefore the acids have to be supplied in the diet. Researchers **Probst (2009)** and **Koivunen et al. (2016)** inform that various diets can cause significant changes in the content of EAA and NEAA in broiler meat. However, our experiment demonstrated that diets with an addition of faba beans and peas have shown a positive effect on the level of EAA and NEAA in broiler meat.

The nutritional value of meat is usually assessed based on parameters such as the content and composition of proteins, levels of AA, and content of fat **(Straková et al., 2002)**. The calculated indicators in meat samples showed that the chemical score, the AA score, the EAAI, and the biological value of tested AA were significantly increased in the PB100 + FBL150 group broilers fed diets supplemented with 150 g.kg$^{-1}$ FBL with 100 g.kg$^{-1}$ PB.

## CONCLUSION

In the present research study, the replacement of protein sources with peas and faba beans was more apparent because the AA composition in the breast muscle, the biological value, and the broiler meat quality have positively changed. It could be concluded that broiler feed rations may comprise peas 'Bruno', 'Pinochio', and faba beans 'Lielplatone' grown in Latvia. The faba beans along with peas could be used to replace part of soybean meal as a source of protein.

There was a significant reduction ($p$ <0.05) in the cholesterol concentration of broiler breast meat from the broilers fed diet PP200 supplemented with 200 g.kg$^{-1}$ PP (110.5 mg.100g$^{-1}$) and FBL100 – with 100 g.kg$^{-1}$ FBL (110.6 mg.100g$^{-1}$) in comparison with the C group, however cholesterol concentration in meat decreased in all the experimental groups (except PB300). The CP content was significantly ($p$ <0.05) higher in breast meat of broilers fed diets FBL100 and PB100 + FBL100.

The significantly higher average EAA levels (6.04, 6.44 and 6.28% of DM) and TAA amounts (12.8, 13.15, and 13.02% of DM) were recorded in experimental finisher stage diets PP200, PB300, and PB100 + FBL150. The chemical score, the AA score, the EAAI, and the biological value of tested AA were significantly increased in group PB100 + FBL100 and PB100 + FBL150 broilers fed diets supplemented with a mixture of 100 – 150 $g.kg^{-1}$ FBL with 100 $g.kg^{-1}$ PB.

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**Conflict of Interest:**

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

**Ethical Statement:**

This article does not contain any studies that would require an ethical statement. The study was restricted to routine on-farm observations and measurements collection that did not inconvenience or stress the animals, meat samples were collected after the birds were slaughtered at a slaughterhouse.

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# COMPARATIVE STUDY OF WEANING PIGS' MUSCLE PROTEINS USING TWO-DIMENSIONAL ELECTROPHORESIS

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## ABSTRACT
The proteostasis system of animals, including various types of protein modification during the growth stage, leads to an almost incomprehensible number of possible forms of protein, and each can regulate numerous functions. In the presented work, the composition of muscle tissue protein from different portions of piglets was studied to understand the main muscle protein formation. Comparative analysis of weaned piglets' main muscle protein from *l. dorsi*, *biceps femoris,* and *brachiocephalicus* were analyzed using two-dimensional electrophoresis. Changes in the staining intensity of protein fractions inherent in different muscles were revealed. As part of this work, candidate groups of pig muscle proteins have been selected. Eleven protein spots were revealed for the longest muscle of the back, and seven for the biceps; the muscles of the neck are characterized by indicators of low protein fraction volume. Among the proteins found, myosin light chains, phosphoglycerate mutase, troponins, and adenylate kinase is most likely present. The obtained results of protein identification in muscle tissues, obtained during the intensive growth period, will allow a more detailed understanding of protein regulation, function, and interactions in complex biological systems, which will subsequently be significantly important for biomonitoring health and predicting farm animals productivity.

**Keywords:** 2-DE; muscle; protein; two-dimensional electrophoresis; pig; piglet

## INTRODUCTION
The increase in demand for lean pork has required the selection of carcasses showing increased muscle mass. The selection for this indicator has significantly improved the meat qualities of most domestic and foreign pig breeds. However, an increase in "meat content" without taking into account other economically useful traits led to a decrease in natural resistance and significant shifts in metabolic processes in the animals, which ultimately affected the quality and organoleptic characteristics of pork **(Fedulova et al., 2018; Benešová et al., 2019; Gorlov et al., 2020)**.

It should be noted that the selection efficiency is assessed mainly by identifying genes that control beneficial traits, as well as polymorphic DNA variants in these genes, which directly affect the phenotype of the animal. In this case, genome and proteome interactions are completely ignored. It is known that an animal's proteostasis system, including folding, modification of the primary structure, and protein degradation, are crucial for the realization of phenotypic traits. At the same time, the huge number of possible post-translational modifications, combined with the multitude of amino acid residues, leads to an almost incomprehensible number of possible protein forms, and each can regulate numerous functions **(Spoel, 2018)**. Thus, the use of proteomic methods in animal husbandry for muscle protein identification will allow a more detailed understanding of protein regulation, function, and interactions in complex biological systems, which will subsequently be vitally important for biomonitoring the health and productivity of farm animals. Also, practical applications in identifying counterfeit meat products could be based on this technique **(Mora, Gallego and Toldrá, 2018)**.

As you know, with the growth and increase in animal live weight, the amount of muscle, adipose, and bone tissue in their body is steadily increasing. However, the growth of these tissues proceeds with unequal intensity. The development of muscle tissue in young animals is due to a high rate of metabolic processes and protein synthesis. Moreover, the main factors that determine the rate of muscle growth are genetic, feeding, muscle activity (exercise), stress hormones, and growth stimulants (natural or synthetic).

As growth slows down and stops, the rates of protein synthesis and degradation are balanced. In conditions of poor nutrition, diseases, including parasitic ones, stress, unfavorable environmental conditions, muscles can atrophy, which means that protein degradation outweighs synthesis **(Purslow, 2017)**.

Until recently, not enough attention has been paid to the development rate of muscle tissue in growing animals to predict the factors that stimulate the growth of animals and determine meat quality.

### Scientific Hypothesis

Identifying the composition of muscle proteins of various parts of a growing animal - weaned piglets - will expand the available information on the formation of muscle tissue to identify quality, safety, and authenticity markers in livestock products.

## MATERIALS AND METHODS

### Object

The object of the study was Vietnamese pot-bellied × Wiesenau weaned pigs from Krolinfo LLC laboratory animal resource center (Likino-Dulyovo, Russia), healthy females 60 – 65 days old. Samples were taken from 9 piglets: *l. dorsi* (longest back muscle), *biceps femoris* (hip biceps), and *brachiocephalicus* (neck muscles) muscle.

### Materials

Chemical reagents: Urea, Thiourea, Ditiothreitol, Sodium hydroxide (NaOH), Glycerol, Sodium dodecyl sulfate (SDS), Tris, Acrylamide, Ammonium persulfate (APS), 2-Propanol, Acetic acid, Bis-acrylamide, Tetramethylethylenediamine (TEMED), Mercaptoethanol, Bromophenol blue, Glycine, Coomassie Brilliant Blue G-250 (PanReac, Spain); Triton X-100 (Helicon Russia); Ampholyte (Serva, Germany) and Phosphoric (V) acid ($H_3PO_4$) (Component-reaktiv, Russia).

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 three replicates.

### Two-dimensional gel electrophoresis (2-DE)

The samples described above were subjected to 2-DE. Proteins were separated by IEF (isoelectric focusing) in the first dimension and SDS-PAGE in the second dimension. This was completed as described by **Matsumoto et al. (2019)** with slight modifications, IEF in the first dimension was performed at 3650 $V.h^{-1}$.

2-DE was performed according to the method of O'Farrell (O'Farrell, 1975) with isoelectric focusing on ampholine pH gradient by Glass Tube-Based IEF. The subsequent detection of the proteins was carried out by staining with Coomassie Brilliant Blue G-250 (PanReac, Spain).

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 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 one-way ANOVA (between gels obtained with a different variation of IEF) by ImageMaster™ 2D Platinum software powered by Melanie 8.0 (GE Healthcare and Genebio, Switzerland).

A $p < 0.05$ was considered to indicate a significant difference. All results are presented as mean ±SD from at least three independent experiments.

The Fold change index was calculated. Fold change index is the ratio between the volume of a protein spot with the highest average value and the lowest average value. It is common to select spots with Fold >2, for instance, to identify those proteins in which treatment causes at least a two-fold spot abundance increase or decrease.

## RESULTS AND DISCUSSION

A study was carried out on three different muscles of *l. dorsi*, *biceps femoris,* and *brachiocephalicus* by two-dimensional electrophoresis to reveal significant differences in protein composition **(Zia et al., 2020)**.

Two-dimensional electrophoretogram fragments with the detected changes are shown in Fig. 1; a total of 18 fractions were selected with Fold index >2 and presented in Table. 1.

Protein spots indicated by numbers 1 and 2 in Figure 1, and presumably myosin light chains fast (MLC1f) **(Murgiano et al., 2010; Yang et al., 2016)** and slow (MLC1s/v), respectively, were quite well manifested in the longest muscle **(Kim et al., 2017; Zou et al., 2018)**. In the *biceps femoris*, only the slow chain was detected, and in the *brachiocephalicus*, a weakly expressed fraction MLC1s/v. **Montowska and Pospiech (2012)** used MLC in their studies as markers in the authentication of meat products made from pork and other meats.

A group of proteins in the range of molecular weights from 50 kDa to 60 kDa, marked with numbers 3 – 7 in Figure 1, were more pronounced in the muscles of the biceps, a smaller amount identified in the longest muscle, and the muscles of the neck trace amounts were detected.

Protein number 8 intensity (Figure 1) decreased evenly from *l. dorsi* to *brachiocephalicus*, the same tendency was observed in fractions 13 – 17, among them troponin is presumably present. The skeletal muscle protein troponin I have already been characterized as a potential thermostable and species-specific biomarker of mammalian muscle tissue in raw meat and meat products **(Zvereva et al., 2015; Mitra et al., 2018; Zuber et al., 2019)**, which makes it promising for identifying muscles of different regions of the animal. The lower spot volume in the neck muscles is possibly associated with the biological function of this muscle since it is less active than *l. dorsi* and *biceps femoris*.

Interesting distribution of protein spots was found in fractions 9 and 10, only protein number 9 was found in the biceps, but fraction 10 (adenylate kinase) was present in a small amount, but its intensity in the large, flat muscle on the back was greater than in the neck muscle **(Lee et al., 2011; Scheffler, Park and Gerrard, 2011; Oliván et al., 2015)**.

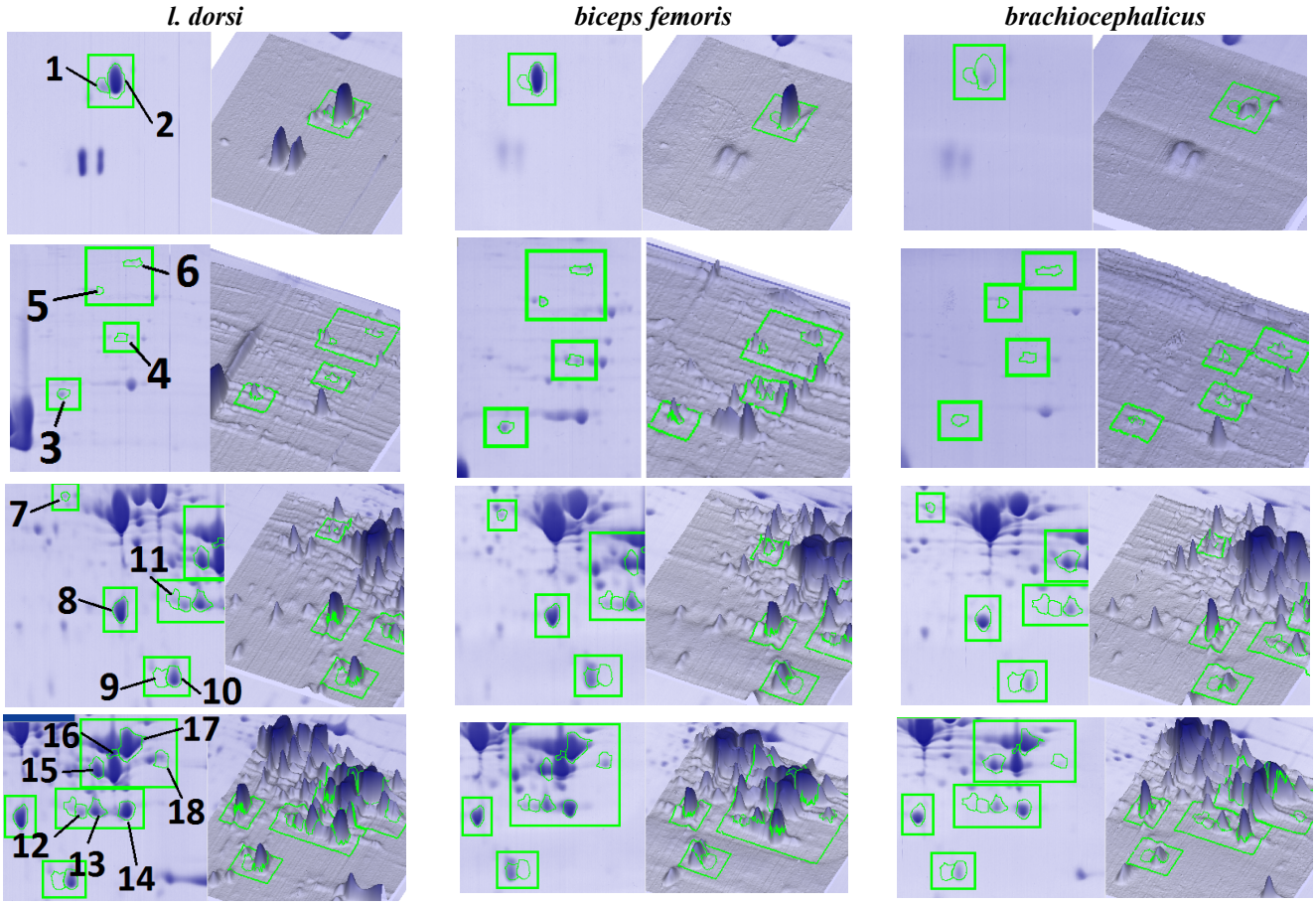


**Figure 1** Fragments 2-DE gels of pig muscle tissue.

**Table 1** Results of the densitometry analysis.

| No of Spot | Muscle tissue | | | Fold |
|---|---|---|---|---|
| | *l. dorsi* | *biceps femoris* | *brachiocephalicus* | |
| 1 | 9.07 ±1.19 x $10^6$ | 2.60 ±1.63 x $10^6$ | 1.46 ±0.36 x $10^6$ | 6.20 |
| 2 | 0.11 ±1.35 x $10^7$ | 8.93 ±1.13 x $10^7$ | 2.78 ±1.77 x $10^7$ | 4.18 |
| 3 | 3.84 ±0.67 x $10^6$ | 2.74 ±1.15 x $10^6$ | 1.02 ±0.37 x $10^6$ | 3.78 |
| 4 | 1.15 ±0.34 x $10^6$ | 2.63 ±0.52 x $10^6$ | 1.18 ±0.57 x $10^6$ | 2.29 |
| 5 | 2.55 ±0.63 x $10^5$ | 1.18 ±0.29 x $10^6$ | 2.72 ±0.67 x $10^5$ | 4.62 |
| 6 | 6.88 ±3.57 x $10^5$ | 2.42 ±0.40 x $10^6$ | 1.42 ±0.47 x $10^6$ | 3.52 |
| 7 | 1.49 ±0.79 x $10^6$ | 5.96 ±1.67 x $10^6$ | 3.49 ±0.22 x $10^6$ | 4.00 |
| 8 | 6.21 ±0.29 x $10^7$ | 5.16 ±0.83 x $10^7$ | 1.75 ±1.52 x $10^7$ | 3.55 |
| 9 | 4.90 ±2.28 x $10^6$ | 1.86 ±1.21 x $10^7$ | 2.08 ±0.16 x $10^6$ | 8.94 |
| 10 | 3.33 ±0.48 x $10^7$ | 1.27 ±0.92 x $10^7$ | 8.07 ±5.80 x $10^6$ | 4.12 |
| 11 | 5.93 ±1.10 x $10^6$ | 5.78 ±1.72 x $10^6$ | 2.03 ±0.49 x $10^6$ | 2.93 |
| 12 | 1.77 ±0.45 x $10^7$ | 8.42 ±2.46 x $10^6$ | 1.69 ±0.54 x $10^6$ | 10.50 |
| 13 | 4.37 ±0.32 x $10^7$ | 3.38 ±1.30 x $10^7$ | 1.24 ±0.34 x $10^7$ | 3.15 |
| 14 | 5.99 ±0.19 x $10^7$ | 5.19 ±1.02 x $10^7$ | 1.84 ±1.53 x $10^7$ | 3.25 |
| 15 | 5.31 ±0.88 x $10^7$ | 4.18 ±0.62 x $10^7$ | 2.52 ±0.99 x $10^7$ | 2.11 |
| 16 | 1.56 ±0.28 x $10^7$ | 7.86 ±2.20 x $10^6$ | 3.90 ±2.56 x $10^6$ | 3.98 |
| 17 | 1.40 ±0.12 x $10^8$ | 1.16 ±0.05 x $10^8$ | 6.86 ±1.51 x $10^7$ | 2.04 |
| 18 | 1.07 ±0.23 x $10^7$ | 1.21 ±0.17 x $10^7$ | 3.71 ±1.17 x $10^6$ | 3.27 |

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. *Vol: The volume of a spot is the sum of the background-subtracted gray values of all pixels delimited by the spot.

The volume of spot number 11 in *brachiocephalicus* was three times less than in *l. dorsi* and *biceps femoris*. The highest Fold value was in fraction 12, which is probably phosphoglycerate mutase, corresponded to 10.5, and is maximally expressed in *l. dorsi* **(Welzenbach et al., 2016; He et al., 2016; Lepczynski et al., 2019)**. Protein number 18 is more strongly expressed in the muscles of the biceps, less intensely noted in *l. dorsi* and a small amount in the neck.

The changes described above in the staining intensity of selected protein fractions may reflect the dynamics of muscle tissue formation in growing animals **(Chen et al., 2018; Li et al., 2020)**. For example, in *l. dorsi*, the most powerful muscle of the spinal column, which determines the movement of the spinal column and head, the maximum amount of intensely colored protein fractions was revealed **(Liu et al., 2014; Paredi et al., 2019)**. Candidate markers for *l. dorsi* can be fractions 1, 3, 8, 10 – 17 (Figure 1, Table 1) **(Zou et al., 2017)**. For the *biceps femoris*, which functionally exhibits actin, the hip extensor, and hock joints, and the flexor of the knee joint, muscle fractions 2, 4 – 7, 9, and 18 can serve as markers (Figure 1, Table 1). The thigh muscle and its protein profile are quite well studied, since various versions of jerky ham are made from pork, such as ham and prosciutto, it is already known that the peptides formed in this muscle have biological functionality (some peptides from MLC1, CK, MYO, TNT, and MHC7 proteins were the most influential) **(Bermúdez et al., 2014; Mora et al., 2016; Chernukha et al., 2018; Zhou et al., 2020)**.

For *l. dorsi* composition, 11 proteins were identified **(Kim et al., 2008; Grujić and Savanović, 2018)**, and 7 proteins for *brachiocephalicus*, besides, the latter showed a tendency of lower intensity protein fractions, some of which are smaller in comparison to *l. dorsi*.

The lowest intensity of staining of protein fractions was noted in *brachiocephalicus*, which is possibly associated with low metabolic processes in this muscle, due to a low functional load.

## CONCLUSION

As part of this work, data were obtained on the variations in muscle protein groups in the muscles of different regions in piglets. For the longest muscle of the back, eleven protein spots with a pronounced intensity of staining were revealed, for the biceps - seven, for the muscles of the neck, low indicators of the spot volume are characteristic. These protein fractions vary across the entire spectrum of intercellular proteins, from heat shock proteins and enzymes of energy metabolism to structural proteins. The identified protein spots can be used as molecular markers of muscle tissue development. However, much remains to be clarified on a structural and molecular basis. This study further revealed the markers of productivity and adaptation of animals, as well as the quality, safety, and authenticity of animal products.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

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# NUMERICAL METHODS AND IMAGE PROCESSING TECHNIQUES FOR MELISSOPALYNOLOGICAL HONEY ANALYSIS

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## ABSTRACT
Pollen analysis is a method used for verification of the botanical and geographical honey origin. Currently, much effort is being made to introduce automated systems with the use of image analysis programs. The automatic analysis is impeded by the insufficient depth of field of objects when using a light microscope, however, this can be avoided by using image reconstruction from images obtained from different focal planes. In this method, testing was performed on the normal focus (NF) and extended-depth-of-focus (EDF) images. These two methods were compared and statistically evaluated. The number of pollen grains and selected morphometric characteristics were compared. For EDF images, a higher number of pollen grains was obtained for the analysis, and except for the length/width ratio, a statistically significant difference was observed in the characteristics of pollen grains between the compared NF and EDF methods.

**Keywords:** Bright field microscopy; extended depth of focus; length/width ratio pollen; morphometry

## INTRODUCTION
One of the important pieces of information for consumers of honey is its geographical and botanical origin **(Švamberk, 2015)**. The way to obtain this information is pollen analysis which is one of the most important examinations of honey, especially in terms of quality control. Nevertheless, sensory and physicochemical analyses are not less important for the correct detection of botanical origin **(Von Der Ohe et al., 2004)**. Highly valued honey are mono-floral ones which, however, should contain at least 45% of the pollen grains of the specified plant species. Nevertheless, there are some exceptions, as some plants are less pollen-producing and therefore the amount of their pollen in honey is lower **(Kolayli et al., 2016)**. Conversely, other plants have a high pollen-producing capacity, thus the pollen content of the single species should be up to 90% **(Oddo et al., 1995)**. To verify the botanical and geographical origin of honey, qualitative pollen analysis has to be applied; while the quantitative analysis is suitable for assessing the extraction and processing of honey (or filtration) **(Von Der Ohe et al., 2004)**.

Pollen analysis is a very demanding method. The examiner performing it must be able to recognize the species or at least the genus of pollen grains contained in a microscopic sample of honey **(Punt et al., 2007)**. The method for the qualitative analysis of honey and its performance is described in the International Commission for Bee Botany of IUBS (International Union of Biological Sciences). According to these recommendations, at least 300 pollen grains should be examined and at least 500 to 1000 pollen grains should be examined to determine the frequency of individual pollen species **(Jones and Bryant, 2001; Von Der Ohe et al., 2004)**. This limit (500 pollen grains and more) is also recommended in the case of bee pollen examination **(Lau, Bryant and Rangel, 2018)**.

Pollen grains differ in appearance, color, and shape. Morphological characteristics of pollen grains can be divided into several groups, according to their shape, size, pollen unit, polarity, symmetry, number and size of apertures, pollen surface, stratification of sporoderms, or exine ornamentation **(Punt et al., 2007)**. Some of these parameters can be measured using an eyepiece scale, but image analysis is currently used more commonly **(Shafiee et al., 2013)**. The most suitable tool for this examination is an electron microscope which is, nonetheless, very expensive and difficult to control in comparison with an optical microscope. When using optical microscopy, a frequent problem is insufficient depth of focus, especially when the sample surface is uneven and part of the object lies outside the selected focal plane. To obtain a sharp image, we can use image reconstruction which involves obtaining images from different focal planes. It is creating a super-sharp image by merging images from multiple focal planes – EDF (extended depth of focus image) **(Wu and Wang, 2009)**. To do this, algorithms are currently used to increase the depth of focus of the image using digital processing. Digital methods of processing the extended depth of focus are based on a set of optical sections that fully cover the studied object. Only a small portion of each section is focused due to the limited depth of focus of the microscope. The purpose of the extended-depth-of-focus algorithms applied is to recover the in-focus pixels from each section

and to create the final single composite image from them. Also, if we record not only the values of the in-focus pixel (intensity) but also the section index from which the in-focus pixels were selected, the three-dimensional structure (3D) information can be preserved. Such extended depth of field algorithms allows the incorporation of 3D information into a single two-dimensional (2D) composite image **(Valdecasas et al., 2001)**.

### Scientific Hypothesis

The scientific hypothesis was to verify whether using an EDF image allows for detecting and identifying more pollen grains than scanning a single focal plane image.

## MATERIAL AND METHODOLOGY

### Samples

In this work, a sample of honey from the market network, which came from the Czech Republic, was tested.

### Description of the Experiment

The amount of 10.0 g of the sample measured to the nearest 0.1 g was used for the examination. This amount was dissolved in conical centrifuge tubes in 20 mL of distilled water tempered at 40 °C. Subsequently, the obtained solution was centrifuged in a centrifuge (Centric 322A, Technica, SLO) for 10 minutes at a speed corresponding to 1000 g. After the centrifugation, the supernatant was removed. Again, 20 mL of tempered distilled water was added and centrifugation was performed. After removing the supernatant again, the remaining sediment was transferred to a microscope slide using a Pasteur pipette and allowed to dry on a heated plate (Vezas spol. ltd, CZE) at 40 °C. Before placing the sample on the slide, a square of 22 × 22 mm had been drawn using a barrier marker (Elite Mini PAP Pen, USA) to prevent the sediment from spilling over a larger area. After the sediment had dried, the sample was mounted with Kaiser's glycerol gelatine.

Subsequent sample scanning was performed with a DFK 23U274 camera (Imaging Source, GER) using an Eclipse Ci-L microscope (Nikon, JPN) with a Prosca III motorized stage (Prior, USA). NIS-Elements AR 5.20 software (Laboratory Imaging, CZE) was used to scan the samples. The program performed a random selection of 100 fields of view, on which the automatic counting of pollen grains was performed. Scanning was performed in the acquisition mode in five focal planes. The distance between the individual focal planes was 8 μm. Thresholding, counting, and measurement of morphometric properties of pollen grains were performed in the ideal focal plane (NF) according to the evaluator's choice and also after merging images from 5 different focal planes after creating an extended depth of focus (EDF) image. The actual scanning and automatic counting of pollen grains were performed in ten replicates per 100 images for each repetition. The two individual methods were then compared and statistically evaluated.

In the next step, the following parameters were measured by the image analysis software of NIS-Elements AR 5.20 (Laboratory Imaging, CZE) for both methods: pollen grain area (basic quantity indicating the size of the object in $\mu m^2$), its length (calculation of the lengths of the central axes of thin objects is used and is given in μm), width (indicating the ratio between the area and length of the object in μm), and MaxFeret90 (indicating the projection length perpendicular to the maximum Feret's projection, Figure 1) **(Laboratory Imaging, 2019)**.

### Statistical Analysis

The data were processed statistically using the MATLAB 2019b (MathWorks, USA). The Anderson-Darling test, Student's t-test, sign test, and Wilcoxon test, two-sample test, and nonparametric Mann-Whitney test were used to evaluate the obtained results.

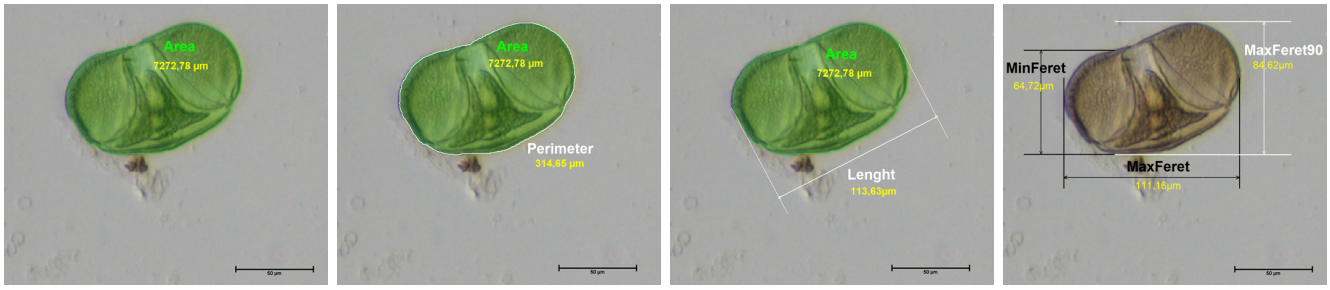
## RESULTS AND DISCUSSION

The analyzed sample was scanned automatically in different focal planes. The sharpest image was selected for NF according to the experience of the human evaluator. For EDF, all the scanned Z levels were combined into one super-sharp image. Z-images were combined into one focused image by picking the focused regions from each frame and the pieces combined. In our study, balanced algorithms of Laboratory Imaging software were used. Algorithms for extended depth of focus were developed in the past 20 years. The primary application of EDF is for transmitted light microscopy systems **(Valdecasas et al., 2001; Tympel, 1997)**.

When merging multiple focal planes, a higher number of pollen grains was recorded in 80% of the measurements. 5397 pollen grains were counted in NF scanning and 5689 pollen grains in EDF scanning for all 10 repetitions on 100 randomly scanned images. This is consistent with the results of a study by **Valdecasas et al. (2001)** who argues that the use of algorithms in digital processing uses extended depths of focus and restores in-focus pixels, thus creating a final composite image from the selected in-focus pixels. The NF and EDF results for all repetitions are summarized in Figure 2.

The numbers of pollen grains recorded by both methods were compared using a paired test, using the difference in the results of individual methods for the same sites instead of the original data. However, the commonly used paired Student's t-test assumes a normal distribution of these differences. This assumption was tested by the Anderson-Darling test described in the study by **Tympel (1997)**. If the assumption of normality is not met, the differences must be tested by one of the nonparametric alternatives, such as the sign test or the Wilcoxon test **(Montgomery and Runger, 2011; Anděl, 2011)**.

By imperfect focusing of the evaluated image, especially smaller pollen grains can completely disappear from the image. A similar problem was addressed in the study by **France et al. (1997)** that focused on the automatic detection of pollen grains and their identification. The author also pointed out a situation whether or not it is a pollen grain or another unidentifiable object. The application of automatic systems and their use to filter objects based on their properties (size and shape) and other information in the image and possible errors are described in the paper by **Ranzato et al. (2007)**.




Area

$$Area = \sum pixel$$

Length

$$Length = \frac{Perimetr + sqrt(Perimetr^2 - 16 * Area)}{4}$$

Width

$$Width = \frac{Area}{\text{Length}}$$

MaxFeret90

**Figure 1** Measured morphometrical criteria.

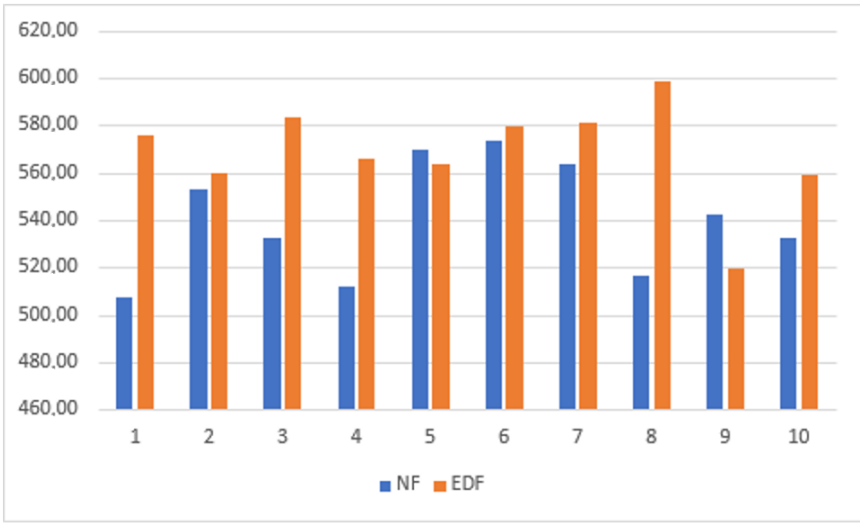



**Figure 2** Number of pollen particles in NF and EDF images for each repetition.

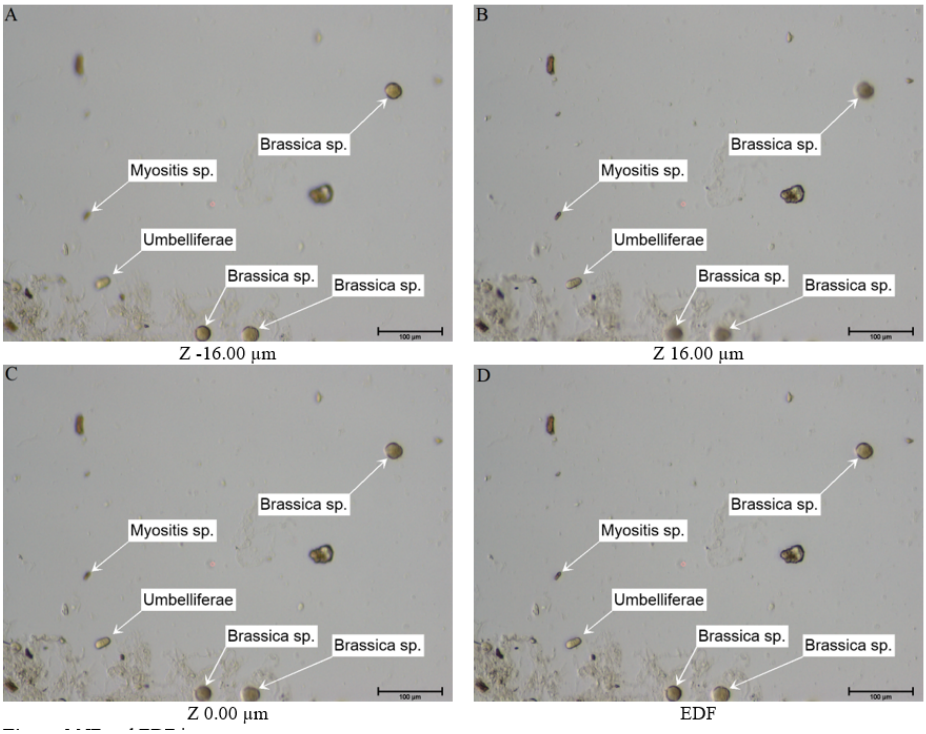



**Figure 3** NF and EDF image. Note: A – C: NF scanning, D: EDF scanning.

In the case of melissopalynological analyses, it is common for both, small as well as large pollen grains, to be present in honey. Small pollen grains typically come from members of the *Myosotis* genus (4 – 10 μm), while large pollen grains are common for conifers (50 – 90 μm). However, the most abundant pollen grains in honey are medium-sized particles (30 – 50 μm). One of the many representatives is, for example, *Brassica napus* **(Oddo et al., 2004)**. If an image contains taxa with pollen grains of different sizes, certain pollen grains are lost in the evaluation from such an image. This error can be observed both when selecting a sharp image by a human evaluator as well as when using automatic focusing systems (Figure 3).

None of the images at different NF levels (Figure 3 C) provides a focused view of all pollen grains. In the case of focusing on the predominant pollen grains of *Brassica* sp. in the image, a strong blurring is evident in the pollen grain of *Myosotis* ssp. (Figure 3 A) and partial blurring in *Umbelliferae* (Figure 3 B) pollen.

As part of the statistical evaluation, it was first tested whether the images obtained by both methods (NF and EDF) detect the same number of pollen grains. For this comparison, all 100 x 10 measured images were considered as a single statistical file. Due to the availability of pairwise comparisons for the number of pollen grains detected by both methods (NF and EDF), it was possible to "filter out" inter-image variability using the paired statistical test. As shown by the histogram in Figure 4, the difference appears to be more pointed than the corresponding normal distribution should be.

A similar deficiency is indicated by the Normal Probability plot (Figure 5). The normal distribution of data was then definitively ruled out by the rejection of the Anderson-Darling test of normality on differences ($p$ <0.001, A-D statistic 35.5644, at a critical value of 0.7513).

The rejection of normality meant that it was not possible to use the standard (parametric) paired variant of the Student's t-test. Thus, to demonstrate a statistically significant difference between the number of grains detected by both methods, it was necessary to use paired nonparametric tests (sign and Wilcoxon test, both with continuity correction). The null hypothesis of these tests in this case was the zero medians of differences (NF-EDF). The sign test rejected the null hypothesis (p-value which contained the first valid digit in the eighth decimal place, $8.5045*10^{-7}$). The result of the Wilcoxon test was then yet by two orders of magnitude stronger rejection of the null hypothesis ($p$ <0.001). Hence, although the difference between the two methods used may seem negligible (EDF finds on average only 0.292 more pollen grains), it is undoubtedly statistically significant and the EDF method detects a higher number of pollen grains.

The amount of pollen grains in honey also varies naturally. Beekeeping practices, most importantly the method of honey extraction, are mainly responsible for the variability of the pollen grains amount in honey. As to concerns about honey adulteration, it is not common to filter honey through sieves with meshes smaller than 0.2 mm. This filtration is only allowed to remove foreign matter **(Ruoff and Bogdanov, 2004)**. However, even honey processed in this way must be designated as filtered honey **(Codex Alimentarius, 2001)**. Other technological steps, such as sedimentation and collection of floating particles on honey, also slightly affect the number of pollen grains in honey. Differences in the number of pollen grains in honey were also noted in a German study which confirmed that the amount of pollen grains in newly produced honey is lower than that in honeybee feed. The pollen concentration is decreased by the honey stomach filtration system of honeybees **(Bryant and Jones, 2001)**. Another reason for the different amounts of pollen grains in honey is also their botanical origin. Some taxa are strongly nectar-producing and poorly pollen-producing, such as Robinia pseudoacacia honey. These honey are naturally characterized by a low content of pollen grains. The opposite example is *Myosotis* honey, which has a significant pollen-producing capacity and low nectar-producing capacity. The content of pollen grains in these honey is therefore high **(Demianowicz, 1964)**.

Thus, the number of pollen grains usable for melissopalynological analysis is not the only criterion for evaluating the suitability of the compared methods. The pollen grains of each botanical taxon have their typical morphometric properties, which may vary according to the botanical species of the plants. The literature describes both, differences between plant species **(Evrenosoğlu and Misirli, 2009)** and between individual varieties **(Hebda and Chinnappa, 1994; Geraci et al., 2012)**. To evaluate the morphology, the parameter of total area, which represents the size of pollen grains **(Pospiech et al., 2019)**, and the parameter of length/width **(Nazeri Joneghani, 2008)** is recommended. Length of polar axis and equatorial diameter evaluations are also commonly used **(D'Albore, 1998)**. The use of these morphological characteristics has found application in both conventional melissopalynological analysis **(Al-Watban et al., 2013; Čeksterytė, Kurtinaitienė and Balžekas, 2013)** as well as in automatic melissopalynological systems based on image evaluation **(Treloar, Taylor and Flenley, 2004**; **Redondo et al., 2015)**. To compare the morphometric properties of the detected pollen grains, the morphometric characteristics of these grains were measured for the first iteration (100 images). The area was determined by the simple sum of the pixels of each pollen grain. Length and width here mean the size of the major (length) and minor (width) axis of the Legendre ellipse of the pollen grain. The construction of a Legendre ellipse is described in detail, for example, in the study by **Flusser, Suk and Zitová (2009)**. Since the number of detected pollen grains was not the same, it was necessary to use a two-sample test for statistical comparison of both methods in this case. Due to the limitations of all observed geometric characteristics, a normal distribution cannot be assumed for any of them (all are limited to 0 below). Therefore, the nonparametric Mann-Whitney test was consistently applied to compare the morphometric parameters **(Montgomery and Runger, 2011; Anděl, 2011)**.

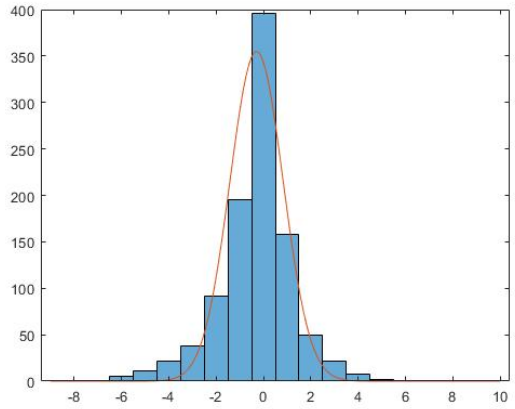



**Figure 4** Histogram of differences in the number of pollen grains obtained by NF-EDF.

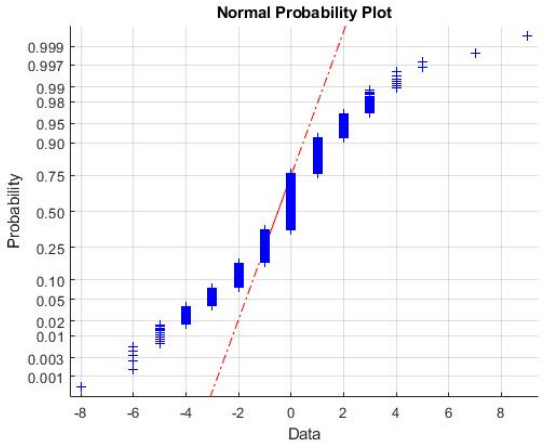



**Figure 5** Normal probability plot of differences in the number of pollen grains NF-EDF.

**Table 1** Medians of the observed geometric parameters of pollen grains.

| Method | Area [µm$^2$] | Length [µm] | Width [µm] | MaxFeret90 [µm] | Length/Width Ratio |
|---|---|---|---|---|---|
| NF | 531.24 | 28.39 | 18.78 | 25.96 | 1.52 |
| EDF | 490.68 | 27.31 | 18.00995 | 25.11 | 1.53 |
| *p*-value | *p* <0.001 | *p* <0.001 | *p* <0.001 | *p* <0.001 | 0.7851 |

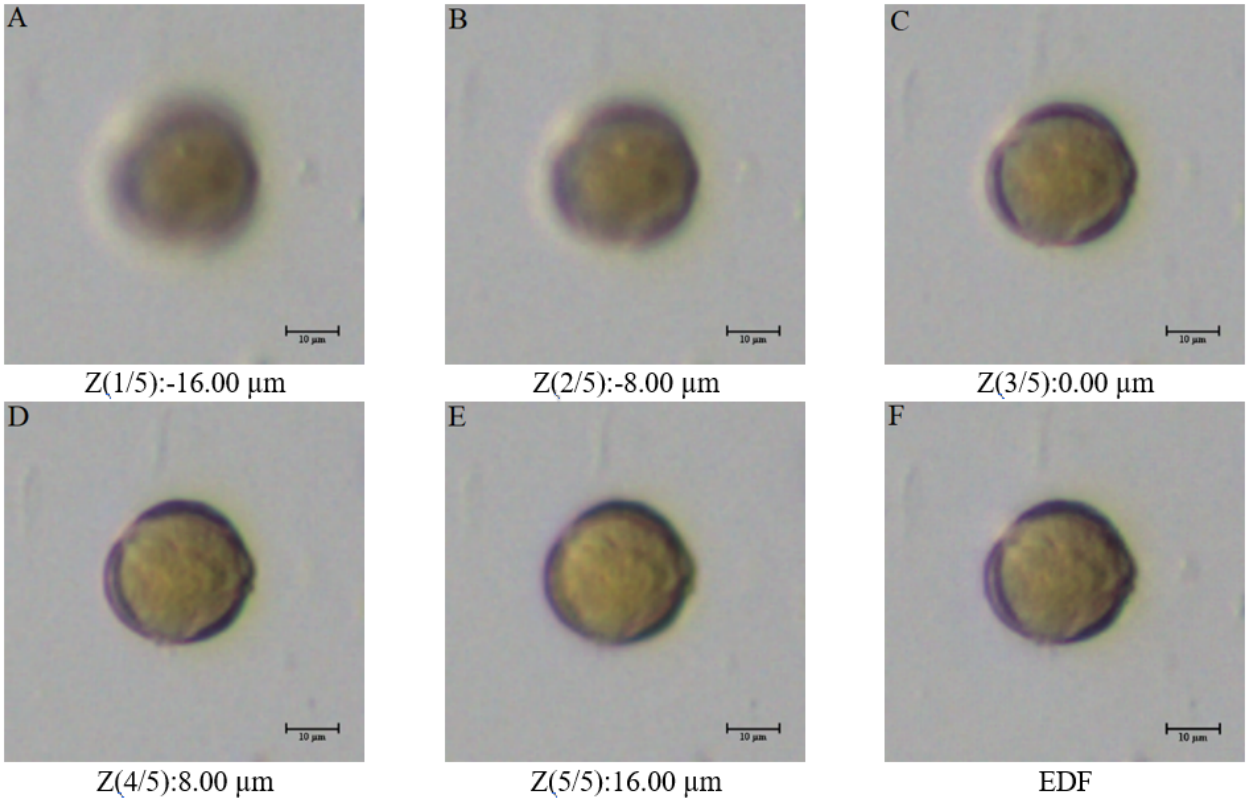



**Figure 6** Pollen sample of Brassica napus. Note: A – E: NF scanning, F: EDF scanning.

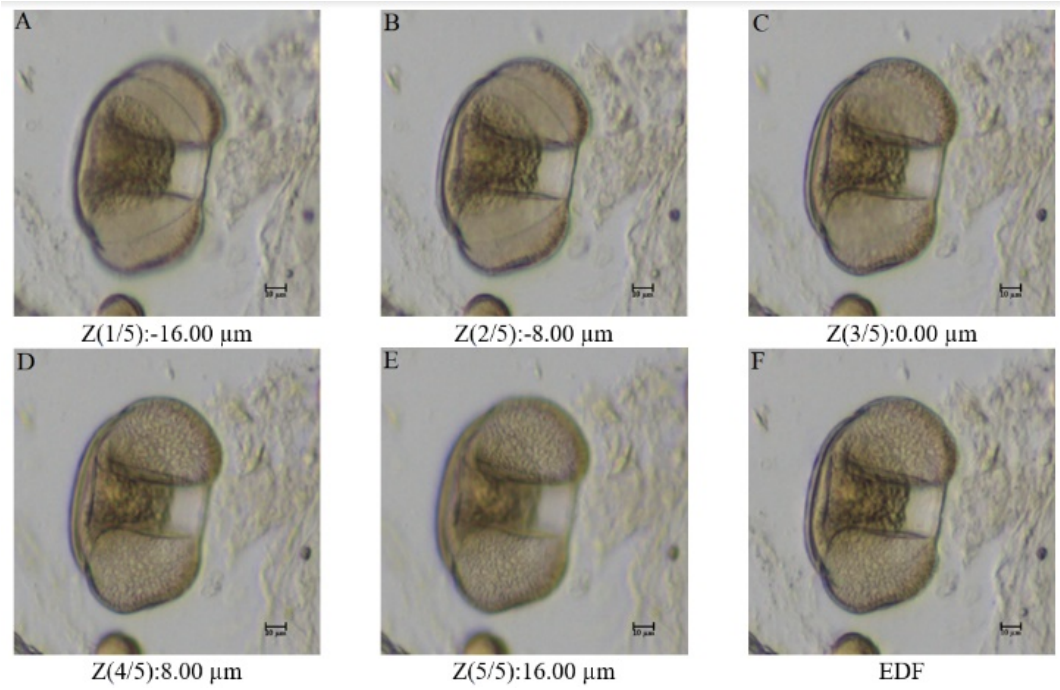



**Figure 7** Pollen sample of Pinus sylvestris. Note: A – E: NF scanning, F: EDF scanning.

All the observed geometric parameters (except the length/width ratio) can be argued that the smaller median indicates that EDF is a better method. In the case of imperfect focusing on NF, the observed pollen grain may contain image information "mixed" with the background, which increases all the monitored morphometric parameters (Figure 6 and Figure 7). Another reason for using the EDF method is the ability of automatic analysis to distinguish pollen grains from other honey impurities. As stated by **France et al. (1997)** in their study, identification of whether it is a pollen grain or not is problematic in automatic processing. The results may be affected by textural properties such as homogeneity, contrast/roughness and grey scatter. These are not only morphometric criteria. Textural properties, including morphometric parameters, can then be used to create classification rules that utilize these texture characteristics, as described by **Li et al., (2004)**. In their study, **Currie et al. (1997)** used only the morphometric parameters (length, width, area, and length/width ratio) of pollen grains, exine, and pollen pores for the discriminant analysis of apple varieties.

Further reduction of the median is caused by more grains detected. The "lost" pollen grains (not detected by the NF method) can be expected to have a smaller length, width, and area.

To compare the suitability of morphometric parameters in NF and EDF, the null hypothesis of the median equality of two statistical sets was tested by the nonparametric Mann-Whitney test. The medians of all observed morphometric characteristics are summarized in Table 1, as well as the *p*-values corresponding to the hypothesis tests.

Hence, it is clear that except for the length/width ratio, there is a statistically significant difference between NF and EDF in all observed geometric characteristics of pollen grains. Since the medians of all statistically significantly different geometric characteristics of EDF are smaller than the medians of NF, it can be argued that the identification of pollen grains in EDF images will be more efficient than in NF images. This rule was not confirmed for the recalculated length/width factor. This result confirms that this factor is a suitable criterion especially for individual evaluation of pollen grains **(Nazeri Joneghani, 2008)** even in the case of differently focused pollen grains in the analyzed image. It can also be assumed that in the case of comparing different focal planes or EDF images, a more suitable parameter is a criterion that is not affected by the texture of the pollen grain. As reported by several authors, it is appropriate to use rather a higher number of measured parameters for the use of image analysis, which will allow more accurate classification of the analyzed pollen grains **(Holt et al., 2011)**, than to choose one or a smaller group of measured parameters only.

## CONCLUSION

In this work, the extended depth of focus obtained from images from different focal planes and normal focused images was compared. The calculation and measurements of morphometric characteristics of pollen grains were used for comparison. The EDF method can detect a larger number of pollen grains in the evaluated images. A statistically significant difference between NF and EDF was demonstrated in all the observed morphometric characteristics of pollen grains except the length/width ratio. Identification of pollen grains in images based on morphometric criteria obtained by the EDF method is more efficient than the identification of pollen grains in images obtained by the NF method. Based on our results, the EDF scanning method is a method more suitable for image-based melissopalynological analysis, providing better results than the technique of scanning a single depth of field in NF.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE INFLUENCE OF THE DENITRIFYING STRAIN OF *STAPHYLOCOCCUS CARNOSUS* NO. 5304 ON THE CONTENT OF NITRATES IN THE TECHNOLOGY OF YOGURT PRODUCTION

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## ABSTRACT

Contamination of food with nitrates is a generally recognized problem. Milk is the basis for the production of many milk mixtures for baby food, and children are considered to be the most vulnerable category to the harmful influence of nitrates. The purpose of the search was to investigate the denitrification of milk with different amounts of nitrates by the denitrifying microorganisms of *Staphylococcus carnosus* in the technology of production of sour-milk products. The denitrification process of *S. carnosus* milk in the amount of 103 CFU.cm$^{-3}$ was found to reduce the nitrate content by an average of 88.0 ±3.9 mg.kg$^{-1}$ and in the samples of the first group was 10.3 ±2.4 mg.kg$^{-1}$, the second 110.7 ±4.1 and the third 214.5 ±6.3 mg.kg$^{-1}$, respectively. In the search of the denitrification process of *S. carnosus* milk in the amount of 104 CFU.cm$^{-3}$, was found that in the ready yogurt in the samples of the first group the amount of nitrates was 1.1 ±0.1 mg.kg$^{-1}$, in the second group 56.4 ±3.5 mg.kg$^{-1}$, and in the third 159.5 ±4.1 mg.kg$^{-1}$ respectively. In the search of the denitrification process of *S. carnosus* milk in the amount of 105 CFU.cm$^{-3}$, was found that nitrates were practically absent in the samples of the first group, the second group did not exceed 10 mg.kg$^{-1}$, and the third was 107.4 ±3.9 mg.kg$^{-1}$. Therefore, received data indicate the possibility of using strain *S. carnosus* No. 5304 for denitrification of milk with a high content of nitrates in the technology of production of fermented milk products, in particular yogurt.

**Keywords:** milk nitrates; milk denitrification; yogurt technology

## INTRODUCTION

Contamination of raw materials, water, and food with nitrates is a generally recognized problem **(Chamandust et al., 2016; Kukhtyn et al., 2018)**. Nitrates of food and water in the organism convert hemoglobin into meth and sulfhemoglobin, causing the development of hypoxia **(**Jiang **et al., 2012; Cockburn et al., 2013)**. Also, food nitrates are considered precursors of highly carcinogenic nitrous compounds **(Winter et al., 2007; Hord et al., 2011)**. The widespread use of nitrogen fertilizers in agriculture causes a large amount of nitrates to be supplied from soil to plants and foodstuffs **(Croitoru et al., 2015; Quijano et al., 2017)**, we often note the significant content of nitrates in drinking water for the population **(Kalaruban et al., 2016; Wang and Chu, 2016)**. Nitrates are also added to foods (cheese, sausage) to inhibit the development of spore-forming microorganisms **(Sungur and Atan, 2013)**.

Children are the most vulnerable and vulnerable to nitrate toxicosis, as the blood enzyme methemoglobin reductase, which restores methemoglobin to hemoglobin, begins to form in humans only at the age of three months **(Yeh et al., 2013)**. Besides, nitrates are poorly utilized in people with gastrointestinal disorders who have impaired intestinal microbiocenosis **(Sindelar and Milkowski, 2012)**. Therefore, children are virtually unprotected from nitrate toxicosis, and people with dysbacteriosis are more sensitive to the influence of nitrates **(Khosrokhavar et al., 2008; Hord et al., 2009)**. Because cow's milk belongs to the main food products, the determination of nitrate content in milk and dairy products is essential for the prevention of chronic influence of nitrates on children.

Various nitrates in cow's milk have been reported in the literature, so researchers in Italy found in the milk the amount of nitrates up to 140 mg.L$^{-1}$ **(Licata et al., 2013)**, while in Iranian cows milk nitrates ranged from 34 to 82.5 mg.L$^{-1}$. **(Chamandust et al., 2016)**. When the content of nitrates is determined in 20 cow's milk samples in Taiwan, it was revealed an average concentration of 14.3 mg.L$^{-1}$ **(Yeh et al., 2013)**, and researchers in Romania in the search of 95 milk samples revealed no more than 5 mg.L$^{-1}$ of nitrates over a four-year research period **(Filip et al., 2019)**. In Ireland under the research of skimmed milk powder was found nitrate concentration in the range from 5 to 120 mg.L$^{-1}$ **(Yeh et al., 2013)**. Thus, literature sources report that milk can be a source of significant input into the organism of nitrate consumers.

According to Regulation (EC) No 1881/2006 **(EC, 2006)** in foodstuffs (dairy mixtures), the permissible nitrate level is 50 to 200 mg.kg$^{-1}$. In the Ukrainian standard for raw milk, cow's milk with a nitrate content of not more than 10 mg.kg$^{-1}$ is authorized for processing **(DSTU 3662, 2018)**. The national standards of the People's Republic of China GB 2761-2017, GB 2762-20171 **(GB, 2017)** regulate the content of nitrites in raw milk, and they should not exceed 0.4 mg.kg$^{-1}$, and in milk powder 4 mg.kg$^{-1}$ **(GB, 2017)**.

Also, no data were found in the literature on the use of denitrifying microorganisms in the technology of dairy products. Researching the influence of microflora on the content of nitrates in the fermentation process of milk is promising since it would allow the production of low-nitrate dairy products.

Consequently, monitoring searches on the determination of nitrates and nitrites content in milk and dairy products will be important to ensure the safety of products and to develop recommendations and technologies for the possible reduction of these harmful substances.

The purpose of the search was to investigate the denitrification of milk with different amounts of nitrates by the denitrifying microorganisms of *Staphylococcus carnosus* in the technology of production of sour-milk products.

### Scientific hypothesis

Is the multiple uses of denitrifying strain *Staphylococcus carnosus* No. 5304 for milk denitrification with excessive content to a safe amount of nitrates in the technology of production of fermented milk products?

## MATERIAL AND METHODOLOGY

### Samples

In the experiments, 15 samples were used of milk with different amounts of nitrates. Milk of the first group (n = 5) had a nitrate content in the range of 100.2 ±3.4 mg.kg$^{-1}$; milk of the second group (n = 5) was with a nitrate content of 200.1 ±5.3 mg.kg$^{-1}$; third (n = 5) – 300.1 ±6.8 mg.kg$^{-1}$.

### Chemicals

To get milk with the amount of nitrates 100, 200 and 300 mg.kg-1 to it was added $KNO_3$. Chemically pure $KNO_3$ (Khimreaktiv, Ukraine) was used. To quantify Staphylococcus carnosus in yogurt was used the medium of yolk-salt agar (Pharmactive, Ukraine). For the production of yogurt was used leaven YoFlex®Acidifixtm 1.0 (Chr. Hansen, Denmark), which contains *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus salivarius subsp thermophilus*. Avirulent, incapable of invasion strain *of Staphylococcus carnosus* No. 5304 was used in the experiments, which is deposited in the Institute of Microbiology and Virology named after D.K. Zabolotnyj (Kyiv, Ukraine).

### Laboratory Methods

The content of nitrates in milk and yogurt was determined by colorimetric method using a cadmium column, followed by photometric determination of azo compounds, formed by the interaction of nitrites with aromatic amines **(GOST 32257, 2013)**. Herewith a LabAnalyt spectrophotometer (Granum, Ukraine) was used for determination of optical density of solutions, laboratory ionometer I-160MI (Micromed, Russia) to determine the pH. Water bath BN-09.2 LabAnalyt (Granum, Ukraine).

The titrated acidity of milk and yogurt was determined by the classical titrometric method **(GOST 3624, 1992)**.

### Description of the Experiment

The technological process of making yogurt involved the introduction of a culture of *Staphylococcus carnosus* in an amount of from $10^3$ to $10^5$ CFU.cm$^{-3}$ of milk, denitrification for 2 hours at a temperature of +37 °C, and subsequent application of sourdough cultures of lactic acid bacteria *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus salivarius subsp. thermophilus* followed by fermentation for 6 h at a temperature of 40 – 42 °C to the acidity of the product of 75 – 85 °T. After fermentation, the product was rapidly cooled to 4 °C and stored for 24 hours. After 2, 4, 8 and 24 hours, the nitrate content, the acidity of the product, and the amount of Staphylococcus carnosus were determined. All research was performed in triplicate.

### 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

Previous research has revealed that 20.7 to 30.2% of batches of raw milk containing nitrates (more than 100 mg.kg$^{-1}$) are coming to processing plants in the winter-spring period, in summer and autumn, the number of such batches of milk ranges from 2.1 to 7.8%. Therefore, we have researched the use of *S. carnosus* in a consortium of *L. bulgaricus* and *S. thermophilus* in the technology of production of fermented milk products as a possible denitrifying microorganism.

For the production of yogurt, an important technological indicator is the titrated acidity, which characterizes the acid-forming properties of lactic acid bacteria. Whereas, we offer additionally before fermentation for milk denitrification with a high content of nitrates to introduce the bacterium culture of *S. carnosus*, therefore, in the first stage of the search, the influence of this type of bacteria was determined on the change in the titrated acidity of sterilized milk, provided that it is used with classic sourdough yogurt (Figure 1).

It was found that the fermentation of milk by the fermentation of *L.bulgaricus* and *S.thermophilus* was carried out following the requirements of the technological instructions since the acidity did not exceed 85 °T after 6 h of fermentation. At the same time, the denitrifying species of *S. carnosus* practically did not ferment lactose, the acidity of milk did not change within 8 hours of fermentation, and after 24 hours was increased to 21 °T. Under the condition of fermentation of milk by a

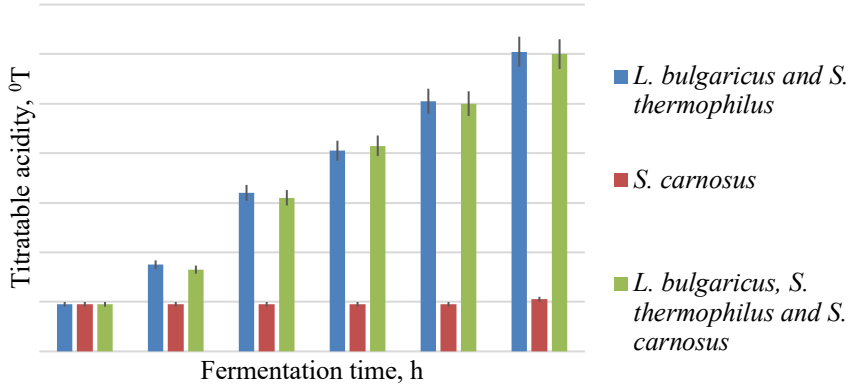


**Figure 1** Change of titrated acidity of sterilized milk by fermentation of *L. bulgaricus* and *S. thermophilus* and in consortium with *S. carnosus* at +40 °C.

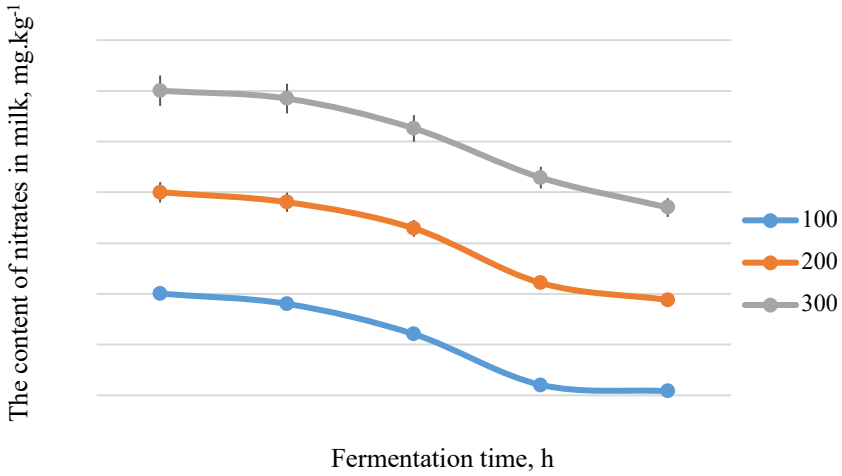


**Figure 2** Dynamics of the process of denitrification of milk with different nitrate content under the influence of *S. carnosus* in the amount of $10^3$ CFU.cm$^{-3}$.

consortium of microorganisms *L. bulgaricus, S. thermophilus* and *S. carnosus*, the dynamics of change of titrated acidity were as if using classical leaven.

The results of researches on the process of denitrification of milk with different nitrates are given with different nitrate content under the influence of *S. carnosus* in the amount of $10^3$ CFU.cm$^{-3}$ revealed (Figure 2), that for the initial amount of *S. carnosus* $10^3$ CFU.cm$^{-3}$ of milk within two hours of denitrification, the nitrate content was decreased by an average of 10 mg.kg$^{-1}$.

As can be seen from Figure 2, for the initial amount of *S carnosus* $10^3$ CFU.cm$^{-3}$ of milk within two hours of denitrification, the nitrate content was decreased by an average of 10 mg.kg$^{-1}$. Over the next two days (for the fourth hour of fermentation), the nitrate content was decreased by 37.4 ±3.1 mg.kg$^{-1}$, and at the end of the yogurt making process, their number was decreased in all samples by 88.0 ±3.9 mg.kg$^{-1}$ and in the samples of the first group was 10.3 ±2.4 mg.kg$^{-1}$, the second 110.7 ±4.1 and the third 214.5 ±6.3 mg.kg$^{-1}$, respectively. It was also found that, after cooling to +4 °C and endurance for 16 h (24 h), the denitrifying process did not stop, as the nitrate content was decreased by 6.2 ±0.3, 16.7 ±1.2 and 29.4 ±2.4 mg.kg$^{-1}$, respectively, possibly related to the nitriductase denitrifying action that accumulated in yogurt during *S. carnosus* development.

The results of searches of the process of milk denitrification are presented with different nitrate contents under the influence of *S. carnosus* in the amount of $10^4$ CFU.cm$^{-3}$ revealed (Figure 3), indicates the similar dynamics of nitrate reduction in milk as in Figure 2.

However, the intensity of the denitrification process at the initial amount of *S. carnosus* $10^4$ CFU.cm$^{-3}$ of milk was faster than its content of $10^3$ CFU.cm$^{-3}$. Thus, during the first two hours of denitrification, the amount of nitrates was decreased by 29.1 ±3.2 mg.kg$^{-1}$, and during the four hours by 81.3 ±2.1 mg.kg$^{-1}$. In the yogurt samples with an initial nitrate content of 100.2 ±3.4 mg.kg$^{-1}$ milk, their amount was 18.1 ±1.4 mg.kg$^{-1}$, and in the second group samples 118.2 ±3.7 mg.kg$^{-1}$, respectively.

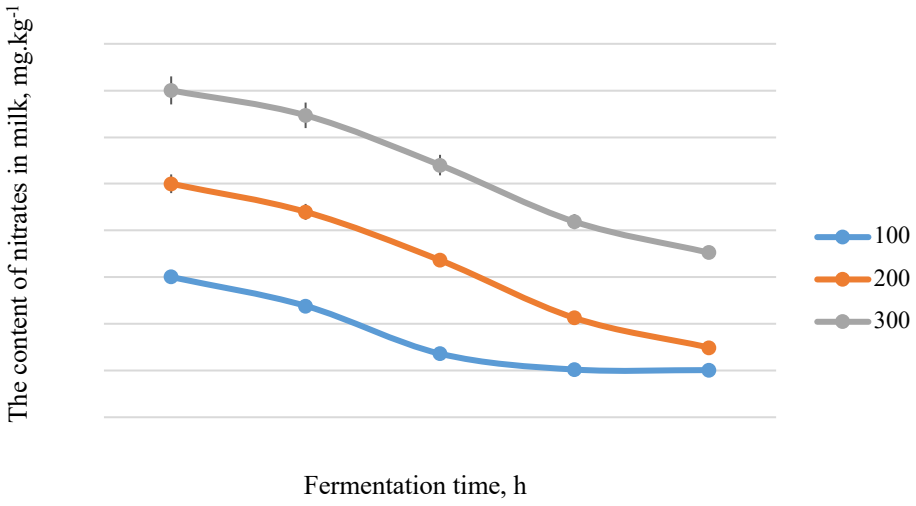


**Figure 3** Dynamics of milk denitrification process with native nitrate content under the influence of *S. carnosus* in the amount of $10^4$ CFU.cm$^{-3}$.

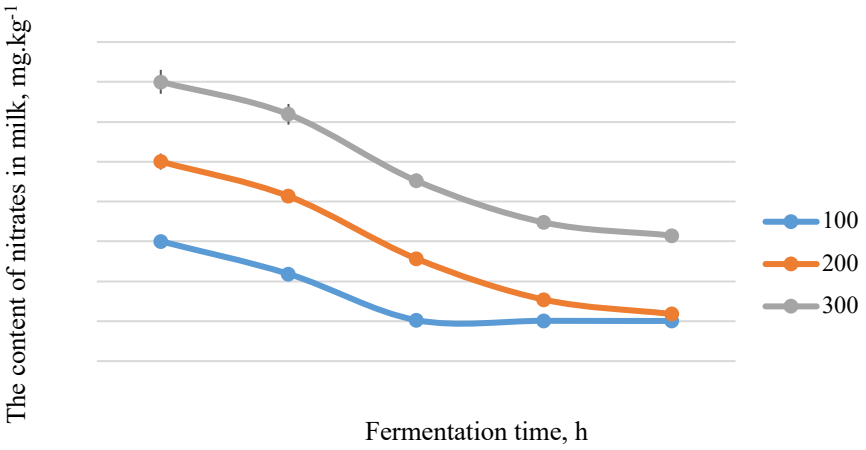


**Figure 4** Dynamics of denitrification of milk with native nitrate content under the influence of *S. carnosus* in the amount of $10^5$ CFU.cm$^{-3}$.

After 8 hours of fermentation the denitrification in the samples of the first group was revealed by 1.1 ±0.1 mg.kg$^{-1}$, in the second group by 56.4 ±3.5 mg.kg$^{-1}$, and in the third group by 159.5 ±4.1 mg.kg$^{-1}$. After 24 hours from the start of fermentation, the amount of nitrates in the samples of the second group was 24.7 ±2.1 mg.kg$^{-1}$, and 126.3 ±4.6 mg.kg$^{-1}$ in the third group.

The results of researches on milk denitrification process with different nitrate content under the influence of *S. carnosus* in the amount of $10^5$ CFU.cm$^{-3}$ (Figure 3), revealed the preservation of the general trend of denitrification of *S. carnosus* nitrates, which are shown in Figures 2 and 3.

Data of Figure 4 indicates the retained general tendencies for denitrification of *S. carnosus* nitrates, which are shown in Figures 2 and 3. However, with the initial amount of *S. carnosus* $10^5$ CFU.cm$^{-3}$ of milk, nitrate content in the samples of the first group was almost undetectable after 8 hours of denitrification, and in the samples of the second group the nitrate content was 27.1±2.3 mg.kg$^{-1}$ of product.

In the finished chilled yogurt, nitrates were practically absent in the samples of the first group, in the second group their amount did not exceed 10 mg.kg$^{-1}$, and in the third was 107.4±3.9 mg.kg$^{-1}$.

In the second stage, our searches were directed to study the activity (reproduction) of *S. carnosus* No. 5304 in sterilized milk in a consortium with lactic acid bacteria *L. bulgaricus, S. thermophilus*, which are used in yogurt production technology (Figures 5 and 6).

It was found that *S. carnosus* is developing well in sterilized milk, during the eight hours of incubation the number of cells is increased by more than 2.5 orders of magnitude. In this case, the growth curves of *S. carnosus* with different initial numbers have almost the same nature of increase. At the same time, the data of Fig. 6 indicate the ostent of the antagonistic action of lactic acid microorganisms used in sourdough for yogurt (*L. bulgaricus, S. thermophilus*) on the development of *S. carnosus* No. 5304.

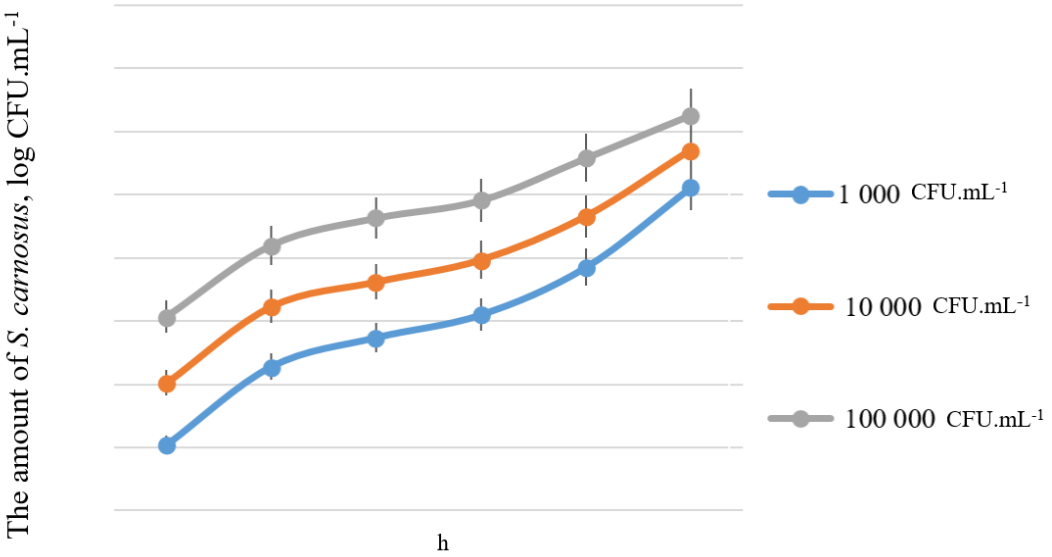


**Figure 5** Reproduction of *S. carnosus* No. 5304 in sterilized milk with different initial amounts at +40 °C.

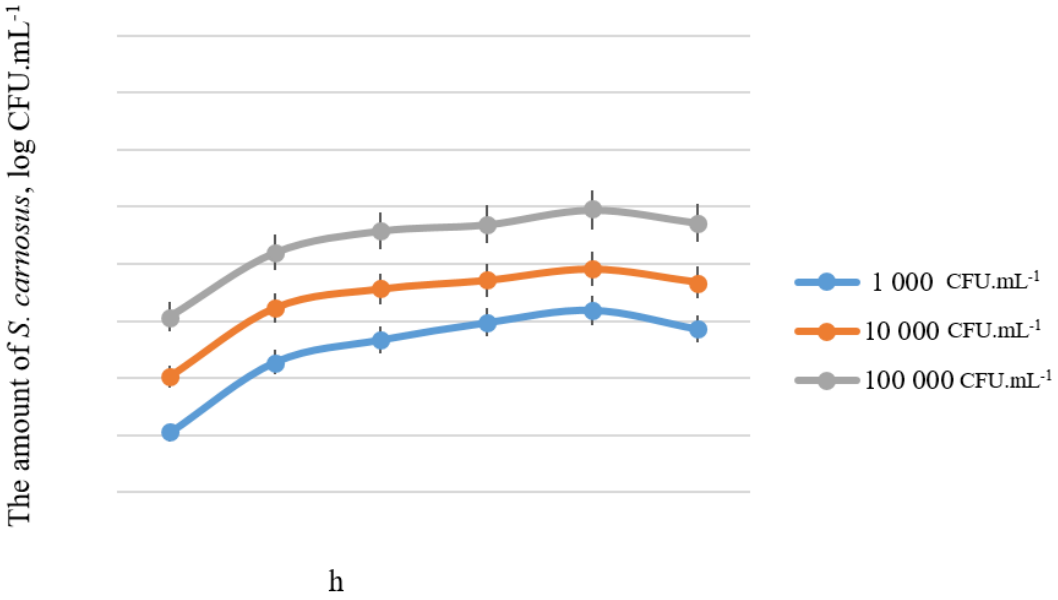


**Figure 6** The influence of fermentation of lactic acid bacteria (*L. bulgaricus*, *S. thermophilus*) on the reproduction of *S. carnosus* in sterilized milk with different initial amounts at +40 °C (Ferment of lactic acid bacteria introduced for 3 h of incubation).

Within 6 hours after the introduction of the leaven, we notice a slowdown in the reproduction of *S. carnosus*, compared with the development of pure culture. At 24 hours the search for staphylococcus reproduction was completely stopped their number even decreased due to the high acidity of the product.

The data obtained indicate that the process of denitrification takes place in the first hours of fermentation, and subsequently the enzymatic activity of staphylococci is suppressed by lactic acid microorganisms.

Nitrates and nitro replacement have a detrimental influence on human health and can accumulate and spread in an alarming amount in the "soil-plant-animal-human" trophic chain **(Bahadoran et al., 2016; Chamandust et al., 2016; Kukhtyn et al., 2018a; Hou et al., 2020)**. Therefore, the production of safe food products is an urgent issue, which is exacerbated by increased consumption and reduced food raw materials. Searches show that there is now a problem of nitrates in raw milk and dairy products **(Licata et al., 2013; Sungur and Atan, 2013; Chamandust et al., 2016)**. Thus, researchers **(Sungur and Atan, 2013)** found that 50% of cheese samples in Turkey had more nitrate levels than allowed, which is specified in the EU Regulation No. 1881/2006 **(EC, 2006)**. Our research also found samples of raw milk with a nitrate content of more than 100 $mg.kg^{-1}$.

At the same time, milk is the basis for the production of many milk mixtures for baby food, and children are considered to be the most vulnerable category to the harmful influence of nitrates. **(Thomson, Nokes and Cressey, 2007; Chetty and Prasad, 2016; Merino et al., 2017)**. In addition, scientists report that in people with chronic diseases of the stomach and intestines, the utilization of nitrates by the microbiome of the large

intestine is slowed down. Therefore, the amount of nitrates required for chronic nitrate poisoning will be less **(Winter et al., 2007; Sindelar et al., 2012; Kalaycıoğlu and Erim, 2019)**.

Therefore, we support the opinion of other researchers **(Hord et al., 2011; Jiang et al., 2012)** about the need to reduce the maximum amount of nitrates in dairy products, especially for baby food. Besides, according to the national standards of the Republic of China, the amount of nitrites is regulated in raw milk, and in the European regulation this rate is absent **(EC, 2006; GB, 2017)**.

Taking into account the above data, the influence of denitrifying bacteria *S. carnosus* on the dynamics of changes in the amount of nitrates in the technology of production of fermented milk products were investigated. Eventually, non-pathogenic denitrifying strains of bacteria can be actively developed along with lactic acid bacteria without changing the organoleptic and physicochemical properties of the product. In particular, researchers **(Bal-Prylypko and Leonova, 2015)** used strains of *S. carnosus* and *S. carnosus* spp. utilis in the technology of production of cooked sausages for utilization of sodium nitrite to safe quantities. Our research found that the denitrifying species of *S. carnosus* does not ferment lactose, as the titrated acidity of milk within 8 hours of fermentation did not change. Therefore, we believe that the development of milk denitrifying species of *S. carnosus* in milk does not influence the activity of the fermenting cultures of lactic acid bacteria *L . bulgaricus* and *S. thermophilus.*

In the search of the process of denitrification of milk with different content of nitrates of *S. carnosus* in the amount of $10^3$ CFU.cm$^{-3}$, it was found that at the end of the technological process of making yogurt (8 h) the amount of nitrates was decreased, on average by 88.0 ±3.9 mg.kg$^{-1}$ and in the samples of the first group was 10.3 ±2.4 mg.kg$^{-1}$, the second 110.7 ±4.1 and the third 214.5 ±6.3 mg.kg$^{-1}$ respectively.

In the search of the denitrification process of *S. carnosus* milk in the amount of $10^4$ CFU.cm$^{-3}$, it was found that 1.1 ±0.1 mg.kg$^{-1}$ of nitrates were found in the prepared yogurt in the samples of the first group, in the second group 56.4 ±3.5 mg.kg$^{-1}$, and in the third 159.5 ±4.1 mg.kg$^{-1}$, respectively. Thus, the received data indicate that an increase in the initial amount of *S. carnosus* denitrifying bacteria in milk before fermentation influences the denitrification intensity.

In the search of the denitrification process of *S. carnosus* milk in the amount of $10^5$ CFU.cm$^{-3}$, it was found that nitrates were practically absent in the samples of the first group, in the second group did not exceed 10 mg.kg$^{-1}$, and the third group was 107.4 ±3.9 mg.kg$^{-1}$. Therefore, received data indicate the possibility of using strain *S. carnosus* No. 5304 for denitrification of milk with a high content of nitrates in the technology of production of fermented milk products, in particular yogurt.

Scientists have mostly investigated the influence of denitrifying bacteria of the genus *Pseudomonas* on the reduction of nitrate content in milk **(Samigullin and Ezhkova, 2004)**, which found no implementation due to organoleptic changes in the product during the development of these bacteria. The influence of technological processing (separation) of milk on the reduction of nitrates in products was also investigated **(Lima et al., 2006)**. In addition, there are literature data on that heat treatment of vegetables and fruits does not significantly affect on reduction of nitrates in finished products **(Min et al., 2012; Habermeyer et al., 2015; Inoue-Choi et al., 2016; Bahadoran et al., 2016)**. However, technological process of vegetable fermentation significantly reduced nitrate content practically to the level of 1 – 5 mg.kg$^{-1}$. **(Kukhtyn et al., 2018b)**. In this search, it was found that *S. carnosus* develops well in milk and exhibits denitrifying properties without altering organoleptic properties. Lactic acid bacteria (*L. bulgaricus, S. thermophilus*) inhibit the development of *S. carnosus*, and in the ready product, its amount is reduced, which is probably due to the high acidity of the product. Therefore, we consider the possibility of using *S. carnosus* strain No. 5304 in the content of the leaven, which could be used for denitrification of milk with a high content of nitrates. The received data are of practical interest in terms of enabling the use of a safe method for processing milk with a nitrate content greater than the maximum amount.

So, as a result of the search, we note that, given the negative influence of nitrates of water r, raw materials and food nitrates on the organism of consumers, monitoring searches of milk content should be carried out. Based on the received data, it is possible to develop a ferment for denitrification of milk with a high content of nitrates.

## CONCLUSION

The denitrification process of *S. carnosus* milk in the amount of $10^3$ CFU.cm$^{-3}$ was found to reduce the nitrate content by an average of 88.0 ±3.9 mg.kg$^{-1}$ and in the samples of the first group was 10.3 ±2.4 mg.kg$^{-1}$, the second 110.7 ±4.1 and the third 214.5 ±6.3 mg.kg$^{-1}$, respectively. In the search of denitrification of milk S. carnosus in the amount of $10^4$ CFU.cm$^{-3}$, it was found that in the ready yogurt in the samples of the first group the amount of nitrates was 1.1 ±0.1 mg.kg$^{-1}$, in the second group 56.4 ±3.5 mg.kg$^{-1}$, and in the third 159.5 ±4.1 mg.kg$^{-1}$, respectively.

In the search of the process of denitrification of *S. carnosus* milk in the amount of $10^5$ CFU.cm$^3$, it was found that nitrates were practically absent in the samples of the first group, the second group did not exceed 10 mg.kg$^{-1}$, and in the third was 107.4 ±3.9 mg.kg$^{-1}$.

The antagonistic influence of lactic acid microorganisms, leaven (*L. bulgaricus, S. thermophilus*) on the development of *S. carnosus* No. 5304 has been revealed. 6 hours after the introduction of the leaven, we notice a slowdown in the reproduction of *S. carnosus*, and for 24 hours the reproduction of staphylococci has completely stopped.

Therefore, the received data indicate the possible use of *S. carnosus* strain No. 5304 in the content of the leaven for denitrification of milk with a high content of nitrates.

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The authors declare that they have no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE MINERAL COMPOSITION OF CHERRY (*PRUNUS CERASUS* MILL.) FRUITS DEPENDING ON THE SCION-STOCK COMBINATION

*Svetlana Motyleva, Galina Upadysheva, Tatyana Tumaeva*

## ABSTRACT

Cherry is a stone fruit crop that is valued for its early fruiting, productivity, and high taste and diet properties. The researches in the sphere of compatible scion-stock combinations selection of cherry crops with high winter hardiness and fruits quality were held in Federal Horticultural Research Center for Breeding, Agrotechnology and Nursery for these crops introduction in industrial gardening of the Central region of Russia. The mineral composition of four cherry cultivars of FHRCBAN selection (Apuchtinskaya, Rusinka, Volochaevka, and Molodezhnaya) grown on rooted and grafted plants (stocks AVCH-2, Izmaylovskiy, and Moskoviya) was studied for the first time. The influence of scion-stock combinations on 11 elements (Na, P, K, Mn, Fe, Mg, Ca, Co, Zn, Se, Mo) accumulation in the fruits was determined. The greatest proportion of mineral substances in cherry fruits belongs to K. Depending on the scion-stock combination its amount is varied from 13.89 mass % (Apuchtinskaya/Moskoviya) to 30.52 mass % (Molodezhnaya/Izmaylovskiy). For all cultivars fruits, the amount of K is 1.1 – 1.3 times higher on stock Izmaylovskiy than on rooted plants. The variation coefficient of K is low (V %) 1.37 – 6.91 for the majority of scion-stock combinations that indicate the stable entry of this element inside the fruit. The most content of all the elements under study was found in the fruits ash of Rusinka, Molodezhnaya, and Volochaevka on stock Izmaylovskiy that indicates the stable physiological state of the plants on these stocks. Results show that the cherry fruits analyzed could be an alternative to the supplementing of a mineral diet.

**Keywords:** *Prunus cerasus* (Mill.); scion-stock combinations; fruit; ash content; EDS analysis

## INTRODUCTION

Stone crops fruits grown in the gardens of the Central region of Russia play an important role in the quality functional nutrition of the population. Most unit weight belongs to cherry fruits. They contain macro and microelements, fiber, minerals, vitamins, bioactive phenolic compounds such as anthocyanins, coumarins, and others. Their consumption has a preventive and curative effect **(McCune et al., 2011; Robinson, Hoying and Dominguez, 2017)**.

The traditional growing of cherry trees in extensive gardens is connected with the growing of large-sized trees with extensional crowns **(Kiprijanovski et al., 2018).** For intensive gardens it is necessary to use scion-stock combinations suitable for close planting and which are characterized by early entry into fruiting and high quality of fruits **(Eremin, 2000; Upadysheva, 2009; Bujdoso, Magyar and Hrotko, 2019; Balducci, Capriotti and Mazzoni, 2019).** The influence of the stock on physiologic, biochemical, and molecular processes in the scion plants is not studied enough till the present time and is an actual subject of researches **(Nimbolkar et al., 2016)**. In the works of some researchers **(Gonçalves et al., 2005; Landi et al., 2014)** the influence of the stock on the physiological-biochemical parameters of the grafted trees leaves (photosynthetic pigments and metabolites composition), biological peculiarities of growth and fruiting was determined **(Jiméneza et al., 2007)**. The stock influences the intake of water, nutrients, and minerals from the soil to determine the intensity of above-ground system growth and photosynthesis, regulation and signalization, growth hormones, gene expression, and phloem proteins transportation. This, in its turn, causes the influence on fruits ripening, the profile formation of carbohydrates, phenolic compounds, and other biologically active substances in the grafted plant fruits **(Spinardi, Visai, Bertazza, 2005; Upadysheva, 2009; Usenik et al., 2010; Prasad et al., 2017; Upadysheva et al., 2018)**.

Literature data point out that some fresh fruits could be a supplementary source for the daily-recommended intake of minerals **(Gogoaşă et al., 2014; Mehari et al., 2015)**.

The important indicator of stonecrops fruits' nutritional value is micronutrients, the content of which also depends on the using stock. However, similar data about cherry trees are extremely few.



**Figure 1** The garden general view in the fruiting period.



**Figure 2** The fruiting of Molodezhnaya on stock Izmaylovskiy.

The researches of cherry scion-stock combinations which are characterized with high technological value and suitable for intensive plantation formation in the Central region of Russia are held in Federal Horticultural Research Center for Breeding, Agrotechnology and Nursery. The clonal rootstocks used are interspecific cherry hybrids: Izmaylovskiy (*Prunus cerasus* x *Prunus fructicosa* x *Prunus maackii*), AVCh-2 (*Prunus fructicosa* x *Prunus maackii*), and Moskoviya (*Prunus cerasus* x *Prunus maackii*). Trees grafted on the Izmaylovskiy and AVCH-2 rootstocks do not form root growth. Trees grafted on the Moskoviya rootstock give many root suckers, which complicates the technology of garden care. Own-rooted trees of the Apuchtinskaya variety are distinguished by low growth, high yield, and are recommended for laying intensive plantings **(Upadysheva and Belikova, 2020)**. Considering all this, the authors of this paper considered it would be worth assessing research was to study the mineral composition of cherry fruits received from scion-rooted and grafted plants depending on the scion-stock combination.

The authors measured the concentration of some essential microelements such as Na, P, K, Mn, Fe, Mg, Ca, Co, Zn, Se, and Mo, eventually assessed the mineral supply of cherry fruits.

**Scientific hypothesis**

Cherry fruits guarantee the intake of biologically active micronutrients in the human organism. The ash composition of cherry fruits depending on the scion-stock combination is not studied.

We checked the influence of the stock on the formation of mineral composition of cherry fruits grown in the conditions of the Moscow region. We suppose that the stock will influence the formation of cherry fruit mineral composition. Our work is devoted to this question studying.

## MATERIAL AND METHODOLOGY

The objects of the research were the fruits of 4 cherry cultivars of Federal Horticultural Research Center for Breeding, Agrotechnology and Nursery selection - Apuchtinskaya, Rusinka, Volochaevka, and Molodezhnaya from scion-rooted and grafted on stocks AVCH-2, Izmaylovskiy, and Moskoviya plants. The cherry trees were grown on the selection plant of FHRCBAN in the Leninskiy district of the Moscow region. The plantation's overall area is 0.6 ha. The garden of intensive type is set out using a 5 x 2.5 m scheme. The soil in the row spacing is under perennial grassing (Figure 1).

The fruits were picked up at the ripeness stage (Figure 2).

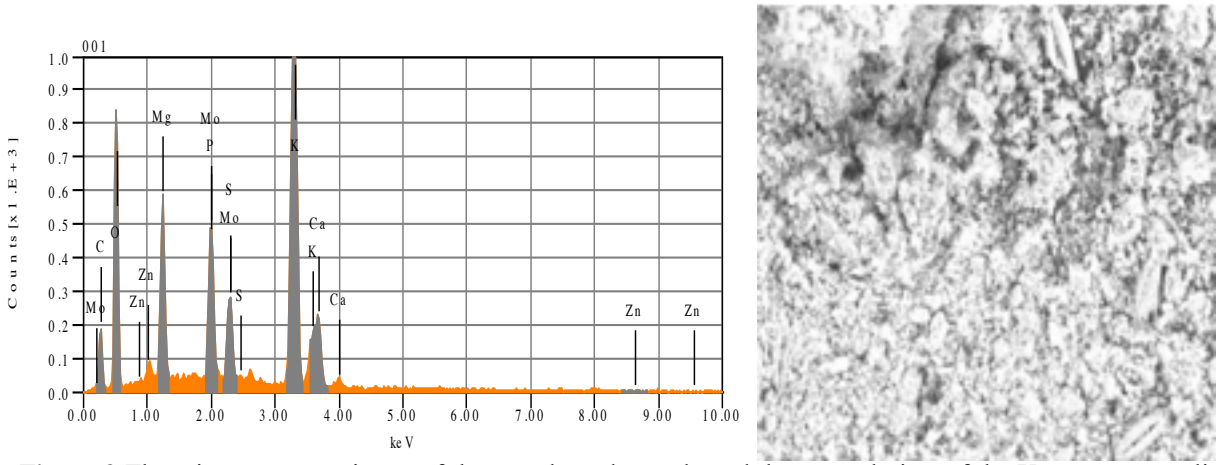


**Figure 3** The microstructure picture of the sample under study and the general view of the X-ray spectrum lines that show the elements presence in the analyzing area. Note: Mg – 6.39; P – 5.73; S – 2.20; K – 26.77; Ca – 5.49 and Zn – 0.13 mass % respectively.

The laboratory research was performed in 2017-2019 at the Laboratory of Physiology and Biochemistry of FHRCBAN.

***Sample preparation***

Cherry fruits were washed in distilled water, dried, separated from stones, selected average samples weighing 10 g, put in porcelain crucibles, dried in drying oven Snol (Russia) at T = 80 °C. The dried samples were mineralized in a muffle furnace Naberterm LVT15/11/B180 (Germany) with a temperature increase of 10 °C/15 min at T = 450 °C 5 hours. The received ash was pounded with a pestle, homogenized in the ultrasound unit, applied on the object table covered with carbonic scotch Agar (Germany), and put in the camera of the electron microscope.

***EDS - analysis***

The chemical composition of the basic ash components (Na, P, K, Mn, Fe, Mg, Ca, Co, 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 solution 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. An energy-dispersive spectrometer allows to carry out the quantitative X-ray microanalysis with the desired analyzing area: in a point or areally. and to receive the maps of elements allocation. X-ray microanalysis data are presented in the form of standard protocols that 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 3.

Taking into consideration the spectrum line 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 um.

**Statistical analysis**

All analyses were **quadruple**-time repetition. Results were expressed as mean values (n = 4) in standard deviation (*SD*). Used the Statistics, version 6 program.

To determine the differences - significance between the data one-way and two-factor experience ANOVA test was used ($p$ <0.05) via the program Statgraphics Centurion.

## RESULTS AND DISCUSSION

The most proportion of mineral substances in cherry fruit belongs to K. Its content depending on cultivar and scion-stock combination varies from 13.89 mass % (Apuchtinskaya/Moskoviya) to 30.52 mass % (Molodezhnaya/Izmaylovskiy), Table 1. In the fruits of all cultivars received from rooted trees the content of K varies insignificantly (21.75 – 24.81) mass %. On stock AVCH-2 the content of K showed a 1.1 times increase in the fruits of Molodezhnaya, 1.2 times in the fruits of Rusinka, and 1.3 times in the fruits of Volochaevka respectively. On stock Moskoviya the content of this element increased insignificantly in Rusinka and Volochaevka, and in the fruits of Molodezhnaya and Apuchtinskaya decreased on 22 and 37% respectively. The variation coefficient of K for the majority of scion-stock combinations is low (V %) 1.37 – 6.91, which shows a stable intake of this element inside the fruits. According to the data of **Heghedűş-Mîndru et al. (2013)**, the content of K in cherry fruits is not only higher than in sweet cherry and plum fruits, but this element is also taken up better. K is a macroelement that is responsible for the regulation of the majority of metabolic reactions that flow in living organisms. The very special role in controlling the homeostasis belongs to K. It controls osmotic pressure transmembrane potential, charges equilibrium, cathode-anion balance, and pH – everything that the homeostasis of cells and tissues consists of. In the ionic form, K can be found in all the organs, tissues, and cell structures in concentrations that exceed the concentration of other ions **(Meathnis et al., 1997)**.

The content of P, Mo, Mg Ca in the fruits of the combinations under study is given in Figure 4.

**Table 1** The pecularities of K accumulation in the cherry fruits depending on stock.

| Scion-stock combination | Defined parameters | | | | |
|---|---|---|---|---|---|
| | K (mass %) | max | min | S | V (%) |
| **Apuchtinskaya own rooted** | 21.99 | 22.31 | 21.19 | 0.538 | 2.45 |
| **Rusinka own rooted** | 21.75 | 22.14 | 21.42 | 0.298 | 1.37 |
| **Volochaevka own rooted** | 22.47 | 22.97 | 21.84 | 0.496 | 2.21 |
| **Molodezhnaya own rooted** | 24.84 | 25.46 | 24.05 | 0.299 | 2.41 |
| **Apuchtinskaya /AVCH-2** | 19.39 | 19.82 | 18.76 | 0.500 | 2.58 |
| **Rusinka/AVCH-2** | 25.92 | 26.17 | 25.33 | 0.395 | 1.52 |
| **Volochaevka/AVCH-2** | 30.03 | 31.24 | 29.08 | 1.01 | 3.35 |
| **Molodezhnaya/ AVCH-2** | 27.17 | 29.14 | 22.11 | 3.392 | 15.33 |
| **Apuchtinskaya /Izmaylovskiy** | 24.46 | 26.41 | 21.34 | 2.281 | 9.34 |
| **Rusinka/ Izmaylovskiy** | 26.23 | 34.12 | 23.31 | 5.273 | 20.10 |
| **Volochaevka/ Izmaylovskiy** | 30.24 | 31.13 | 29.94 | 0.534 | 1.76 |
| **Molodezhnaya / Izmaylovskiy** | 30.52 | 32.14 | 29.87 | 1.041 | 3.52 |
| **Apuchtinskaya /Moskoviya** | 13.86 | 14.28 | 13.19 | 0.511 | 3.64 |
| **Rusinka/Moskoviya** | 23.76 | 24.19 | 23.24 | 0.494 | 2.10 |
| **Volochaevka/Moskoviya** | 23.18 | 25.14 | 21.22 | 1.602 | 6.91 |
| **Molodezhnaya/Moskoviya** | 19.49 | 23.22 | 14.25 | 2.043 | 20.89 |

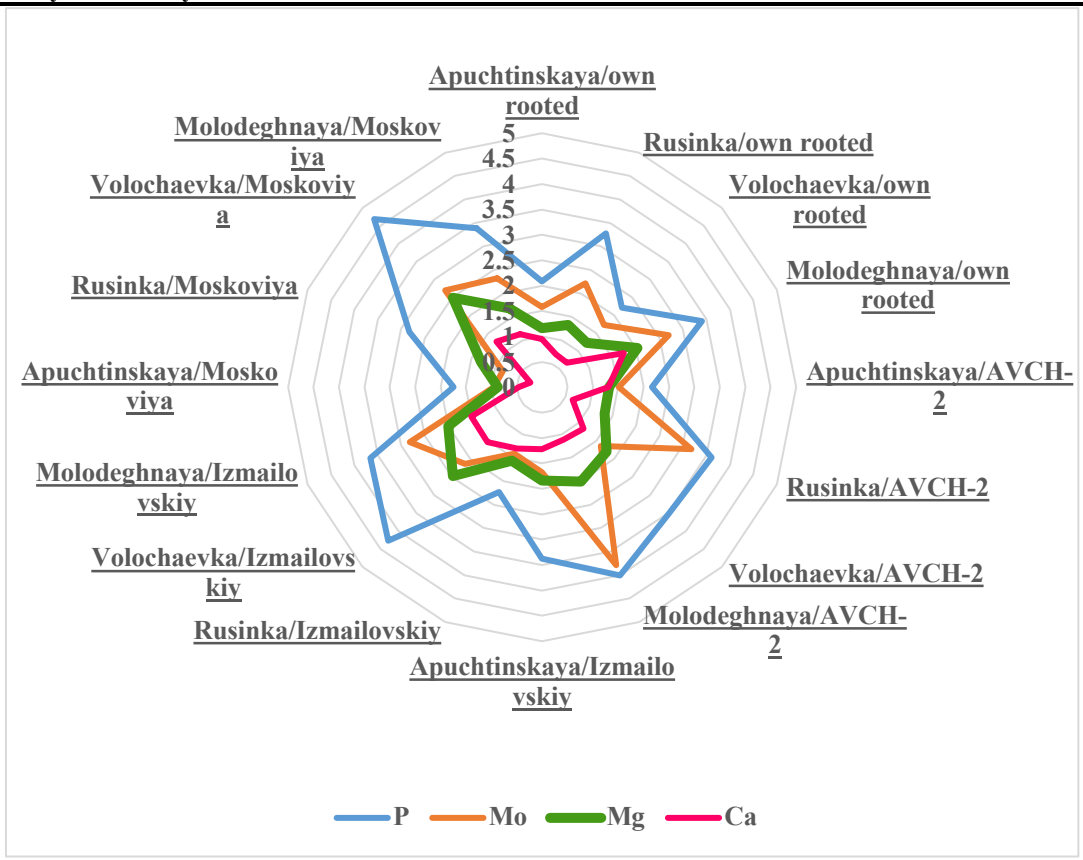


**Figure 4** The content of P, Mo, Mg and Ca in the cherry fruits on different stocks, mass %.

The most content of P is determined in the fruits of scion-stock combinations Volochaevka/Moskoviya, Molodezhnaya/Izmaylovskiy, Volochaevka/Izmaylovskiy, Rusinka/AVCH-2, Volochaevka/AVCH-2, and Molodezhnaya/AVCH-2. The proportion of Mo in cherry fruits is 0.5 – 2 times lower than P.

The most content of Mo was marked in the fruits of the following combinations: Molodezhnaya/Izmaylovskiy (3.2 mass %), Molodezhnaya/AVCH-2, and Volochaevka/AVCH-2 (3.6 mass %).

The content of Mg is at 1.2 – 2.5 mass % level in the cherry fruits. In the cherry fruits on stocks Moskoviya and Izmaylovskiy, the proportion of Mg is 2 – 2.5 mass %.

The most proportion of Ca (1.5 – 1.7 mass %) was stated in the cherry fruits of rooted plants of Molodezhnaya, combinations Molodezhnaya/Izmaylovskiy and Volochaevka/Izmaylovskiy. Scion-stock combinations Apuchtinskaya/AVCH-2 and Apuchtinskaya/Izmaylovskiy are also specific in the content of Ca. The minimal content of P, Mo, Mg, and Ca was marked in the fruits of Apuchtinskaya and Volochaevka roots, Apuchtinskaya/Moskoviya combination.

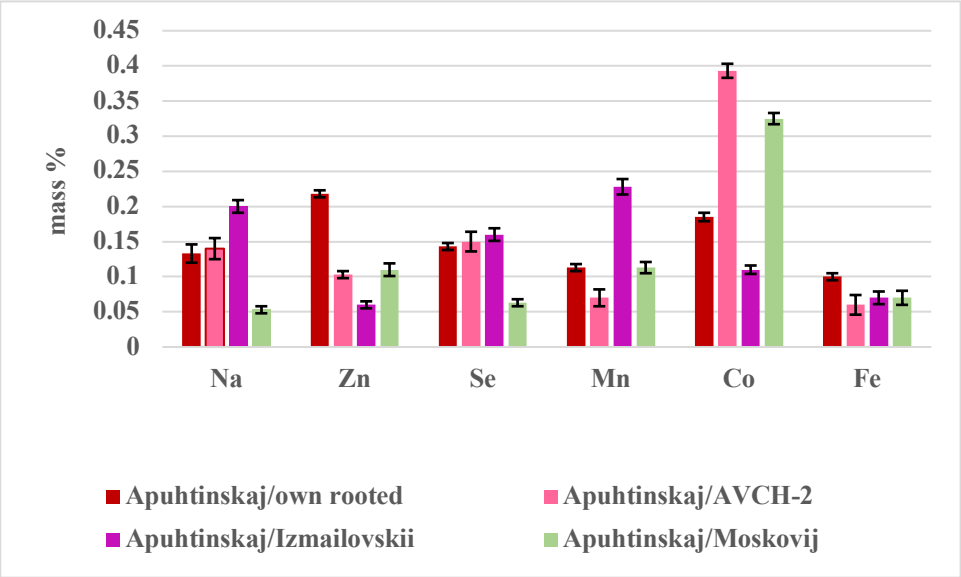


**Figure 5** The content of Na, Zn, Se, Mn, Co and Fe in the cherry fruits of Apuchtinskaya on different stocks.

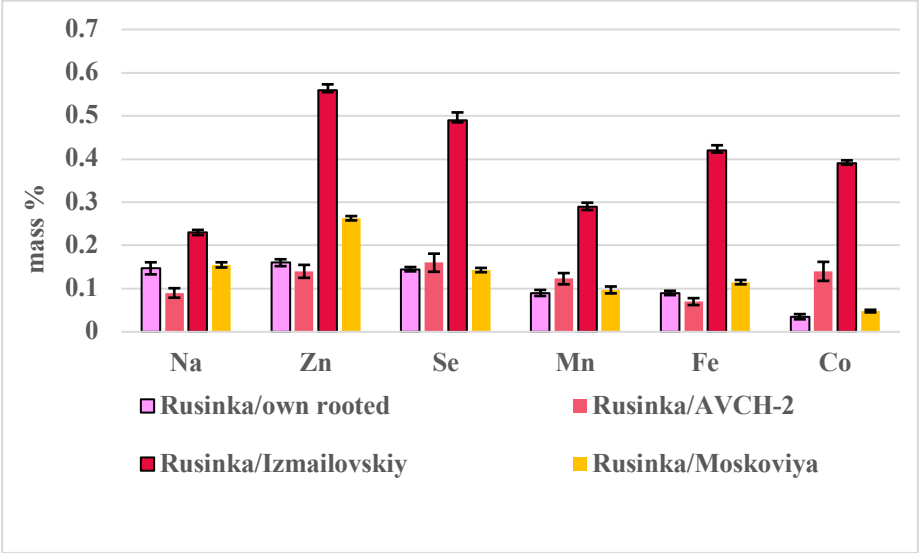


**Figure 6** The content of Na, Zn, Se, Mn, Co, and Fe in the cherry fruits of Rusinka on different stocks.

The received data about the content of Ca, Mg, and P in cherry fruits correlate with the data published in the works **(Papp et al., 2008; Kalyoncu, Ersoy and Yılmaz, 2009)**.

High concentrations of K, Mg, and Ca in the fruits of *Laurocerasus officinalis* Roem. and the content of Mn, Fe, and Zn, comparable with our data, are noted in the work **(Kolayli et al., 2003; Mahmood et al., 2012)**.

**Usenik et al., (2005)** the effects of rootstock on sweet cherry mineral composition (N, P, K, Ca, Mg, S, B, Cu, Fe, Mn, Mo, and Zn) was studied in 'Lapins' grafted on ten different rootstocks. The highest influence of rootstock was observed concerning P, K, Mg, and Fe.

P plays a very important role in cell energetics. Mg is necessary for the processes of regeneration and renewal of cells, tissues, and organs. It activates a large number of enzymes that take part in the processes of $CO_2$and N assimilation. In cytosol Mg counter-balances organic compounds (sugars groups, nucleotides, organic and amino acids). Mg is necessary for the keeping up of the cathode-anion balance and pH regulation **(Avtsyn et al., 1991)**.

The content of Na, Zn, Se, Mn, Co, and Fe in the fruits of Apuchtinskaya is given in Figure 5.

Scion-stock combination Apuchtinskaya/Izmaylovskiy is specific in the high content of Na, Se, and Mn. In the fruits of Apuchtinskaya/Moskoviya and Apuchtinskaya/AVCH-2 combinations, the proportion of Co is 0.32 mass %, that shows twice the increase than in the fruits of rooted plants. The content of Fe in the fruits of Apuchtinskaya grafted trees is lower than at roots and does not exceed 0.1 mass %.

The fruits of Rusinka picked up from grafted trees have a richer mineral composition in comparison with rooted plants (Figure 6). Thus, the content of Na is 1.5 – 2 times, Zn – 1.5 times, and Fe – 0.6 times higher. Cherry of the Rusinka variety on the Izmailovsky rootstock has the highest trace element content. The Zn content is 3.2, Se in 2.8, Mn in 2.5, Fe in 3.8, and Co in 8 times higher in comparison with the fruits of rooted trees.

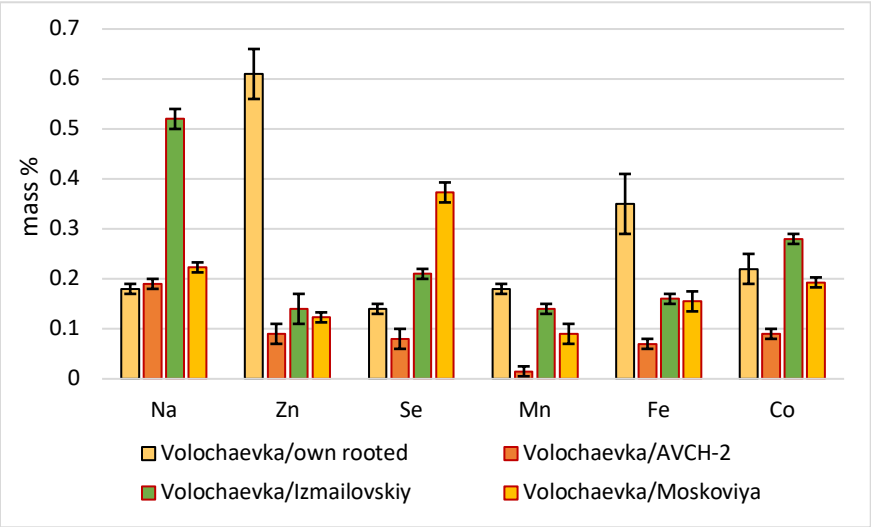


**Figure 7** The content of Na, Zn, Se, Mn, Co and Fe in the cherry fruits of Volochaevka on different stocks.

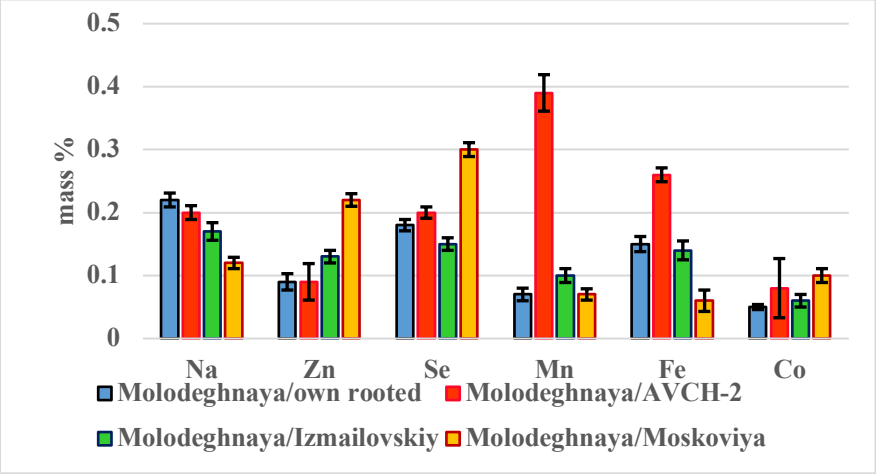


**Figure 8** The content of Na, Zn, Se, Mn, Co, and Fe in the cherry fruits of Molodezhnaya on different stocks.

In the cherry fruits of Volochaevka (Figure 7) on stock Izmaylovskiy, the content of Na is 2.5 times and Se and Co – 0.8 times higher; on stock Moskoviya in the fruits, the content of Se is 2.6 times higher than in cherry roots.

Researchers **(Jurikova et al., 2012)** provide data on the low Na content in *Prunus tomentosa* fruits as compared to the Mg and Ca content.

In the fruits of Molodezhnaya (Figure 8) on stock AVCH-2, the content of Mn is 3 times, Fe – 1.5 times higher and the content of Se and Co is insignificant. In the cherry fruits of the scion-stock combination, Molodezhnaya/Moskoviya Zn is accumulated 2 times and Se – 1.5 times higher in comparison with the fruits of rooted trees. Se is one of the most important trace elements. Interest in selenium is mainly due to its powerful antioxidant activity. In the human body, selenium plays an important role in the functioning of the immune system. The work **(Serradilla et al., 2016)** indicates that cherry is a source of Se.

Fe is an essential microelement: it has a respiratory function and makes up hemoglobin and certain enzymes. It plays a role in cell energy, in the processes of oxi-reduction, and the synthesis of nucleic acids; it supports the human body's defense system against the aggressiveness of infectious factors. Assimilating iron is linked to the presence of copper, cobalt, and manganese, as well as to that of vitamins C **(Ismail et al., 2011; Naeem et al., 2017)**.

Mn a microelement that enhances the activity of many enzymes and vitamins of Complex B has beneficial effects on the nervous system reducing irritability and increasing the ability to memorize. Zincum, a microelement essential mainly in the maintenance of reproduction and immune system health **(Slavin and Lloyd, 2012)**. Mn an essential microelement involved, together with Zincum and copper, in some enzymatic processes. Cu, an essential microelement, is involved, together with Zincum and manganese, in numerous enzymatic systems; Co, a microelement that makes up vitamin B12, playing an essential role in the making up of blood red cells and being also known for its vasodilating properties **(Avtsyn et al., 1991; Ishida, 2004)**, was determined in much smaller amounts than Fe, Zn, Mn.

The order of accumulation of elements in fruits according to average data for the same for varieties Rusinka and Apuchtinskaya and has the form: K > P > Mo > Mg > Ca > Mn > Zn > Se > Co > Na > Fe. In the fruits of varieties Molodezhnaya and Volochaevka, the order is different.

In the fruits Molodezhnaya – K > P > Mo > Mg > Ca > Na > Se > Fe > Zn > Mn > Co.

**Table 2** Table 2: Correlation coefficient between 11 trace elements matrix for the 11 elements in the ash pollen of cherry fruits.

| Elements | K | P | Mo | Mg | Ca | Na | Mn | Fe | Si | Zn | Co |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **K** | 1 | 0.53 | 0.55 | 0.63 | 0.29 | 0.01 | -0.41 | -0.01 | -0.01 | -0.04 | 0.05 |
| **P** | | 1 | 0.84 | 0.54 | 0.32 | -0.09 | -0.46 | -0.48 | -0.51 | -0.55 | -0.42 |
| **Mo** | | | 1 | 0.59 | 0.39 | -0.52 | 1 | -0.22 | -0.50 | -0.25 | -0.19 |
| **Mg** | | | | 1 | 0.78 | 0.09 | -0.05 | -0.3 | -0.08 | -0.42 | -0.11 |
| **Ca** | | | | | 1 | -0.42 | 0.14 | -0.03 | 0.29 | -0.34 | 0.18 |
| **Na** | | | | | | 1 | 0.23 | -0.14 | 0.15 | -0.19 | -0.02 |
| **Mn** | | | | | | | 1 | 0.61 | 0.56 | 0.17 | 0.75 |
| **Fe** | | | | | | | | 1 | 0.38 | 0.73 | 0.53 |
| **Si** | | | | | | | | | 1 | -0.01 | 0.58 |
| **Zn** | | | | | | | | | | 1 | 0.12 |
| **Co** | | | | | | | | | | | 1 |

In the fruits Volochaevka – K > P > Mo > Mg > Ca > Zn > Na > Se > Fe > Co > Mn. The genotypes we studied to be important sources of nutritive minerals. Thus, cornelian cherry consumption can contribute to a well-balanced diet. The varietal differences in the accumulation of mineral elements in fruits of different cherry varieties on different rootstocks that we established are consistent with the data available in the scientific literature, for example, research by **(Gozlekci et al., 2017)**, have found that the order of accumulation of nutritive minerals depending cherry genotypes. In the article, they give the following series of accumulation of elements K > Ca > P > Mg > Na > Fe > Mn > Zn > Cu. In the article **(Mitić et al., 2012)** cherry cultivars were analyzed using inductively coupled plasma optical emission spectrometry. The four elements are very abundant (K, Na, Ca, and Mg), and four are not abundant (Cu, Fe, Mn, and Zn). The order of the content of these elements in fruits of two cherry varieties is K > Mg > Ca > Na > Fe > Zn > Mn.

Calculated correlation coefficients between elements (Table 2).

There is a high correlation between elements, for example, P and Mo (r = 0.84); Mg and Ca (r = 0.78) and Mo and Mn (r = 1).

The mean correlation is between K and Mg (r = 0.63); Mn and Fe (r = 0.61); Mo and Mg (r = 0.59); Si and Co (r = 0.58); Mn and Si (r = 0.56); K and Mo (r = 0.55); P and Mg (r = 0.54); K and P and Fe and Co (r = 0.53); Mo and Na (r = 0.52).

Weak correlation found between Mo and Ca (r = 0.39); P and Ca (r = 0.32); K and Ca and Ca and Si (r = 0.29); Na and Si (r = 0.25); Na and Mn (r = 0.23); Ca and Mn (r = 0.14).

## CONCLUSION

The results of 11 mineral element accumulation that characterizes the micronutrient composition of cherry (*Prunus cerasus* Mill.) fruits depending on the used scion-stock combination and on rooted plants are presented in the present paper. As a result of the researches, the influence of a cultivar and a stock on micro- and macroelements accumulation was determined and the limits of their content change depending on the used stock were stated. The most proportion of mineral elements in cherry fruits belongs to K. The variation coefficient of K is low (V %) 1.37 – 6.91 for the majority of scion-stock combinations that show a stable intake of this element in the fruits. Descending series of the content of the elements under study is the following: K > P > Mo > Mg > Ca > Na > Zn > Se > Mg > Zn > Se > Mn > Fe > Co.

Stocks Izmaylovskiy and AVCH-2 conduced high accumulation of P and Mo in Molodezhnaya, Volochaevka, and Rusinka and higher accumulation of Zn, Se, Mn, and Fe in the fruits of Rusinka and Molodezhnaya in comparison with the content of these elements in the fruits of rooted plants and on other stocks. The fruits of Apuchtinskaya on stock Izmaylovskiy are specific in a higher content of Na and Mn in comparison with rooted trees fruits and Co, Zn, and Se on stocks AVCH-2 and Moskoviya. Using the data of laboratory researches it can be stated that the optimal stocks that conduce mineral elements accumulation in the fruits of Rusinka, Volochaevka, and Molodezhnaya are Izmaylovskiy and AVCH-2. In the fruits of roots trees, the content of elements is minimal. In the fruits of Apuchtinskaya on rooted plants, macro- and microelement composition of all elements under study is richer than on scion-stock combinations. In the fruits of Apuchtinskaya on rooted plants, macro- and microelement composition of all elements under study is richer than on scion-stock combinations. The results give new information about the influence of a stock on the formation of cherry fruits mineral composition and confirm that fresh cherry fruits can be additional sources of macro and micronutrients.

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The authors declare no conflict of interest.

**Ethical Statement:**

This article does not contain any studies that would require an ethical statement.

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# THE MICROPERFORATED PACKAGING DESIGN AND EVALUATION OF SHELF LIFE OF FRESH-SLICED MUSHROOMS

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## ABSTRACT

Fresh mushrooms have a very short shelf life, of 1 – 3 days because of their high respiration rate and lack of cuticles that protect the plant from external factors. In the case of fresh-sliced mushrooms, they will be more susceptible to spoilage reactions due to the increase in respiration rate as a result of a broader surface area. Conventional packaging materials can not meet the requirements for modified atmosphere packaging of fresh-sliced mushrooms. One of the techniques to extend the fresh-cut produce shelf life is the passive modification of modified atmosphere packaging technology. For highly respiring fresh-cut produce such as fresh-sliced mushrooms, the permeability properties of the polymeric materials might not be enough to provide an equilibrium gas concentration in the passive modification of modified atmosphere technology. In this case, the microperforated packaging materials can be used for passive modification of fresh-cut produce. But the microperforation process needs a design for the application of the appropriate number and diameter of microholes to meet the requirements of passive modified atmosphere packaging. For this reason in this research, the design of the microperforation process to be used in passive modified atmosphere packaging was based on the diameter and the number of microholes, and the shelf life of fresh-sliced mushrooms was determined. The samples were stored at 15 °C/80% RH, and pH, color, weight loss, textural, sensorial, and microbial analysis were performed periodically during storage. It was determined that the empirical equation used in this research can be applied to microperforated packaging design for fresh-sliced mushrooms. The shelf life of the fresh-sliced mushrooms packaged with microperforated packaging material was 8 days, while it was less than 7 days (4, 5, or 6 days) when packaged with non-microperforated packaging material. This result shows that the use of microperforated packaging material is effective in extending the shelf life of fresh-sliced mushrooms.

**Keywords:** modified atmosphere packaging; passive modification; fresh-sliced mushrooms; microperforation; shelf life

## INTRODUCTION

Fresh mushrooms are known as very perishable commodities, with a short shelf life of 1 – 3 days at ambient temperature. Due to their fine and porous epidermal cell structure, their respiration rate is higher compared to those of the other vegetables and fruits (200 – 500 $mg.kg^{-1}.h^{-1}$ at 20 °C) **(Kader, 1986; Warwick and Tsureda, 1997; Singh et al., 2010)**. Their short shelf life can be attributed to the high respiratory rate of fresh mushrooms and also their lack of cuticles to protect them from physical or microbial changes or dehydration **(Mau, Miklus and Beelman, 1993; Lee, 1999; Villaescusa and Gil Muñoz, 2003; Kamal et al., 2015)**. Slicing mainly accelerates the spoilage reactions since it leads to an increase in the respiration rate and creates a broader surface area susceptible to dehydration, browning, and microbial growth **(Brennan and Gormley, 1998)**. This situation is also observable for fresh-sliced mushrooms. Several reactions such as browning, wilting, slaking, loss of water, textural deformations, loss of aroma, off-flavor occur more rapidly in the fresh-sliced mushrooms, and in this way, the shelf life of these products becomes shorter **(Iqbal et al., 2009; Singh et al., 2010; Kamal et al., 2015)**.

Conventional polymeric films are unfavorable for the packaging of fresh-sliced mushrooms because their ratio of $CO_2$ to $O_2$ diffusion rates is high and this is not suitable for products requiring high $CO_2$ and low $O_2$ concentrations due to increased risk of anaerobiosis **(Oliveira et al., 1998; Fonseca, Oliveira and Brecht, 2002)**. For this reason, microperforated polymeric materials may be an option to maintain the optimum gas concentrations in the package since diffusion rates of $O_2$ and $CO_2$ are very close to each other **(Brody, 2005; Gonzalez-Buesa et al., 2009; Toivonen, Brandenburg and Luo, 2019; Cliff et al., 2010; Forney and Yaganza, 2011; Hussein, Caleb and Opara, 2015)**.

Microperforated films (perforation diameter <200 µm) are commonly used in the modified atmosphere packaging (MAP) of fresh food products with high respiration rates; for example, minimally processed fruits and vegetables **(González et al., 2008)**. The microperforations in the polymeric film allow a much higher exchange of gases than

conventional polymeric films. The $O_2$ concentrations in the headspace of the package used for the fresh-sliced mushrooms have been maintained below 5%. This value must be higher than 1% to inhibit the growth of anaerobic and pathogenic microorganisms. The $CO_2$ concentration in the headspace should be higher whereas higher concentrations than 12% might lead to physiological disorders and browning in mushrooms **(Parentelli et al., 2007)**. Hence the use of microperforated packages for fresh-sliced mushrooms might be an alternative solution for the attainment of the desired gas combinations and the preservation of the quality characteristics of these products. So far, a limited number of studies concerning the extension of the shelf life of the fresh-cut produce mushrooms by using perforated polymeric films in packaging have been reported in the literature. Several scientists **(Oliveira et al., 2012a)** studied the optimum MAP conditions of the sliced mushrooms in packages perforated by a needle (each perforation has a diameter of 0.33 mm) to achieve the best headspace gas composition, for maintaining quality and extending the shelf life of fresh-sliced mushrooms. In this study, they packed fresh-sliced button mushrooms in a tray covered with cellophane film and monitored the headspace gas composition and the quality characteristics of weight loss, pH, firmness, and color at storage conditions of 10 °C for 3 days. As a result, they found that the optimum packaging conditions as; 110 g of sliced mushrooms and 2 perforations in the film, leading to 3.6% of $O_2$ and 11.5% of $CO_2$ in the headspace gas composition, after 3 days at 10 °C. In another research **(Oliveira et al., 2012b)**, the authors examined the effect of temperature and number of perforations on the package on the quality of fresh-sliced mushrooms. Sliced mushrooms were packed in a tray, covered with cellophane film, and stored for 7 days at four levels of temperature (0, 5, 10, and 15 °C) and three levels of perforations at each temperature ranging the number of perforations from 1 (58 perforations per $m^2$) to 6 (349 perforations per $m^2$). Headspace gas composition and quality parameters (weight loss, pH, firmness, and color) were measured throughout the storage period. They found that at least one perforation was needed to avoid null $O_2$ concentration that might lead to anaerobic respiration and the potential resulting in the growth of pathogens. They also showed that the needle diameter used (0.33 mm) was too big to use at lower temperatures, therefore a smaller needle diameter which might yield gas composition close to the optimal range is recommended. Fresh-sliced mushrooms had a shelf life of 1, 2, 4, and 7.5 days at 15, 10, 5, and 0 °C, respectively, under optimum MAP conditions. In another study **(Pogorzelska-Nowicka et al., 2020)**, the authors studied the effect of high oxygen atmosphere (80% $O_2$) in conjunction with Polysulfone films of different levels of microperforations (6, 12, 23, 25 holes with a diameter of 90 μm, 91 μm, 90 μm, and 143 μm respectively) on antioxidant capacity, volatile compounds profile, sensory acceptance, and quality of fresh mushrooms stored at 20 °C for 5 days. Some authors **(Rivera et al., 2010)** studied the design of modified atmosphere packaging suitable for *Tuber melanosporum* and *Tuber aestivum* truffles that extend their shelf life and their availability as a fresh product. They found that the microperforated film with 2 holes (90 × 50 μm) was the best to be used in modified atmosphere packaging of fresh truffles. The quality and microbiological characteristics of fresh truffles, packaged in this film revealed that the microbial counts of *Pseudomonads* and *Enterobacteriaceae* were decreased, the weight loss was reduced, the typical hard texture was maintained, and the development of mycelium growth was delayed, enabling good scores for aroma and flavor, and therefore prolonging the shelf life of *T. melanosporum* and *T. aestivum* truffles to 28 and 21 d, respectively.

To maintain the quality and extend the shelf life of fresh-sliced mushrooms packaged with the microperforated packaging materials during the storage period, the design of the perforation process is necessary. This design must be performed carefully by taking the possible effects of several parameters in the perforation into consideration. The size and the number of microperforations in the polymeric packaging material are the key parameters for the microperforation process. In a polymeric film, the gas transmission rate (TR) is directly proportional to the area of the perforation. The $O_2$ or $CO_2$ transmission rates of a microhole with a mean diameter can be calculated by using the initial $O_2$ and $CO_2$ concentrations in the headspace and the equilibrium gas composition, the number of perforations required in a packaging film can be calculated with the $O_2$ or $CO_2$ transmission rates **(González et al., 2008)**.

## Scientific Hypothesis

The hypothesis of this study was to determine whether the shelf life of freshly sliced mushrooms could be extended by using microperforated packaging materials compared to non-perforated packaging materials in passive modified atmosphere packaging. The microperforation process should be designed according to the requirements of gas compositions of the headspace of passive modified atmosphere packaging and the respiration rate of the product. Therefore the objectives of this research are (i) to optimize the number of microperforations performed by laser perforation technique in packaging material of fresh-sliced mushrooms at a storage temperature of 15 °C to which the product can be exposed during storage in the market, (ii) to determine the shelf life of the fresh-sliced mushrooms packaged with this optimized microperforated packaging material by analyzing the headspace gas concentrations, pH, weight loss, color, hardness, microbiological and sensorial properties of the mushrooms.

# MATERIAL AND METHODOLOGY

## Samples

White button mushrooms (*Agaricus bisporus*) were supplied by the Gülen Mushroom Farm (Antalya, Türkiye) and transported to Ege University (Izmir, Türkiye) at 4 ±2 °C and 80% ±1 RH. Transparent, heat sealable, and antifog biaxially oriented polypropylene (BOPP) with a film thickness of 30 μm was used as the packaging material provided by the Bak Ambalaj Inc. (Izmir, Türkiye).

## Chemicals

Plate Count Agar (Merck, United States), Pseudomonas CFC Agar (Merck, United States), Pseudomonas CFC Selective Supplement (Merck, United States), and Peptone from Casein (Merck, United States) were used in microbiological analysis.

## Instruments

The changes in the oxygen and carbon dioxide concentrations during storage in the headspace of the packaging materials were measured by the headspace analyzer (PBI Dansensor Checkmate, Ringsted, Denmark). The microperforation process of the packaging films was performed using UV Laser equipment (Laseral, Izmir, Turkey) having F100 optical and 355 nm lenses. The diameters of the holes were determined via a stereomicroscope (Leica CLS 150 XE, Germany) and their image was monitored by scanning electron microscopy (SEM Thermo Scientific Apreo II). The pH measurements were made by the device HANNA HI83141 (Rhode Island, USA). The color changes and Hunter color values ($L^*$, $a^*$ and $b^*$) of the sliced mushrooms were determined using Hunter colorimeter (Colorflex, CFLX 45-2 model Colorimeter, Hunter Lab, Reston, VA). The hardness of the samples was analyzed using the texture analyzer (Stable Micro System, Txt Plus, UK) equipped with a cylindrical probe (P/R 36).

## Laboratory Methods

Oxygen and water vapor permeability tests were conducted by the standardized methods **(ASTM D3985, 2017)** at 25 °C and 0% RH and **(ASTM F1249, 2020)** at 37.8 °C, 90% RH respectively. The TAMB count was performed as described by the method described in the **ISO 4833-1 (2013)**. The principle of the count of *Pseudomonas* spp. is based on the method described by **ISO 13720 (2010)**.

## Sample preparation

The mushrooms were dried and sliced at a thickness of 0.5 cm after being separated manually and washed to eliminate the specks of dirt. Then, they were first placed into the trays made of polystyrene foam (each tray contains 125 g of mushrooms). These trays were placed in pouches of 21 x 29.7 cm made of microperforated and non-perforated BOPP. The pouches were heat-sealed afterward. The samples were stored for 10 days in a climate cabinet (Nüve- TK 120, Turkey) at 15 ±1 °C and 80% ±1 RH. The shelf life analysis was conducted periodically on days 0, 2, 4, 7, 8, 9, and 10 of the storage.

## Determination of the respiration rate

The respiration rate of the mushrooms was determined by monitoring the change in the gas concentration inside the leak-proof packaging material throughout the entire storage process of the product. For this purpose, sliced mushroom samples (each of 200 g) were stored in the glass jars of 5 L at 15 °C for 24 hours. The initial and final gas concentrations were measured by the headspace analyzer (PBI Dansensor Checkmate, Ringsted, Denmark). The respiration rate in terms of $O_2$ and $CO_2$ were calculated using equation (1) and equation (2) respectively.

$$R_{m1} = \left(A_i - A_f\right) x \frac{V}{100} \quad (1)$$

$$R_{m2} = C_f \times \frac{V}{100} \quad (2)$$

Where:
$R_{m1}$, $R_{m2}$: Respiration rate; $A_i$: Initial $O_2$(%) concentration (21%), $A_f$: Final $O_2$(%) concentration; $C_f$: Final $CO_2$ concentration (%), V = Volume of the jar, (mL).

## Microperforated packaging design

### *Determination of the diameter of the micro holes*

The standardization of the micro perforation was carried out by the adjustment of the point time (PT) which determines the focusing time of the beams and the optimal diameter and number of the holes. Several PT's were tried in nine replicates and the optimum PT has been determined to obtain standard diameter and circular holes. The diameters of the holes were determined via a stereomicroscope (Leica CLS 150 XE) and their image was monitored by scanning electron microscopy (SEM Thermo Scientific Apreo II, USA). Minimum three replicates were performed for this process.

### *Determination of the number of holes concerning the respiration rate*

The number of holes micro perforated on the packaging film was calculated by considering the following parameters: The $CO_2$ transmission rate of the microhole, the headspace volume, the $CO_2$ volume in excess in the headspace. The $CO_2$ transmission rate was calculated by using the mean diameter of the measurements and equation (3) **(González et al., 2008)**.

$$TR = a_1 {A_h}^{a_2} \quad (3)$$

Where;
TR = $CO_2$ or $O_2$ transmission rate (mL $day^{-1}$); $a_1$, $a_2$ are the constants for $O_2$ or $CO_2$; $A_h$ = area of each microhole ($\mu m^2$).

## Determination of the shelf life of the fresh-sliced mushrooms

Headspace, pH, weight loss, color, hardness, microbiological, and sensory analyses were performed to determine the shelf life of the mushrooms. Each analysis was conducted in triplicate. The storage process of microperforated packaged samples was performed with two replicates.

### *Headspace analysis*

The PBI Dansensor Checkmate (Ringsted, Denmark) digital analyzer was used to determine the $O_2$ and $CO_2$ concentrations in the headspace.

### *pH*

10 g of sample was homogenized with distilled water of 50 mL and then the measurement was performed with a pH meter.

### *Weight loss*

It was determined gravimetrically and expressed in terms of percentage. The weight loss was calculated using the following equation:

$$\Delta M = \frac{M_i - M_t}{M_t} \times 100 \quad (4)$$

Where:
$\Delta M$, $M_i$, and $M_t$ denote the percentage change in the weight loss, initial weight, and weight at the instance of measurement respectively.

***Color***

The color analysis was carried out by assessing L*, a*, and b* values. The instrument was calibrated with black and white standard plates before the analysis. The reported data are the mean of eight measurements made for each sample. Total color difference (ΔE) and browning index (BI) were calculated by using the equations below.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (5)$$

$$BI = \frac{100(x-0.31)}{0.172} \quad (6)$$

$$x = \frac{a+1.75L}{5.645L+a-3.012b} \quad (7)$$

***Hardness***

Five slices of each sample lot were analyzed in terms of hardness. The test velocity and the compression rate were adjusted as 1.5 $mm.s^{-1}$ and 75% respectively. The hardness of the sample was expressed in terms of the maximum force (g). The analysis was conducted in triplicate.

## Microbiological analysis

The microbiological growth during the storage of the samples was observed to determine the shelf life of the mushrooms. To detect the initial amount of microorganisms in mushrooms, total aerobic mesophilic bacteria, *Pseudomonas* spp., *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes,* and *E.coli* O157 analysis were performed on day 0. During the shelf life, total aerobic mesophilic bacteria and *Pseudomonas* spp. counts, which are the parameters affecting the shelf life of mushrooms, were determined. The mushroom samples were prepared in ten-fold dilutions and homogenized by using appropriate diluents.

***Total aerobic mesophilic bacteria count (TAMB)***

Briefly, 10 g of mushroom samples were added into the stomacher bags containing 90 mL 0.1% peptone solution. The samples were serially diluted in 0.1% peptone solution to the appropriate concentration after homogenization. The analysis was performed by the pour plate method using Plate Count Agar (PCA, Merck). The colonies were counted and the CFU $g^{-1}$ was calculated after incubation of the plates at 30 ±1 °C for 72 hours.

***Pseudomonas spp. count***

10 grams of the samples were weighed into Stomacher bags under aseptic conditions, and decimal serial dilutions were prepared until $10^{-7}$. 0.1 mL of each decimal dilution were spread over the surface of Pseudomonas CFC Agar (Merck) plates and incubated at 25 °C ±1 °C for 48 h ± 2 h. After incubation, Petri dishes containing 30-300 colonies were counted.

## Sensory evaluation

The sensory characteristics of the sliced mushrooms were assessed in terms of texture, odor, appearance, and overall acceptability by five trained panelists. For the evaluation, a scoring test including the hedonic scale ranging between 1 and 5 was used. The samples having a score higher than or equal to 3 were evaluated as acceptable in the sensory tests.

## Statistical Analysis

The experimental data were analyzed using the SPSS software **(IBM Corp., 2013)**. Homogeneity of the variance tests was performed by the Levene statistics. The statistically significant differences among the sample groups were determined using one-way ANOVA. The tests concerning the differences between the sample groups with non-homogenous distribution were carried out using Mann-Whitney U tests. The standard deviations of the means were computed in a spreadsheet (Microsoft Excel, Office 2016, Microsoft Corporation).

# RESULTS AND DISCUSSION

## Determination of the respiration rate

The respiration rates of the fresh-sliced mushrooms after 24 hours of storage were measured 1925.27 ±159.6 and 1782.38 ±105.291 mL ($kg^{-1}.24h^{-1}$) for $O_2$ and $CO_2$ respectively.

## Determination of the diameter and number of the microholes

For standardization in terms of the uniformity of the microholes, other parameters except for the point time (PT) were kept constant. The optimized PT was determined as 0.9 to obtain uniform microholes each having a diameter of 25.6 ±3.96 µm. This value was used in the calculation of the number of microholes. The SEM image of such a microhole with a diameter of 25.22 µm was presented in Figure 1.

The TR of the $CO_2$ and $O_2$ were calculated using Equation (3) for a single hole with a mean diameter of 25.6 µm as 28.97 and 32.29 $mL.day^{-1}$ respectively. The headspace volume was determined as 310 mL which was calculated by subtracting the volume occupied by 125 g mushrooms (130 mL) from the total volume of the polystyrene foam tray (440 mL). In the literature, it was reported that the value for the $CO_2$ transmission rate should not exceed 12% **(Parentelli et al., 2007)**. This rate was corresponding to 37.2 mL in our experiments. The TR of $CO_2$ for 125 g of mushrooms was calculated 222.8 mL by using the mean value for $CO_2$ (1782.38 $mL.kg^{-1}.day^{-1}$). The volume of $CO_2$ in excess in the package was computed 185.6 mL by subtracting the maximum allowable $CO_2$ rate in the package (37.2 mL) from the TR of $CO_2$. This value (185.6 mL) refers to the volume of $CO_2$ that must be discharged from the package per day. The number of microholes was calculated as 6.41 (approx. 6) by dividing 185.6 mL by the $CO_2$ permeability of a single microhole (28.97 $mL.day^{-1}$).
The design of UV laser perforation was performed with 6 microholes and shown in Figure 2.

## Determination of the permeability of the BOPP films

To reveal the effectiveness of the microperforation, the permeability tests were conducted for both non-perforated and microperforated samples. The oxygen transmission rate (OTR) and water vapor transmission rate (WVTR) of the BOPP films were measured for microperforated samples as 3184.9 ±89.9 $cc.m^{-2}.day^{-1}$ and 31.0 ±1.3 $g.m^{-2}.day^{-1}$ and non-perforated samples as 1300.0 ±25.15 $cc.m^{-2}.day^{-1}$ and 6 ±0.24 $g.m^{-2}.day^{-1}$ respectively.

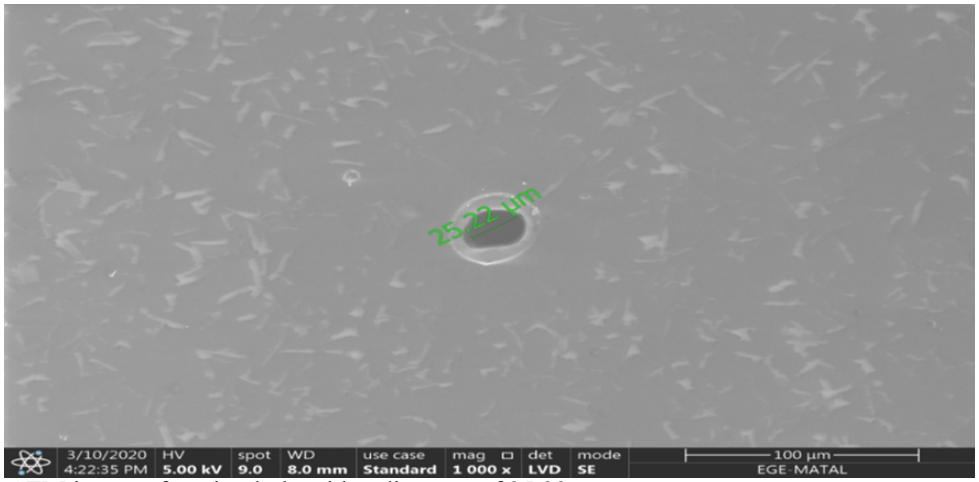


**Figure 1** The SEM image of a microhole with a diameter of 25.22 µm.

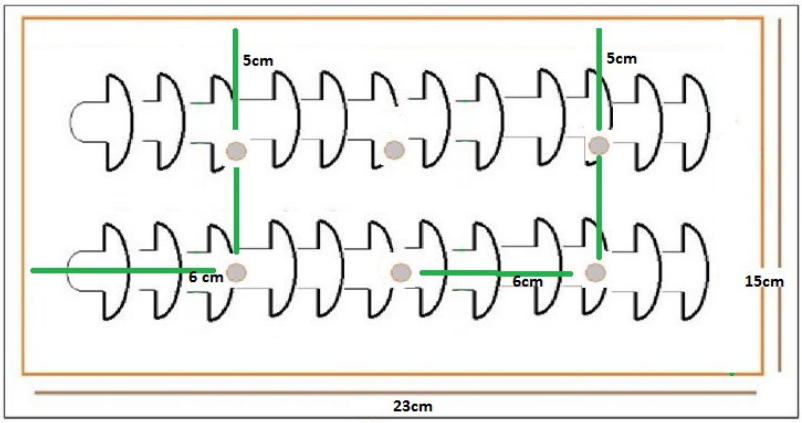


**Figure 2** The schematic design of the microperforated BOPP films showing the microperforated holes on the package.

The microperforation seemed to be successful since the OTR and WVTR values of the BOPP films increased drastically in this way. The effect of the enhancement in the permeability on the shelf life of the mushrooms was described in detail under the title "Determination of the shelf life of the fresh-cut mushrooms" section.

## Validation of the empirical TR formula using permeability test results

To validate the empirical formula described in the research **(González et al., 2008)**, the TR of $O_2$ for 6 microholes was calculated as 193.7 mL (32.29 mL.day$^{-1}$ x 6). Using Eq. (3) with the dimensions of BOPP films (0.21 x 0.297 m) and the $O_2$ transmission rate (3184.9 cc.m$^{-2}$.day$^{-1}$) determined experimentally, the volume of $O_2$ was calculated 198.6 mL.day$^{-1}$ for 6 microholes which were indicating that the theoretical and experimental TR of $O_2$ were close to each other and the empirical formula was applicable in this case.

## Determination of the shelf life of the fresh-sliced mushrooms

To determine the shelf life of the mushrooms, the samples were stored at 15 °C, 80% RH for 10 days in microperforated and non-perforated BOPP packages. Before starting with the analysis on the day of the experiment, the photos of each sample were taken and presented in Figure 3.

### *The quantitative changes in the headspace gas composition during storage*

The deterioration of the mushrooms might be caused by the impact of several anaerobic conditions, which also depend on the $O_2$ level in the headspace and accelerate the spoilage when the $O_2$ level becomes less than 1%, which may result in the tissue softening and undesired odors **(Parentelli et al., 2007)**. **Almenar et al. (2007)** and **De Reuck, Sivakumar and Korsten (2009)** revealed that extremely low oxygen levels in the headspace might trigger anaerobic reactions which result in off-odors and off-flavors. Thus, the $O_2$ level measured in the headspace might be a good indicator for microbial spoilage. On the other hand, $CO_2$ was claimed to have an important role in the retention of textural characteristics, when it is present at high concentrations in the headspace **(Gonzalez-Fandos et al., 2001; Simón, Gonzáles-Fandos and Tobar, 2005)**. The quantitative changes in the headspace gas composition were presented in Figure 4.

As seen in Figure 4, the $O_2$ level in the headspace declined to 0.45% after 4 days of storage in the non-perforated samples, whereas it fell below 1% (0.4%) after 9 days of storage in the microperforated samples. Consequently, one might state that the microbiological spoilage might have started after 4 days in the non-perforated samples, and after 9 days of storage in the microperforated group.




**Figure 3** Microperforated (MP) and non-perforated (NMP) packaged samples during storage period of 0th, 2 nd, 7th, 8th, 9th days.

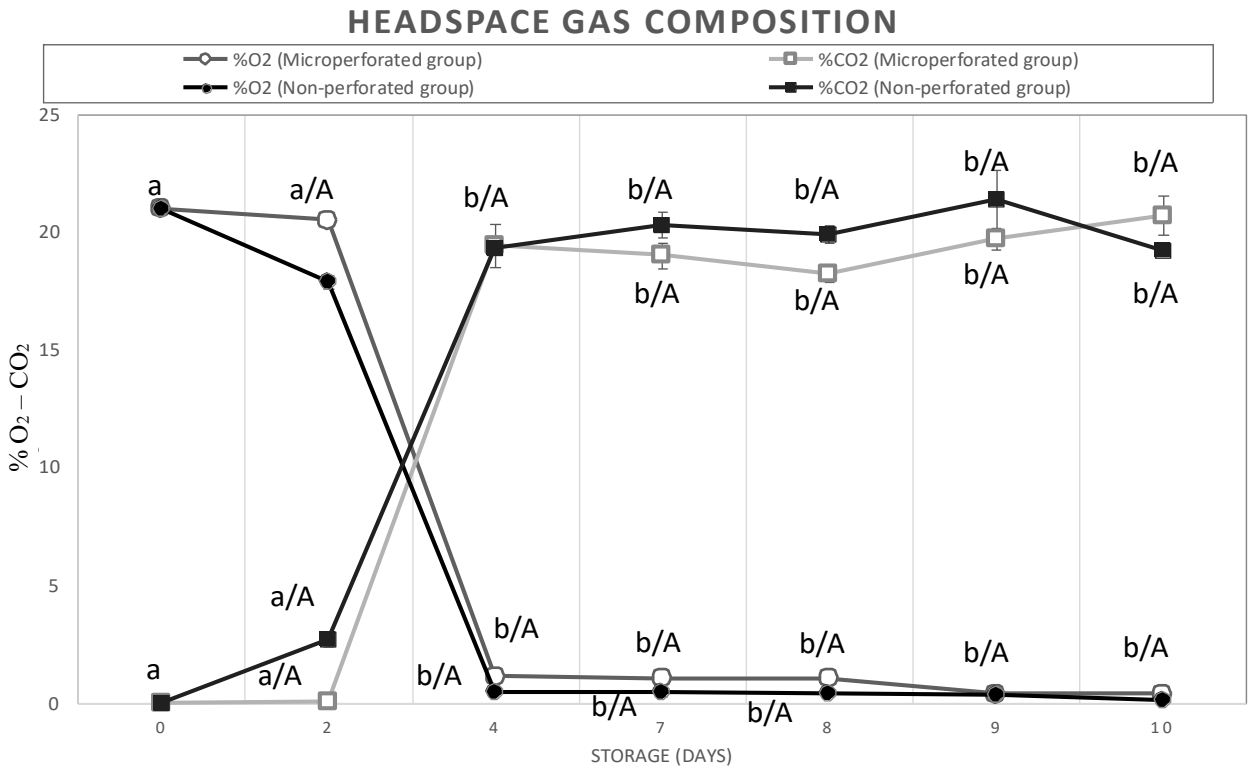


**Figure 4** Headspace gas compositions of microperforated and nonperforated packages. Note: Different letters show the difference among the sample groups concerning the storage period. Different capital letters indicate the difference between the samples.

***pH***

The changes in the pH of the samples during the storage period were observed and the measurements were summarized in Table 1. No statistical difference could be detected among the samples in both groups concerning the storage period. The storage time and/or the microperforation process did not statistically affect the pH value. The pH values ranged from 6.69 to 6.92 and 6.86 in the non-perforated and microperforated samples respectively. These results are in agreement with those of several studies **(Jaworska et al., 2010)**.

Over the 10 days of storage, the slight increase in the pH (1.9% and 3.2% for microperforated and non-perforated respectively) of the mushrooms was also reported by several studies **(Tao et al., 2006)**. On the other hand, in another study **(Oliveira et al., 2012b)**, it was reported a slight decrease in the pH from 6.74 to 6.5 after 7 days of storage. However, the pH values of the control samples were slightly higher than those of microperforated samples. This result was also stated by several scientists **(Gholami, Ahmadi and Farris, 2017)**.

**Table 1** pH values of fresh-sliced mushrooms during the storage period.

| pH | Storage period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** | **2** | **4** | **7** | **8** | **9** | **10** |
| **Microperforated** | 6.70 ±0.09$^{a/A}$ | 6.76 ±0.04$^{a/A}$ | 6.80 ±0.08$^{a/A}$ | 6.84 ±0.03$^{a/A}$ | 6.86 ±0.01$^{a/A}$ | 6.83 ±0.01$^{a/A}$ | 6.83 ±0.02$^{a/A}$ |
| **Non-perforated** | 6.70 ±0.09$^{a/A}$ | 6.84 ±0.04$^{a/B}$ | 6.93 ±0.04$^{a/B}$ | 6.95 ±0.01$^{a/B}$ | 6.91 ±0.01$^{a/B}$ | 6.92 ±0.01$^{a/B}$ | 6.92 ±0.01$^{a/B}$ |

Note: Different small letters show the difference among the sample groups for the storage period. Capital letters indicate the difference between the sample groups ($p \leq 0.05$).

**Table 2** Weight loss of fresh-sliced mushrooms during the storage period.

| Weight loss (%) | Storage period (days) | | | | | |
|---|---|---|---|---|---|---|
| | **2** | **4** | **7** | **8** | **9** | **10** |
| **Microperforated** | 0.81 ±0.01$^{a/A}$ | 1.00 ±0.08$^{b/A}$ | 1.21 ±0.11$^{c/A}$ | 1.60 ±0.01$^{d/A}$ | 2.02 ±0.07$^{e/A}$ | 2.80 ±0.11$^{f/A}$ |
| **Non-perforated** | 1.22 ±0.28$^{a/A}$ | 1.34 ±0.18$^{a/A}$ | 1.62 ±0.01$^{a/A}$ | 2.01 ±0.16$^{a/A}$ | 3.2 ±0.65$^{b/A}$ | 3.59 ±0.81$^{b/A}$ |

Note: Different small letters show the difference among the sample groups for the storage period. Capital letters indicate the difference between the sample groups ($p \leq 0.05$).

**Table 3** Color values of fresh-sliced mushrooms during the storage period.

| Color | Storage period (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **0** | **2** | **4** | **7** | **8** | **9** | **10** |
| **ΔE (MP)** | - | 9.59$^{a/A}$ | 13.74$^{ab/A}$ | 18.51$^{b/A}$ | 25.26$^{c/A}$ | 39.21$^{d/A}$ | 42.53$^{e/A}$ |
| **ΔE (NP)** | - | 17.89$^{a/B}$ | 25.00$^{b/B}$ | 23.32$^{b/B}$ | 34.78$^{c/B}$ | 45.11$^{d/B}$ | 46.45$^{e/B}$ |
| **BI (MP)** | 19.86$^{a/A}$ | 28.79$^{b/A}$ | 31.94$^{c/A}$ | 41.13$^{d/A}$ | 48,12$^{e/A}$ | 56.96$^{f/A}$ | 70.96$^{g/A}$ |
| **BI (NP)** | 19.86$^{a/A}$ | 34.30$^{b/B}$ | 48.12$^{c/B}$ | 50.44$^{d/B}$ | 58.00$^{e/B}$ | 78.79$^{f/B}$ | 80.79$^{g/B}$ |
| **L* (MP)** | 86.18 ±1.44$^{a/A}$ | 77.40 ±0.94$^{b/A}$ | 73.06 ±0.75$^{c/A}$ | 69.18 ±0.88$^{d/A}$ | 61.03 ±1.70$^{e/A}$ | 47.42 ±1.83$^{f/A}$ | 44.41 ±0.36$^{f/A}$ |
| **L* (NP)** | 86.18 ±1.44$^{a/A}$ | 68.83 ±2.28$^{b/B}$ | 62.49 ±0.23$^{c/B}$ | 65.00 ±0.94$^{d/B}$ | 51.68 ±1.19$^{e/B}$ | 41.95 ±0.32$^{f/B}$ | 40.44 ±0.71$^{f/B}$ |

MP: Microperforated samples, NP: Non-perforated samples Note: Different small letters show the difference among the sample groups for the storage period. Capital letters indicate the difference between the sample groups ($p \leq 0.05$).

***Weight loss***

The weight loss during the storage period has been considered as one of the main factors influencing the microbial spoilage of the mushrooms. Regardless of the type of packaging material, the spoilage proceeds along the storage period. For most of the fruits and vegetables, a weight loss of 5 – 6% indicates a significant reduction in their commercial value due to the spoilage **(Guillame et al., 2010)**. The changes in the weight loss of the mushrooms stored in non-perforated and microperforated packaged samples were presented in Table 2.

As presented in Table 2., the weight loss in the non-perforated and microperforated mushrooms was below 5% indicating that there was no decrease in the commercial value of the mushrooms during the storage period **(Mahajan et al., 2007)**. Also, no significant difference was determined between the microperforated and non-perforated groups during each storage period. This result is in agreement with those of the previous research **(El-Seifi et al., 2019)**. No statistical difference for the storage period could be detected among the non-perforated mushroom samples until the 9$^{th}$ day of storage. On the other hand, a statistically significant difference was determined among the microperforated samples for each storage period ($p$ <0.05). The weight loss on the 10$^{th}$ day of the storage period was lower in the microperforated mushrooms (2.80%) than that of the non-perforated samples (3.59%). The scientists **(El-Seifi et al., 2019)** also reported a similar result for the dill in PP packages with 8 microholes of diameter (40 μm) after 7 days at 0 ºC plus 4 days at 10 ºC storage.

***Color***

The Hunter Lab (L*, BI, and ΔE ) values of the non-perforated and microperforated fresh-sliced mushroom samples were shown in Table 3. As presented in Table 3., the L* values tended to decrease throughout the entire storage period in both groups of samples (non-perforated and microperforated). The results are consistent with the data of some other studies **(Oliveira et al., 2012b; Gholami, Ahmadi and Farris, 2017)**. The L* values appeared to be higher in the microperforated samples than those of the non-perforated ones. This issue was evident for the undesired browning reactions that might have taken place in the non-microperforated samples. For all storage periods examined in this study, there was a statistical significance between the microperforated and non-perforated groups in the L$^{*}$, BI, and ΔE values ($p$ <0.05). The most important factor which might have an impact on the customer preferences for the mushrooms

(*Agaricus bisporus*) is to be considered as the appearance and the browning in the tissue after being harvested. The enzymatic activities and microbial contamination might be responsible for the browning on the surface of the mushrooms **(Nerya et al., 2006)**. Hence, the total color difference (ΔE), the lightness (L* ), and the browning index (BI) have been regarded as the most critical parameters during the storage and the changes in these values were presented in Table 3.

As presented in Table 3., the browning index and the total color difference values for both samples increased throughout the 10 day storage period. The L* values decreased considerably along the storage period in both groups. However, the L* values of non-perforated samples were lower compared to those of the microperforated ones during all storage periods. The difference in the L* values between microperforated and non-perforated samples was found statistically significant for each period ($p$ <0.05). The BI values of both groups increased during the entire storage period. However, a statistically significant difference between the two groups was detected in all storage periods ($p$ <0.05). BI values were determined as 70.96 and 80.79 and ΔE values were determined as 42.53 and 46.45 for microperforated and non-perforated samples respectively on the 10$^{th}$ day of storage. BI values increased approximately by 257% and 307% since the beginning of the storage. In several studies **(Oliveira et al., 2012b; Pogorzelska-Nowicka et al., 2020)** such an increase in the BI values was observed along the storage period. The browning index and total color difference values of the microperforated samples were lower than compared to those of the non-perforated samples which might be associated with the microbial spoilage and enzymatic reactions that started earlier in the non-perforated samples. Several authors **(Lin et al., 2017)** reported that high $CO_2$ concentration might give rise to damage in the cap surface tissue of the mushrooms which also stimulates the browning and leads to higher BI values. On the 4$^{th}$ day, the $CO_2$ concentrations in the package were close to 20% for microperforated and non-perforated packages. Hence, the increase in the BI values might be associated with the rise in the $CO_2$ concentration.

***Hardness***

The textural characteristics of the mushrooms can be harshly influenced due to enzymatic activity and water loss during storage **(Gholami, Ahmadi and Farris, 2017)**. Loss of turgor and softening of the tissues were reported as the main causes of the poor textural quality in the mushrooms by several authors **(Guillame et al., 2010; Liu et al., 2010; Khan et al., 2014; Qin et al., 2015)**. The hardness is the most common feature used for the description of quality attributes for the button mushrooms (*Agaricus bisporus).* The higher the hardness, the better the sensory properties of the mushrooms **(Gholami, Ahmadi and Farris, 2017)**. The changes in the hardness of the fresh-sliced mushrooms in the nonperforated and microperforated packaging films during the storage were presented in Table 4.

The aging process, the loss in the tissue hardness, and the overripening of the mushrooms lead to tissue softening characterized by a spongious texture **(Villaescusa and Gil Muñoz, 2003)**. As expected, the hardness values of both groups of samples declined throughout the storage period. As shown in Table 4, no statistically significant changes in the hardness values of the microperforated samples could be observed during the first four days of storage. On the other hand, the decrease in the hardness values of non-perforated samples was higher than in those of the microperforated ones during each storage period. Therefore, one might state that the softening process occurred more obviously in the non-perforated samples. This result might be evident for the beneficiary effect of microperforation in terms of the retardation of tissue softening.

After 10 days of storage, the decline in the hardness was approximately 38% and 66% for the microperforated and non-perforated samples respectively. The loss in the hardness might be associated with the loss of cell urgency caused by the cell disruption, intercellular expansion of the pileal surface **(Zivanovic, Buescher and Kim, 2000)**, the protein and polysaccharide degradation which also affect the cell permeability **(Oliveira et al., 2012a)**. In the study **(Pogorzelska-Nowicka et al., 2020)**, it was reported a higher decline (72.5%) in the firmness of the mushrooms in perforated packages after 7 days of storage at 15 °C. They used a macro-perforation design with 6 holes of a diameter of 330 microns which could allow more tissue softening due to the higher transmission rates of oxygen as a result of wider surface area.

***Microbiological analysis***

Investigation of the pathogenic microorganisms was carried out at the beginning of the storage (day 0).

**Table 4** Hardness values of fresh-sliced mushrooms during the storage period.

| Storage period (Day) | Hardness (g) | |
|---|---|---|
| | Microperforated | Non-perforated |
| **0** | 34351.87 ±1031.40$^{a/A}$ | 34351.87 ±1031.40$^{a/A}$ |
| **2** | 33458.6 ±178.42$^{a/A}$ | 26519.53 ±300.25$^{b/B}$ |
| **4** | 33215.47 ±531.52$^{a/A}$ | 25290.98 ±170.80$^{bc/B}$ |
| **7** | 28276.83 ±565.52$^{b/A}$ | 25184.28 ±1003.84$^{bc/B}$ |
| **8** | 27770.93 ±797.67$^{b/A}$ | 24343.85 ±1286.35$^{c/B}$ |
| **9** | 21797.1 ±472.70$^{c/A}$ | 19599 ±266.04$^{d/B}$ |
| **10** | 21368.83 ±460.99$^{c/A}$ | 11678.33 ±208.50$^{e/B}$ |

Note: Different small letters show the difference among the sample groups for the storage period. Capital letters indicate the difference between the sample groups ($p$ ≤0.05).

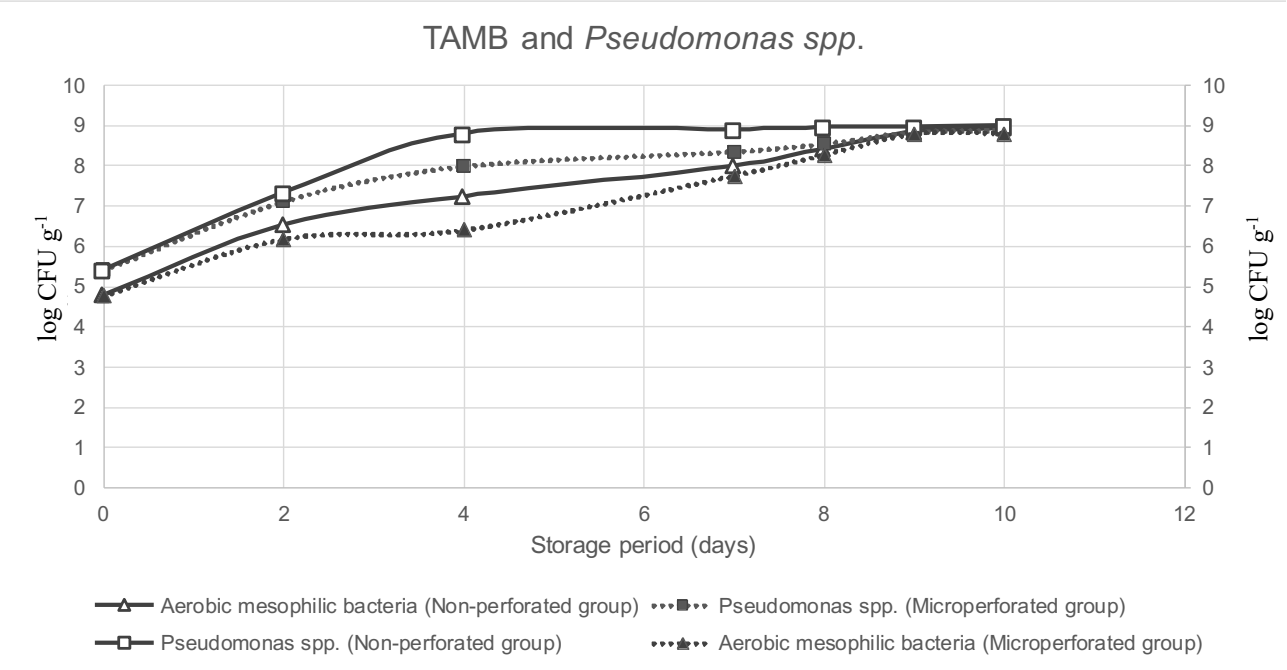


**Figure 5** Count of Total aerobic mesophilic bacteria and *Pseudomonas* spp. (log CFU $g^{-1}$) of fresh-sliced mushrooms during the storage period.

No pathogenic microorganisms were detected in the fresh-sliced mushroom samples.

The shelf life of the mushrooms was severely influenced by the growth of total aerobic mesophilic bacteria and *Pseudomonas* spp. Thus, analysis of the total aerobic mesophilic bacteria count and *Pseudomonas* spp. counts were performed along with the shelf life. The results were presented in Figure 5.

As shown in Figure 5, since the initial microbial load was over 5 log CFU.$g^{-1}$ the number of total microorganisms rapidly increased during the first days of storage. The total count of *Pseudomonas* spp. increased from 5.40 (day 0) to 8.88 log CFU.$g^{-1}$ in the microperforated packaged samples at the end of the storage period (day 10), while a quite rapid increase in the number of *Pseudomonas* spp. was observed in the non-perforated packaged samples on the 4$^{th}$ day by reaching a value of 8.78 log CFU.$g^{-1}$. On the other hand, the TAMB count increased more slowly compared to the count of *Pseudomonas* spp. and reached in the microperforated and non-perforated packaged samples 8.79 log CFU.$g^{-1}$ and 8.92 log CFU.$g^{-1}$ respectively at the end of the storage. On the other hand, the TAMB count increased more slowly compared to the count of *Pseudomonas* spp. and reached in the microperforated and non-perforated packaged samples 8.79 log.CFU $g^{-1}$ and 8.92 log CFU.$g^{-1}$ at the end of the storage. The studies concerning the determination of the shelf life of the fresh-sliced mushrooms reported similar results to our findings and revealed that *Pseudomonas* spp. was regarded as one of the main factors leading to microbial spoilage of mushrooms **(Gonzalez-Fandos et al., 2000; Srivastava et al., 2010; Wang et al., 2017)**. Unfortunately, no legislative regulation specific to the microbial spoilage is available about the fresh-cut produce except for the pathogenic microorganisms. However, basically above the level of 8 log CFU.$g^{-1}$ can be considered as the inception of deterioration, even though no risks in terms of food safety might arise at this level and it is important for food quality because *Pseudomonas* spp. are saprophytic microorganisms that lead to deterioration in mushrooms. As a result of the microbial spoilage, several qualitative defects such as changes in color, undesired odor, and softening might be encountered which affect the consumer demand **(Simón, González-Fandos and Tobar, 2005)**. Based on the counts of *Pseudomonas* spp. and TAMB, we can conclude that the microbial deterioration started on the 7$^{th}$ day of storage in the microperforated packaged samples whereas it started on the 4$^{th}$ day of storage in the non-perforated packaged samples. Tissue softening due to microbial spoilage and/or drip loss occurred in the microperforated packaged samples on the 7$^{th}$ day according to the hardness values. On the other hand, there was a remarkable decrease in the hardness in the non-perforated samples on the 2$^{nd}$ day.

***Sensory evaluation***

The changes in the sensory characteristics of the samples during storage were presented in Table 5.

As summarized in Table 5, the scores of the odor, texture, appearance, and overall acceptability decreased with the storage period for each group of samples. However, the scores for the sensorial attributes of the microperforated packaged samples were higher than the non-perforated packaged samples. The sensorial attributes were maintained for microperforated packaged samples until the 9$^{th}$ day of storage and on this period the sensorial scores were below 3 which is the limiting value to accept the food in 5 points hedonic scale for the sensorial attributes.

**Table 5** Sensory evaluation of fresh-sliced mushrooms during the storage period.

| Storage period (Day) | Sensory scores | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Texture | | Odor | | Appearance | | Overall Accept. | |
| | MP | NP | MP | NP | MP | NP | MP | NP |
| **0** | 5.0 ±0.00 | 5.0 ±0.00 | 5.0 ±0.00 | 5.0 ±0.00 | 5.0 ±0.00 | 5.0 ±0.00 | 5.0 ±0.00 | 5.0 ±0.00 |
| **2** | 4.6 ±0.49 | 4.2 ±0.40 | 4.4 ±0.49 | 3.8 ±0.75 | 4.8 ±0.40 | 3.8 ±0.75 | 5.0 ±0.00 | 3.8 ±0.40 |
| **4** | 4.6 ±0.80 | **4.2 ±0.40** | 4.2 ±0.40 | **4.0 ±0.89** | 4.2 ±0.40 | **3.0 ±0.63** | 4.4 ±0.49 | **3.6 ±0.80** |
| **7** | 4.2 ±0.40 | 2.8 ±0.75 | 4.0 ±0.89 | 2.4 ±0.49 | 3.4 ±0.63 | 2.4 ±0.49 | 3.6 ±0.80 | 2.6 ±0.49 |
| **8** | **3.2 ±0.40** | 2.2 ±0.40 | **3.4 ±0.80** | 2.4 ±0.80 | **3.0 ±0.49** | 2.2 ±0.40 | **3.4 ±0.80** | 2.2 ±0.40 |
| **9** | 2.2 ±0.98 | 1.4 ±0.49 | 2.4 ±0.49 | 1.6 ±0.80 | 2.8 ±0.40 | 1.6 ±0.49 | 2.8 ±0.40 | 1.6 ±0.49 |
| **10** | 2.2 ±0.40 | 1.2 ±0.40 | 2.2 ±0.40 | 1.4 ±0.80 | 2.2 ±0.40 | 1.2 ±0.40 | 2.2 ±0.40 | 1.4 ±0.80 |

Note: MP: Perforated samples NP: Nonperforated samples. Different small letters show the difference among the sample groups for the storage period. Capital letters indicate the difference between the sample groups ($p \leq 0.05$).

Hhowever the sensorial properties of non-perforated packaged samples were acceptable until the 4th day of storage. Since we couldn't analyze on the 5th or 6th day of the storage, it is not clear if the non-perforated packaged samples were acceptable or not on these days. However the non-perforated packaged samples weren't accepted because of their poor sensorial attributes all including texture, odor, appearance, and overall acceptance on the 7th day of storage. For this reason, it is not clear whether the non-perforated packaged samples were acceptable or not after the 4th day of storage. Several scientists **(Eastwood and Burton, 2002)** reported that the consumers mostly prefer the mushrooms with fewer spots and better appearance even though the aged mushrooms have a better taste. Thus, the overall acceptability of the consumers depends much more on the color, texture, and appearance than the flavor of the mushrooms. Based on the sensorial properties, we can conclude that the shelf life of fresh-sliced mushrooms can be extended to 8 days with microperforated packaging. Whereas the shelf life of fresh-sliced mushrooms was less than 7 days (4, 5, or 6 days). It was shown that the microperforation process was effective to keep the sensorial properties and extending the shelf life of fresh-sliced mushrooms as stated by **(Dhalsamant et al., 2015)**.

## CONCLUSION

The microperforation process of BOPP films extended the shelf life of the fresh-sliced mushrooms concerning the non-perforated packaged samples. The shelf life of the fresh-sliced mushrooms in microperforated packaging material of BOPP was 8 days seasonally. Although the microbial deterioration starts on the 7th day of the storage period in the microperforated packaged samples, these samples were evaluated as acceptable for their sensorial attributes on the 8th day of the storage period. The shelf life of the non-perforated packaged fresh-sliced mushrooms was determined to be less than 7 days (4, 5, or 6 days) based on the sensorial evaluation since their sensorial scores were above 3 on the 4th day and below 3 on the 7th day of the storage period.

The design of the microholes in the perforation process becomes crucial since the number and diameter of the holes are effective on the respiration rate and hence the shelf life of the fresh-sliced mushrooms. The empirical formula was based on elliptic dimensions of 40 × 30 µm and 350 × 110 µm which was a unique radial and comparatively smaller dimension in our experiments. Thus, the OTR was calculated using this diameter. To obtain more accurate results an estimation by extrapolation of this value to fit in the empirical formula ought to be performed. This evaluation would promise to the scientists to create more effective and novel designs of microperforated packaging materials in the future which might also prolong the shelf life of other foods.

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# MICROBIOLOGICAL, CHEMICAL AND ORGANOLEPTIC EVALUATION OF FRESH FISH AND ITS PRODUCTS IRRADIATED BY GAMMA RAYS

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## ABSTRACT

The present study evaluated the microbiological, chemical, and organoleptic aspects of irradiated fresh fish and its products to extend their shelf life. Fresh fish and its products were irradiated at three doses (1.5, 3.0, and 4.5 kGy) used for preservation to study the effects of irradiation on their microbiological properties, fatty acid composition, and organoleptic properties. Irradiated fresh bolti fish, smoked herring, and smoked mackerel were evaluated microbiologically, chemically, and organoleptically. Radiation treatment not only reduced the counts of aerobic bacteria, fecal streptococci, molds, and yeasts but also destroyed all the *Staphylococcus aureus* cells, improving the hygienic quality of the fresh and smoked fish samples. Irradiation increased the peroxide, acid, and thiobarbituric acid values, but they remained within acceptable levels. No new fatty acids or other artifacts due to irradiation were observed. Irradiation of 4.5 kGy greatly reduced the organoleptic quality scores of fresh bolti fish, indicating that the optimum radiation dose for this fish was 3.0 kGy. Smoked herring and mackerel could be irradiated with up to 4.5 kGy without adverse effects on their organoleptic properties.

**Keywords:** microbiological; chemical; organoleptic; fish; irradiated

## INTRODUCTION

Currently, irradiated food (up to 10 kGy) has become acceptable and is commercially available in many countries worldwide **(Lacroix and Ouattara, 2000; Demartini et al., 2019)**.

Radiation sterilization, which is analogous to the processing techniques used for other meats (beef, poultry, and seafood), is also a new and promising method applicable to the processing of precooked meats (enzyme inactivation). Radiation sterilized foods can be stored for a long time (years) without refrigeration **(Duliu, Ferdes, and Ferdes, 2004; Sedeh et al., 2007; Fallah et al., 2008; Kakatkar et al., 2017)**. Many studies of potential techniques for the commercial radiation sterilization of seafood have been carried out because radiation extends the shelf life of the product and improves the hygienic quality of the seafood **(Chouliara et al., 2004; Brennan, 2005; Özkan et al., 2007)**.

The lipids in the fish muscle are different from those of other animal tissues due to their high contents of polyunsaturated fatty acids **(Al-Kahtani et al., 1996; Javanmard et al., 2006; Özden and Erkan, 2010)**.

The high polyunsaturated fat content can be considered the main reason for the oxidative rancidity of fish, which adversely affects its flavor. Irradiation has been reported to increase 2-thiobarbituric acid-reactive substances (TBARS) in aerobically packaged raw poultry meat **(Du et al., 2000; Turgis et al., 2008)**.

Irradiation has shown considerable promise in the microbial decontamination of fresh fish and fish products, extending their shelf lives and improving their hygienic quality **(Farkas, 2006; Mbarki et al., 2009)**. However, the acceptability of irradiated fish products is dependent on the chemical composition of the fish, particularly the lipids. Many investigators have found that irradiation in the presence of oxygen can accelerate lipid oxidation and induce oxidative rancidity; hence, lipid quality and fatty acid composition can be altered **(Byun et al., 2008; Mbarki et al., 2009)**. The progressive use of gamma radiation in the field of fish and fish product preservation makes it important to evaluate radiation processes and their effects on the qualities of these products **(Badr, 2012)**.

Bolti (*Tilapia nilotica*) is one of the most important and preferred freshwater fish. Smoked fish products have received more attention due to their pleasant taste. The most important types of smoked fish are herring and mackerel **(WHO, 2000; Nickelson et al., 2001)**.

Fresh and smoked fish products can be contaminated with many pathogenic microorganisms that affect their shelf life **(Moini et al., 2009; Rostamzad et al., 2010)**.

The main aim of the present study was to evaluate the effects of gamma irradiation on the microbiological, chemical, and organoleptic qualities of fish (fresh bolti fish as well as smoked herring and smoked mackerel).

### Scientific hypothesis

H1: Radiation is considered a safe method of preserving food from microbial deterioration and consequently prolonging its marketing period.

H2: Gamma irradiation we will be using for the fresh fish and smoked improving the hygienic quality and increasing its shelf life without adverse effects on its organoleptic properties.

## MATERIAL AND METHODOLOGY

Three types of fish, fresh bolti (*Tilapia nilotica*), smoked herring, and smoked mackerel, were used in this experiment. Fresh bolti fish samples were obtained from the Dammam fish market in the eastern province of Saudi Arabia. This market is one of the main sources of fish in the kingdom, while smoked herring and smoked mackerel were obtained from the market in Riyadh, Saudi Arabia. The fish samples were wrapped with non-perforated plasticized polyvinyl chloride (PVC) film, which is a stretch film with high transparency, 12-micron thickness, self-clinging, and the following rates of permeability: $CO_2$ >100 $cm^3/m^2/24$ hr; $O_2$ 1800 $cm^3/m^2/24$ hr; and water vapor 700 $g/m^2/24$ hr.

### Irradiation process

Fish samples in bags were irradiated for 30 Minutes. The samples temperature was when irradiation at 2 – 3 °C ±1 °C were exposed to 1.5, 3.0, and 4.5 kGy from cobalt 60 in a Gammacell 220 at King Abdul Aziz City for Science and Technology (KACST) in Riyadh (Model Gammacell 220 from MDS; Nordion Initial Canada Activity source (Co-60) was 24.000). All fish samples were stored at 3° C ±1 °C (90 – 92% RH).

### Microbiological tests

The total aerobic bacterial counts were enumerated on agar (Merck, Darmstadt, Germany) plates as described by **Maturin and Peeler (2001)**. The quantification of fecal streptococci was done through the MPN technique using the Dextrose Azide Broth **(APHA, 1998)**, while *Staphylococcus aureus* was counted on Baird-Parker medium as described by **Tallent et al. (2001)**. The total molds and yeasts were counted on malt extract agar plates **(Tournas et al., 2001)**.

### Lipid extraction

The parts of fish were homogenized and 5 g of the homogenized sample was mixed well with 10 g cleaned sea sand and 20 g anhydrous sodium sulfate, and then percolated for 6 hours with a hexane-acetone mixture (2:1) in a glass column with a Teflon stopcock. After evaporation of the solvent from the percolate (600 mL) under vacuum (all chemicals were from Fischer, USA), the fish lipids extracted were weighed **(AOAC, 1990)**.

### Lipid quality attributes

The peroxide value (as equivalents/kg lipid) and the acid value were determined as described by **AOAC (1990)**. The thiobarbituric acid value was determined according to performed as described by **Lynch and Frei (1993**).

### Fatty acid profiles

The fatty acid composition was determined by a gas-liquid chromatography apparatus according to the method by **AOAC (1990)**.

### Organoleptic tests

The appearance, odor, texture, and taste of the fish samples were served to a taste panel of 10 members using a hedonic scale of 1 to 9 and the dishes were rated as 9 for excellent, 6 for good and below 4 as poor or unacceptable. **(WHO, 2000)**.

### Statistical Analysis

The obtained data are reported as the mean ±*SD* and were statistically analyzed using SPSS program version 22.0 (SPSS, 2018, SPSS Inc., Chicago, Illinois, USA). Significant differences were evaluated by Duncan's multiple range test (DMRT), with differences considered significant at $p < 0.05$.

## RESULTS AND DISCUSSION

The results of the microbiological tests are shown in Table 1. A comparison of the fresh bolti fish and smoked fish (herring and mackerel) indicated that most microbial counts were higher for fresh bolti fish and lowest for smoked mackerel. The use of salt and the smoking process might have affected the microbial counts. Irradiation caused a substantial reduction in all the microorganisms evaluated, and the reduction percentage was proportional to the irradiation dose. The lowest irradiation dose used (1.5 kGy) decreased the total aerobic bacterial counts of the fresh bolti fish by $7.5 \times 10^3$, while it decreased the total aerobic bacterial counts of smoked fish (herring and mackerel) by $4.4 \times 10^3$ and $2.0 \times 10^3$, respectively.

The higher reduction in the total aerobic bacterial count for fresh bolti fish might be due to the direct effect of the radiation as well as indirect effects resulting from water radiolysis, which is greater in fresh fish than in smoked fish. The highest irradiation dose used, i.e., 4.5 kGy, reduced the total aerobic bacterial counts by approximately 99.9% (<100).

Considering that Staphylococcus aureus is the main food poisoning microorganism, irradiation, even at the lowest dose, destroyed almost all the cells of this dangerous microorganism (<100). It could be concluded that irradiation doses used were sufficient to substantially reduce the counts of all the microbial species investigated and improve the hygienic quality of both fresh and smoked fish, rendering these products safe for human consumption. Many other investigators reported substantial reductions in the microbial counts on fish and fish products as a result of irradiation **(Noomhorm et al., 2003; Duliu, Ferdes and Ferdes, 2004; Özkan et al., 2007; Demartini et al., 2019)**.

It has been reported that irradiation doses of 1, 3, and 5 kGy significantly reduced the total viable counts of microorganisms on rainbow trout fillets **(Moini et al., 2009; Kakatkar et al., 2017)**.

**Mendes et al. (2005)** and **Nickelson et al. (2001)** found that the mesophilic bacterial counts of irradiated shrimp, crab, and fish were lower than those of nonirradiated samples during storage at 4 °C.

**Table 1** Microbiological quality of fresh and smoked fish and before and after irradiation.

| Microbiological quality (Fresh bolti fish) | Fresh bolti fish | | | |
|---|---|---|---|---|
| | 0 kGy | 1.5 kGy | 3 kGy | 4.5 kGy |
| Total aerobic bacteria | $8.2 \times 10^5$ | $7.5 \times 10^3$ | $5.0 \times 10^2$ | $1.1 \times 10^2$ |
| Fecal streptococci | $3.0 \times 10^3$ | $3.2 \times 10^2$ | <100 | <100 |
| *Staphylococcus aureus* | $3.3 \times 10^2$ | <100 | <100 | <100 |
| Yeasts | $8.0 \times 10^3$ | $6.1 \times 10^2$ | $1.0 \times 10^2$ | <10 |
| Molds | $0.7 \times 10^2$ | $3.3 \times 10$ | <10 | <10 |

| Microbiological quality (Smoked herring and Smoked mackerel) | Smoked herring | | | | Smoked mackerel | | | |
|---|---|---|---|---|---|---|---|---|
| | 0.0 kGy | 1.5 kGy | 3 kGy | 4.5 kGy | 1.5 kGy | 0.0 kGy | 3 kGy | 4.5 kGy |
| Total aerobic bacteria | $6.2 \times 10^4$ | $4.4 \times 10^3$ | $2.0 \times 10^2$ | $7.1 \times 10$ | $2.0 \times 10^3$ | $3.0 \times 10^4$ | $4.1 \times 10^2$ | $6.0 \times 10$ |
| Fecal streptococci | $1.9 \times 10^2$ | $1.1 \times 10$ | <100 | <100 | $1.1 \times 10$ | $7.0 \times 10^2$ | <100 | <100 |
| *Staphylococcus aureus* | $7.2 \times 10^2$ | <100 | <100 | <100 | <100 | $2.2 \times 10^2$ | <100 | <100 |
| Yeasts | $7.4 \times 10^3$ | $5.6 \times 10^2$ | $5.0 \times 10$ | <10 | $8.0 \times 10$ | $7.1 \times 10^2$ | $2.1 \times 10$ | <10 |
| Molds | $1.5 \times 10^3$ | $7.3 \times 10$ | <10 | <10 | <10 | <10 | <10 | <10 |

**Table 2** Effect of irradiation on the peroxide values, acid values and thiobarbituric acid values (TBA) of fish products.

| Parameters | Fresh bolti fish | | | | Smoked herring | | | | Smoked mackerel | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 kGy | 1.5 kGy | 3 kGy | 4.5 kGy | 0.0 kGy | 1.5 kGy | 3 kGy | 4.5 kGy | 1.5 kGy | 0.0 kGy | 3 kGy | 4.5 kGy |
| Peroxide value | 5.7 ±0.26 | 7.9 ±0.13 | 9.8 ±0.22 | 14.3 ±0.05 | 7.5 ±0.55 | 9.2 ±0.33 | 11.1 ±0.58 | 15.9 ±0.10 | 8.3 ±0.70 | 9.9 ±0.90 | 12.5 ±0.60 | 13.7 ±0.22 |
| Acid value | 0.8 ±0.05 | 0.96 ±0.04 | 1.2 ±0.03 | 1.87 ±0.03 | 1.15 ±0.23 | 1.52 ±0.21 | 2 ±0.22 | 2.55 ±0.19 | 1.37 ±0.18 | 1.7 ±0.13 | 2.11 ±0.21 | 2.7 ±0.15 |
| TBA | 0.178 ±0.04 | 0.211 ±0.07 | 0.25 ±0.08 | 0.592 ±0.04 | 0.279 ±0.46 | 0.372 ±0.23 | 0.446 ±0.33 | 0.635 ±0.23 | 0.353 ±0.04 | 0.415 ±0.04 | 0.481 ±0.03 | 0.722 ±0.06 |

Note: Results are presented as mean ±*SD* (n = 3). Values are significantly differen ($p$ <0.05).

**Table 3** Relative percentages of fatty acids in nonirradiated and irradiated fish products.

| Fatty acids | Carb No. | Fresh Bolti Fish | | | | Smoked Herring | | | | Smoked Mackerel | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.0 kGy | 1.5 kGy | 3.0 kGy | 4.5 kGy | 0.0 kGy | 1.5 kGy | 3.0 kGy | 4.5 kGy | 0.0 kGy | 1.5 kGy | 3.0 kGy | 4.5 kGy |
| Lauric | 12.0 | 00.12 | 00.12 | 00.34 | 00.18 | 00.31 | 00.38 | 00.34 | 00.44 | 00.20 | 00.14 | 00.24 | 00.16 |
| Myristic | 14.0 | 06.56 | 09.41 | 09.64 | 11.17 | 09.81 | 09.70 | 09.61 | 09.91 | 08.00 | 10.01 | 08.52 | 09.75 |
| Pentadecanoic | 15.0 | 01.70 | 00.92 | 00.92 | 00.79 | 00.82 | 00.21 | 00.65 | 00.65 | 01.11 | 01.33 | 01.60 | 01.12 |
| Palmitic | 16.0 | 28.65 | 28.22 | 27.70 | 27.79 | 00.21 | 00.15 | 00.87 | 01.50 | 00.24 | 00.21 | 00.20 | 00.24 |
| Palmitoleic | 16.1 | 18.01 | 18.10 | 18.00 | 17.75 | 19.13 | 18.57 | 17.26 | 14.40 | 19.63 | 18.00 | 16.20 | 15.20 |
| Heptadecanoic | 17.0 | 03.55 | 5.75 | 05.33 | 05.85 | 00.50 | 00.12 | 00.75 | 00.95 | 07.61 | 08.75 | 07.70 | 08.90 |
| Heptadecanoic | 17.1 | 0.3.68 | 3.00 | 04.12 | 04.28 | 00.83 | 00.12 | 00.19 | 01.34 | 02.35 | 01.26 | 01.31 | 00.72 |
| Stearic | 18.0 | 0.6.25 | 6.00 | 06.29 | 08.45 | 00.92 | 00.57 | 00.95 | 00.97 | 00.52 | 00.67 | 00.22 | 00.71 |
| Oleic | 18.1 | 21.71 | 20.01 | 19.70 | 16.93 | 03.20 | 03.11 | 03.62 | 03.66 | 06.35 | 06.66 | 05.14 | 05.26 |
| Linoleic | 18.2 | 05.16 | 4.51 | 03.89 | 02.32 | 18.43 | 18.70 | 18.03 | 19.00 | 20.51 | 17.29 | 17.29 | 24.00 |
| Linolenic | 18.3 | 00.55 | 00.55 | 00.68 | 00.55 | 19.01 | 25.00 | 18.27 | 22.14 | 08.61 | 08.22 | 18.60 | 17.10 |
| Arachidic | 20.0 | 0.1.34 | 1.20 | 01.07 | 01.10 | 02.00 | 02.03 | 02.52 | 02.82 | 02.74 | 03.13 | 02.71 | 02.92 |
| Gadoleic | 20.1 | 01.00 | 00.95 | 01.18 | 01.30 | 00.92 | 01.66 | 04.00 | 09.06 | 05.31 | 04.73 | 04.78 | 08.32 |
| Eicosadienoic | 20.2 | 00.26 | 00.28 | 00.28 | 00.23 | 23.38 | 21.70 | 23.0 | 13.22 | 14.69 | 18.82 | 15.90 | 05.27 |
| Eleostearic | 20.3 | 00.24 | 00.24 | 00.30 | 00.37 | 00.70 | - | - | - | 00.27 | 00.50 | 00.22 | 00.31 |
| Arachidonic | 20.4 | 01.12 | 01.10 | 00.50 | 00.85 | 00.31 | - | - | - | 01.90 | 00.20 | - | - |
| T. sat * | | 48.17 ±1.23 | 51.42 ±2.62 | 51.29 ±2.05 | 55.33 ±2.75 | 14.57 ±2.05 | 13.16 ±1.65 | 15.69 ±0.65 | 17.24 ±0.58 | 20.42 ±1.23 | 24.24 ±2.62 | 21.19 ±0.63 | 23.80 ±0.45 |
| T. Unsat ** | | 51.73 ±2.75 | 48.78 ±2.27 | 48.95 ±1.10 | 44.58 ±0.50 | 85.41 ±1.37 | 88.86 ±2.27 | 84.37 ±2.03 | 82.82 ±2.62 | 79.62 ±2.52 | 75.68 ±1.37 | 78.90 ±1.57 | 62.21 ±0.57 |

Note: Results are presented as mean ±*SD* (n = 3). Values are significantly differen ($p$ <0.05).

**Table 4** Organoleptic evaluation of nonirradiated and irradiated fresh and smoked fish.

| Parameters | Fresh bolti fish | | | | Smoked herring | | | | Smoked mackerel | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 kGy | 1.5 kGy | 3 kGy | 4.5 kGy | 0.0 kGy | 1.5 kGy | 3 kGy | 4.5 kGy | 1.5 kGy | 0.0 kGy | 3 kGy | 4.5 kGy |
| Appearance | 8.5 ±0.01 | 8.4 ±0.06 | 8.5 ±0.04 | 8.3 ±0.04 | 8.6 ±0.01 | 8.6 ±0.04 | 8.7 ±0.03 | 8.2 ±0.03 | 8.9 ±0.01 | 8.7 ±0.04 | 8.7 ±0.04 | 8.5 ±0.03 |
| Odor | 8.2 ±0.04 | 8 ±0.07 | 8 ±0.07 | 7.3 ±0.11 | 8.5 ±0.02 | 8.3 ±0.04 | 8 ±0.07 | 8 ±0.09 | 8.7 ±0.01 | 8.4 ±0.02 | 8.4 ±0.02 | 8 ±0.04 |
| Texture | 8.7 ±0.01 | 8.6 ±0.04 | 8.3 ±0.05 | 8.3 ±0.11 | 8.3 ±0.04 | 8.5 ±0.05 | 8.2 ±0.07 | 8 ±0.11 | 8.3 ±0.05 | 8 ±0.04 | 8 ±0.04 | 7.8 ±0.07 |
| Taste | 8.4 ±0.03 | 8.2 ±0.07 | 8 ±0.03 | 8 ±0.12 | 8.5 ±0.02 | 8.5 ±0.04 | 8 ±0.05 | 8 ±0.05 | 8.8 ±0.01 | 8.5 ±0.04 | 8.3 ±0.05 | 8.2 ±0.05 |

Results are presented as mean ±SD (n = 3). Values are significantly differen ( $p<0.05$) .

The effects of irradiation on the fish lipid quality attributes are presented in Table 2. Generally, irradiation increased the peroxide value, acid value, and thiobarbituric acid value (TBA) of both fresh and smoked fish, but the levels were still indicative of acceptable quality.

Irradiated fresh bolti fish and smoked fish (herring and mackerel) showed significantly higher peroxide values (14.3, 15.9 and 13.9 meq$O_2$.kg$^{-1}$ at 4.5 kGy) than the control samples (5.7, 7.5 and 8.3 meq$O_2$.kg$^{-1}$), respectively, and the peroxide values were positively correlated with the dose used. The results of this study are in agreement with the findings of other studies that have reported an increase in oxidation activity and lipid peroxidation as a result of radiation treatment of fish and fish products **(Ahn et al., 2000; Byun et al., 2008; Rostamzad et al., 2010).** In contrast, **Javanmard et al. (2006)** reported no significant ($p>0.05$) differences in the peroxide value between irradiated and control chicken meat after irradiation.

TBA values revealed that irradiation caused an increase in lipid oxidation in fish samples. The rate of the increase was correlated with the irradiation dose, and the highest increase was observed in the thiobarbituric acid values (0.592, 0.635, and 0.722 mg MDA.kg$^{-1}$ at 4.5 kGy), indicating that the peroxides and hydroperoxides degraded into low-molecular-weight compounds. Similarly, some researchers have shown increases in TBA values during irradiation of various fish and fish products **(Byun et al., 2008; Turgis et al., 2008)**. In contrast, **Chun et al. (2010)** reported no significant differences in the TBARS values with both increasing irradiation doses and increasing storage periods in chicken breasts.

Comparing fresh bolti, smoked herring and smoked mackerel revealed that the greatest increase in lipid quality attributes occurred in fresh bolti fish, and the smallest increase occurred in smoked mackerel fish.

The fatty acid compositions of fresh bolti fish and smoked fish (herring and mackerel) are shown in Table 3. The compositions of the irradiated and nonirradiated samples were qualitatively similar since no new fatty acids or other artifacts were generated by irradiation. Among all the fatty acids, palmitoleic acid (C16:1) (17.75, 14.40, and 15.20% at 4.5 kGy and 18.01, 19.13, and 19.63% in the control, respectively) was the most abundant fatty acid in all the irradiated and nonirradiated samples. The least abundant fatty acid was lauric acid (C12:0) (0.18, 0.44, and 00.16% at 4.5 kGy and 0.12, 0.31, and 0.20% in the control, respectively) in all the irradiated and nonirradiated samples. The relative percentage of total polyunsaturated fatty acids for all fats in the fish samples decreased slightly with increasing radiation dose (44.58, 82.82, 62.21% at 4.5 kGy and 51.73, 85.41, 79.62% for the control), suggesting the potential for the oxidation of unsaturated compounds by irradiation.

Fresh bolti fish, which are considered lean fish, have a lower percentage of unsaturated fatty acids than the other tested fish, suggesting that the higher susceptibility of the fat in bolti fish to oxidation by irradiation may be due to the high content of monounsaturated fatty acids that are easily oxidized.

There were no significant differences ($p>0.05$) in the levels of all fatty acids, saturated fatty acids, or unsaturated fatty acids between the control and irradiated fish samples at 1.5, 3.0, and 4.5 kGy. Therefore, the irradiation process had no significant effect ($p>0.05$) on the fatty acid composition.

**Javan and Motallebi (2015)** reported an increase in fatty acid oxidation with increasing dose of gamma irradiation in their study of the effects of different doses of gamma radiation (0, 0.75, 1.5, 2.25, 3, 3.75, and 4.5 kGy) on the fatty acid composition of rainbow trout fillets.

**Oraei et al. (2011)** also reported that different irradiation processes and doses of radiation (1, 3, and 5 kGy) had no significant effects ($p>0.05$) on the fatty acid composition of rainbow trout fillets.

**Al-Kahtani et al. (1996)** reported the influence of irradiation on the chemical components of tilapia and Spanish mackerel, and radiation doses of 1.5 – 10 kGy caused a decrease in some fatty acids. **Erkan and Özden (2007)** reported that the total fatty acid contents in the muscle of nonirradiated sea bream were lower than in sea bream irradiated with 2.5 kGy and higher than in sea bream irradiated with 5 kGy.

**Mbarki et al. (2009)** reported that low-dose irradiation had no adverse effect on the nutritionally important polyunsaturated fatty acids in Mediterranean horse mackerel.

**Özden and Erkan (2010)** reported that the total saturated and total monounsaturated fatty acids in irradiated sea bass increased at 2.5 and 5 kGy, and the total polyunsaturated fatty acid contents in irradiated samples were higher than that in nonirradiated samples.

The organoleptic scores (for appearance, odor, texture, and taste) for fresh fish and smoked fish are shown in Table 4). Smoked mackerel earned the highest scores, while fresh bolti fish earned the lowest scores. Irradiation reduced the organoleptic scores of all the tested fish samples, but the reduction was not significant. The reduction was proportional to the radiation dose, and the largest reduction

was observed for fresh bolti fish, especially in odor and taste. The average appearance scores for the nonirradiated samples were 8.5, 8.6, and 8.9. For the irradiated samples, the scores were 8.3, 8.2, and 8.5 at 4.5 kGy. Both the irradiated and nonirradiated samples were in acceptable conditions. Considering the organoleptic evaluation, the optimum radiation dose for fresh bolti fish is less than or equal to 3.0 kGy. Smoked herring and smoked mackerel can be irradiated up to 4.5 kGy without adversely affecting their chemical and organoleptic properties.

Our results agreed with the results of **Prakash et al. (2014) and Badr (2012)**, who reported that irradiated and nonirradiated dry fish were in acceptable condition. **WHO (2000)** and **Ahmed et al. (2009)** showed that radiation doses up to 5 kGy had a significant effect on the visual qualities, decay rate, color, and texture of minimally processed foods. **Alam, Ahmed and Shahin (2009)** showed good correlations between bacterial populations and the overall acceptability scores with the shelf life of hilsa.

## CONCLUSION

The irradiation of food enhances the safety and the hygienic qualities of fresh and smoked fish products because of its high efficacy for inactivating pathogenic and spoilage microorganisms without deteriorating the quality of the product.

According to all the obtained data, gamma irradiation, especially 4.5 kGy, can be applied for microbial control and improving the safety of smoked fish, and increasing its shelf life without adverse effects on its organoleptic properties. Also, the current study showed that irradiation of 4.5 kGy greatly reduced the organoleptic quality scores of fresh bolti fish, indicating that the optimum radiation dose of this fish is 3.0 kGy.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE EFFECT OF KEFIR-SPIRULINA ON GLYCEMIC STATUS AND ANTIOXIDANT ACTIVITY IN HYPERGLYCEMIA RATS

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## ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia. It is caused by impaired insulin secretion or by insulin receptor insensitivity. DM and its complications are often related to increases in the level of oxidative stress. Spirulina is a nutrient-dense food that contains an abundance of antioxidant compounds. In combination with kefir, it may serve as both a nutrient-rich diet and an antioxidant agent that can prevent complications of diabetes. This study aims to investigate the nutritional content of kefir-spirulina and its effect on glycemic status and antioxidant activity in streptozotocin-nicotinamide (STZ-NA) induced diabetic rats. A total of 30 male Sprague Dawley rats were divided into five groups: normal control (K1), diabetic control (K2), pioglitazone treatment (K3), kefir combined with 1% spirulina treatment (P1), and kefir combined with 2% spirulina treatment (P2). All rats were induced by STZ-NA, except for the normal control. Before and after the 28 days of intervention, blood samples were taken and analyzed for fasting plasma glucose, postprandial glucose, and SOD activity. The nutritional content, ethanol content, and total antioxidant capacity of kefir-spirulina were also analyzed. The diabetic rats that were fed with kefir-spirulina (P1 and P2) had a significant decrease in both fasting and postprandial plasma glucose ($p$ <0.001) compared to the diabetic control rats. The decrease of plasma glucose in K2 is comparable to the control rats treated with the diabetic drug pioglitazone (K3). The activity of SOD in diabetic rats fed in P1 and P2 were higher ($p$ <0.001) than in untreated diabetic rats (K2). The IC50 of kefir-spirulina was 42 – 43 ppm. It was concluded that kefir combined with spirulina has high nutrition and antioxidant capacity, which is proven to be capable of controlling glycemic status and enhancing antioxidant status in a diabetic rat model.

**Keywords:** kefir; spirulina; antioxidant; hyperglycemia; SOD

## INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia, impaired insulin secretion, insulin resistance, and β cell dysfunction. The common characteristic symptoms include thirst, polyuria, polyphagia, blurring of vision, and weight loss **(WHO, 1999)**. The International Diabetic Federation (IDF) reported that approximately 451 million individuals suffered from DM in 2017, and this number is predicted to increase to 693 million in 2045 **(Cho et al., 2018)**. The mortality rate in 2017 of individuals aged 20 – 99 was 5 million, and this figure will continue to increase worldwide, creating a global health burden.

Hyperglycemia induces free radicals production and defects in the endogenous antioxidant defense mechanism, resulting in oxidative stress **(Moussa, 2018; Schultz Johansen et al., 2005)**. Free radicals such as reactive oxygen species (ROS) can be generated through many processes, including glucose auto-oxidation, change of redox balance, reduction of antioxidant concentration, and failure of antioxidant enzyme activity. ROS leads to oxidative damage to various biomolecules in the cell, such as proteins, lipids, and nucleic acids. ROS oxidizes the polyunsaturated fatty acids (PUFA) in the cell membranes and generate hydrogen peroxides. These hydrogen peroxides could undergo fragmentation, creating reactive intermediates, such as prostaglandin, isoprostane, and malondialdehyde (MDA) **(Catalá, 2009)**. The accumulation of ROS could induce macromolecules' glycation and may cause chronic complications in DM such as retinopathy, nephropathy, atherosclerosis, and coronary heart disease **(Schultz Johansen et al., 2005; Matough et al., 2012)**.

The high levels of ROS are commonly accompanied by the impairment of various antioxidant enzymes, such as catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) **(Moussa, 2018)**. These enzymes are critical in clearing the free radicals generated in the body. SOD, for example, is an essential antioxidant enzyme capable of preventing damage to macromolecular components of cells and repairing free radical compounds **(Valko et al., 2007)**.

The clinical management of DM controls glycemic status by consuming diabetic drugs either orally or by injection. However, this approach could increase complications such as hyperinsulinemia and infection **(Gedawy et al., 2018;**

**Santaguida et al., 2005)**. Limitation in drug treatment encourages an alternative approach to DM management. There has been a growing body of evidence that functional foods and probiotics have beneficial effects on DM management **(Venkatakrishnan, Chiu and Wang, 2019)**. Various foods rich in nutrients and bioactive compounds such as fruits, vegetables, legumes, probiotics, and fermented foods have shown promising results in controlling glycemic status and decreasing oxidative stress **(Barengolts et al., 2019; Habib and Parvin, 2008)**.

Kefir is fermented milk containing approximately 52 types of lactic acid bacteria and yeast **(Pogačić et al., 2013)**. It is widely used as a medication to treat various illnesses in middle Asian countries **(Kýlýc et al., 1999)**. Kefir acts as antioxidants that are capable of enhancing SOD, GSH-Px, and catalase activity **(Judiono, Djokomoeljanto and Hadisaputro, 2011)**.

In this study, we aimed to explore kefir's potential as functional food for controlling glycemic status and antioxidant status in DM modeled rats. To enhance the kefir activity and nutrition, we combined kefir with spirulina, nutrient-rich microalgae. Having high protein content (60 – 70%) **(Sadeghi et al., 2018)** and various bioactive compounds **(Wu et al., 2005)**, spirulina has shown effectivity in controlling blood glucose **(Sadeghi et al., 2018; Senthil, Balu and Murugesan, 2013)** and the level of oxidative stress **(Niccolai et al., 2019; Layam and Reddy, 2007)**.

### Scientific hypothesis

This study hypothesizes that a combination of kefir and spirulina could serve as a novel functional food in DM management through controlling glycemic status and antioxidant capacity in a diabetic rat model.

## MATERIALS AND METHODOLOGY

### Statement of ethics

This research obtained ethical approval with Ethical Clearance 152/EC/H/KEPK/FK-UNDIP/XII/2019 Faculty of Medicine, Diponegoro University, Semarang, Indonesia.

### Chemicals and reagents

All the chemicals used were of the highest purity grade. Streptozotocin (STZ) and nicotinamide (NA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Chloric acid, sulphuric acid, potassium sulfate, copper sulfate, ethanol, methanol, demineralized water, potassium hydroxide, and acetone were purchased from Merck. A kit for the glucose assay (GOD-PAP) was procured from DiaSys, Holzheim, Germany. A kit for estimation of SOD activity was procured from BioVision, Inc.

### Preparation of kefir-spirulina

Spirulina was obtained from PT Neoalgae Sukoharjo, Central Java. Spirulina generally contains 60 – 70% total protein, including essential amino acids such as leucine, isoleucine and, valine. It has 4 – 7% lipids, essential fatty acids such as linoleic and γ-linolenic acid, and ω-3 and ω-6 polyunsaturated fatty acids. It also contains provitamin A, vitamin B12, and β-carotene **(Koru, 2012)**.

Kefir-spirulina was made by combining 1% cow's milk with either 1% (w/v) or 2% (w/v) spirulina, followed by pasteurization. Kefir grain (5%) was added to the solution after it cooled down, followed by incubation at room temperature (25 – 37 °C) for 24 hours (Figure 1). After separating the grain from the solution, the kefir-spirulina mixture was analyzed for its nutritional content, ethanol content, and antioxidant capacity.

### Nutrient analysis

Nutrient analysis was performed according to the standard official methods of the Association of Official Analytical Chemists procedures 2005 **(AOAC, 2005)**. Nutritional parameters such as moisture, protein, fat, ash, carbohydrate, and fiber were determined.

#### *Moisture content*

Approximately 2 g of the sample (W1) was placed on a dish and dried in an oven for 5 hours at 95 – 105 °C. After drying, the dish was transferred to a desiccator to cool, and the sample was subsequently reweighed (W2). The moisture content was calculated as:

$$\text{Moisture}(\%) = \frac{(\text{W1} - \text{W2})}{\text{W1}} \times 100\%$$

W1 = weight (g) of the sample before drying,
W2 = weight (g) of the sample after drying.

#### *Ash content*

A drying method was used to assay the ash content. Briefly, 1 g of the sample was placed in a silica crucible. The sample was spread in an even layer and placed in a muffle furnace. The furnace temperature was allowed to reach a temperature of 550 °C for approximately 2 hours or until the sample turned white or slightly grey. The ash content was calculated as follows:

$$\text{Ash}(\%) = \frac{\text{Weight of ash}}{\text{Weight of the sample}} \times 100\%$$

#### *Crude fat content*

Fats were determined by the intermittent Soxhlet extraction method. A flask was weighed as an initial weight. A 2 g sample was placed in the fat sleeve. The sample and 200 mL of chloroform were added to the Soxhlet flask apparatus. The flask was then placed in an oven at 105 °C for 2 hours, then cooled in a desiccator. The percent crude fat was determined using the following formula:

$$\text{Crude Fat}(\%) = \frac{\text{Wt. of ether extract}}{\text{Wt. of sample}} \times 100\%$$

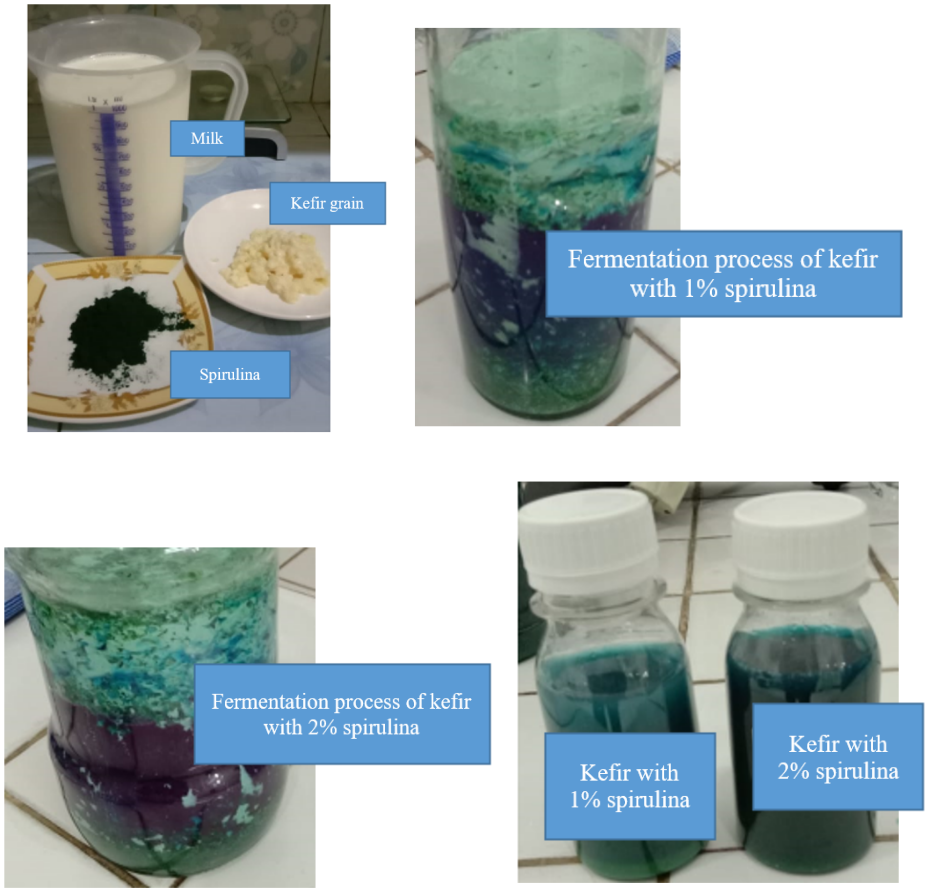


**Figure 1** Fermentation process of kefir with 1% and 2% of spirulina.

***Crude protein content***

Protein content in the sample was determined by the Kjeldahl method according to **(Afifah et al., 2019)** with some modification. A 2 g sample was digested by adding 15 mL of concentrated H2SO4 and two tablets of catalyst. The solution mixture was boiled until the solution was clear. The solution was filtered into a volumetric flask and connected for distillation. Ammonia was steam distilled from the solution, to which 50 mL of 45% sodium hydroxide solution had been added. 150 mL of the distillate was collected in a flask containing 100 mL of 0.1 N HCl and methyl red indicator. The ammonia-containing distillate reacted with the acid, and the excess acid in the flask was estimated by back titration against 2.0 M NaOH.

***Carbohydrate***

Total carbohydrate content was calculated as an approximation percentage with the following formula:

$$(\%)\ \text{Carbohydrate} = 100\% - [\text{Protein}(\%) + \text{Moisture}\ (\%) + \text{Ash}\ (\%) + \text{Fat}\ (\%)$$

***Dietary fiber content***

The sample (2 – 3 g) was mixed with 50 mL of 1.25% $H_2SO_4$ in a flask for 30 minutes. An amount of 50 mL NaOH (3.25%) was added to the mixture, then boiled. The mixture was filtered through a Buchner funnel containing Whatman paper and washed with 1.25% $H_2SO_4$, hot water, and 96% ethanol. The residue left in the Whatman paper was weighed.

$$\textbf{\textit{Crude fiber}}\ (\%) = \frac{\boldsymbol{W1 - W2}}{\boldsymbol{W1}} \times \mathbf{100\%}$$

***Ethanol content***

The ethanol content in the sample was determined using high-performance liquid chromatography (HPLC), according to **(Dias et al., 2020)**. Water as a mobile phase was used together with column YMC-Triat C8 (150 mm × 4.6 mm, particle size: 5 µm). 20 µL of the sample was run with 1 $mL.min^{-1}$ flowrate at 40 °C for 15 minutes.

***Antioxidant activity***

Antioxidant activity was determined based on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. The kefir sample was diluted in methanol absolute and mixed well. A series of sample concentrations of 5 ppm, 10 ppm, and 20 ppm were diluted with methanol absolute up to 5 mL, after which 1 mL of 0.4 mM DPPH solution was added. The mixture was shaken vigorously and incubated at room temperature for 30 min in the dark,

and the absorbance was measured at 517 nm. Trolox with various concentrations was used to make a standard curve.

$$\% \text{ Inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

The concentration of kefir-spirulina providing 50% inhibition($IC_{50}$) was calculated from the plotted graph of kefir-spirulina concentration and percentage of inhibition. The $IC_{50}$ value was then estimated using the fitted line **(Hendel, Larous and Belbey, 2016)**.

$$Y = a * X + b$$
$$IC_{50} = \frac{(0.5 - b)}{a}$$

## Animal experiment

The sample used in this study was a male Sprague Dawley *Rattus novegicus* aged 8 weeks, weighing approximately 150 – 200 g. The animals were acclimatized for one week in a temperature-controlled room (20 – 25 °C) and maintained on a 12 h light/12 h dark cycle. They were allowed free access to a standard commercial diet (Comfeed II, Japfa) and tap water. A total of 30 male Sprague Dawley rats were divided into five groups: normal control (K1), diabetic control (K2), pioglitazone treatment (K3), kefir combined with 1% spirulina treatment (P1), and kefir combined with 2% spirulina treatment (P2). All rats were induced by streptozotocin-nicotinamide (STZ- NA), except for the normal control. Nicotinamide (NA) was dissolved in saline solution and given intraperitoneally (i.p.) at a dose of 110 mg.kg$^{-1}$ body weight. After 15 minutes, the rats were induced by 45 mg.kg$^{-1}$ body weight of streptozotocin (STZ) in citrate buffer (i.p.). The rats considered having diabetes if three days after induction the fasting blood glucose was more than 126 mg.dL$^{-1}$ and plasma glucose were more than 200 mg.dL$^{-1}$. Groups P1 and P2 received kefir-spirulina via gavage with a total dose of 3 mL.200g$^{-1}$ body weight per day orally for 28 days. Group K3 received the diabetes drug pioglitazone at a dose of 0.27 mg.200g$^{-1}$ body weight per day orally for 28 days.

Before and after the 28 days of intervention, blood samples were taken through the retro-orbital sinus and were analyzed for fasting plasma glucose, postprandial glucose, and SOD activity.

### *Blood glucose test*

Fasting and postprandial blood glucose were determined using the Glucose Oxidase Phenol 4-Aminoantipyrine Peroxidase (GPO-PAP) according to the kit (DiaSys, Holzheim, Germany). Briefly, 10 µL of blood plasma was mixed with 1000 µL of reagent, then incubated for 20 minutes at 20 – 25 °C. The absorbance was read at 505 nm **(System, 2012)**.

### *Analysis of SOD activity*

SOD activity was measured via the WST method using a kit by BioVision. Briefly, 20 µL of blood plasma was mixed with 200 µL of WST solution, 20 µL of dilution buffer, and 20 µL of the SOD working solution. The mixture was then incubated for 20 minutes at 37 °C, and absorbance was read at 450 nm **(SOD Assay, 2019)**.

## Statistical analysis

Statistical analyses were performed using SPSS version 24 **(IBM, 2016)**. The obtained data were analyzed using Shapiro-Wilk since the data were normally distributed. The paired t-test and one-way ANOVA were used to determine the effect of experimental treatments. Differences were considered to be significant if *p*-values less than 0.05 ($p$ <0.05). In the case of variance homogeneity $p$ >0.05, post-hoc LSD was used; otherwise, post-hoc Tamhane was used.

# RESULTS AND DISCUSSION

## Nutrient content

Table 1 showed the nutrient content of the two formulations of kefir-spirulina. The water content was 93.82% and 92.67% for formulas A and B, respectively. The high water content in the two formulations was due to the clear part of the kefir used in the formulation, while the curd was separated. Our result is in line with several other studies that showed that kefir generally contained 89 – 90% water **(Sarkar, 2007; Plessas et al., 2017)**.

The ash content for the kefir formulation was similar, at 0.76% (formula A) and 0.72% (formula B). Our results were comparable with several other studies that found ash content was around 0.7 – 1.1% **(Arslan, 2014; Plessas et al., 2017; Rosa et al., 2017)**.

The two kefir formulations prepared in this study contained 4.02% and 5.53% protein, almost twice the minimum amount suggested by CODEX STAN 234-2003 **(FAO, 2010)**. It has been shown previously that kefir usually contains 3% of protein **(Sarkar, 2007; Plessas et al., 2017; Magalhães et al., 2011)**. The improvement of the protein content of the kefir prepared in this study is likely due to the addition of spirulina, which is rich in protein (circa 60 – 70% of its dry weight) **(Wu et al., 2005)**. The addition of 2% spirulina to the kefir showed higher protein content than the addition of 1% spirulina.

The carbohydrates content of kefir-spirulina for formula A and B were 1.56% and 1.12%, respectively. These results are lower than previous studies indicating that kefir contains carbohydrates between 3.8% and 4.7% **(Arslan, 2014)**. The fiber content in this study's kefir formulations was 2.49% (formula A) and 6.40% (formula B). Fiber is a nonstarch polysaccharide with high nutritional value; the high fiber content in spirulina will slow down the glucose absorption in the ileum, thereby lowering postprandial glucose **(Hernández-Alonso et al., 2017; Chandalia et al., 2000)**.

Fat content in the kefir formulations was 1.04% (formula A) and 0.59% (formula B). The low level of fat found in our kefir formulations was presumably due to lipase activity, which was produced by lactic acid bacteria (BAL). This study's fat content is relatively higher compared to the 0.2% that was shown in previous studies **(Sarkar, 2007; Plessas et al., 2017)**. Fat content in the kefir could vary depending on the type of milk used in the fermentation process **(Otles and Cagindi, 2003)**.

**Table 1** The nutritional content of the two formulations of kefir-spirulina.

| Formulation | Water | Protein | Fat | Ash | Carbohydrate | Fiber |
|---|---|---|---|---|---|---|
| | | | (%) | | | |
| (A) Kefir with 1% spirulina | 93.82 ±0.17 | 4.02 ±0.22 | 0.20 ±0.05 | 0.76 ±0.04 | 1.56 ±0.22 | 2.49 |
| (B) Kefir with 2% spirulina | 92.67 ±0.47 | 5.53 ±0.22 | 0.29 ±0.07 | 0.72 ±0.08 | 1.12 ±1.17 | 6.40 |

**Table 2** Ethanol content and antioxidant activity of kefir-spirulina.

| Formulation | Ethanol (%) | $IC_{50}$ (ppm) |
|---|---|---|
| (A) Kefir with 1% spirulina | 0.59 | 43.65 ±0.15 |
| (B) Kefir with 2% spirulina | 0.65 | 42.00 ±0.08 |

**Table 3** The average body weight of the experimental animals.

| Group | Body weight (g) ±SD | | | $p^b$ |
|---|---|---|---|---|
| | Pre-treatment | Post-treatment | Delta pre-post | |
| K1 (Normal control) | 187.67 ±12.59 | 199.33 ±22.28 | 11.66 ±17.68 | 0.249 |
| K2 (Diabetic control) | 166.17 ±4.17 | 151.00 ±4.73 | -15.17 ±1.33 | 0.026* |
| K3 (Diabetic + Pioglitazone) | 166.17 ±10.87 | 173.67 ±15.82 | 12.50 ±21.57 | 0.173 |
| P1 (Diabetic + Kefir with 1% spirulina) | 170.67 ±17.69 | 172.17 ±7.88 | 1,50 ±23.85 | 0.462 |
| P2 (Diabetic + Kefir with 2% spirulina) | 188.67 ±32.44 | 198.67 ±24.24 | 10.00 ±37.54 | 0.893 |
| $p^a$ | 0.023* | 0.001* | 0.066 | |

Note: [a]Wilcoxon; [b]Kruskal-Wallis; *Significant ($p$ <0.05).

**Table 4** Fasting blood glucose levels ($mg.dL^{-1}$).

| Group | Average fasting blood glucose ± SD | | | $p^b$ |
|---|---|---|---|---|
| | Pre-treatment | Post-treatment | Delta pre-post | |
| **K1 (Normal control)** | 69.37 ±1.25 | 71.17 ±2.51 | 1.80 ±1.91 | 0.069 |
| **K2 (Diabetic control)** | 263.42 ±2.98 | 265.53 ±2.43 | 2.10 ±1.31 | 0.011* |
| **K3 (Diabetic + Pioglitazone)** | 268.05 ±3.27 | 99.35 ±2.90 | -168.70 ±4.41 | 0.000* |
| **P1 (Diabetic + Kefir with 1% spirulina)** | 270 ±3.03 | 150.62 ±1.43 | -119.44 ±1.96 | 0.000* |
| **P2 (Diabetic + Kefir with 2% spirulina)** | 266.98 ±4.36 | 105.89 ±3.61 | -161.09 ±7.11 | 0.000* |
| $p^a$ | 0.000* | 0.000* | 0.000* | |

Note: [a]One-Way ANOVA; [b]Paired t-test; *Significant ($p$ <0.05).

**Table 5** Postprandial blood glucose ($mg.dL^{-1}$).

| Group | Average postprandial blood glucose ± SD | | | $p^b$ |
|---|---|---|---|---|
| | Pre-treatment | Post-treatment | Delta pre-post | |
| **K1 (Normal control)** | 77.92 ±1.80 | 81.26 ±1.98 | 3.34 ±0.97 | 0.000* |
| **K2 (Diabetic control)** | 278.58 ±3.25 | 274.06 ±2.64 | -4.52 ±2.64 | 0.010* |
| **K3 (Diabetic + Pioglitazone)** | 281.38 ±3.74 | 110.71 ±3.77 | -170.67 ±5.23 | 0.000* |
| **P1 (Diabetic + Kefir with 1% spirulina)** | 283.52 ±1.74 | 170.05 ±2.96 | -112.47 ±1.62 | 0.000* |
| **P2 (Diabetic + Kefir with 2% spirulina)** | 280.24 ±4.36 | 126.18 ±4.03 | -154.12 ±7.09 | 0.000* |
| $p^a$ | 0.000* | 0.000* | 0.000* | |

Note: [a]One-Way ANOVA; [b]Paired t-test; *Significant ($p$ <0.05).

**Table 6** SOD Activity ($U.mL^{-1}$).

| Group | Average SOD activity ± SD | | | $p^b$ |
|---|---|---|---|---|
| | Pre-treatment | Post-treatment | Delta pre-post | |
| **K1 (Normal control)** | 84.89 ±3.37 | 83.60 ±3.87 | -1.29 ±1.36 | 0.068 |
| **K2 (Diabetic control)** | 18.61 ±2.45 | 25.00 ±3.09 | 6.39 ±4.99 | 0.026* |
| **K3 (Diabetic + Pioglitazone)** | 24.48 ±4.26 | 72.95 ±3.06 | 48.47 ±5.63 | 0.000* |
| **P1 (Diabetic + Kefir with 1% spirulina)** | 25.00 ±5.32 | 50.82 ±3.74 | 25.81 ±5.93 | 0.000* |
| **P2 (Diabetic + Kefir with 2% spirulina)** | 23.43 ±4.07 | 65.30 ±4.06 | 41.86 ±6.82 | 0.000* |
| $p^a$ | 0.000* | 0.000* | 0.000* | |

Note: [a]One-Way ANOVA; [b]Paired t-test; *Significant ($p$ <0.05).

## Ethanol content

One of the main products of kefir fermentation was ethanol. The kefir-spirulina prepared in this study has a relatively low ethanol content (Table 2), which was 0.59% (formula A) and 0.65% (formula B). Ethanol in the kefir is usually in the range of 0.5 – 2.0% **(Rosa et al., 2017; Karaçalı, ÖzdemİR and Çon, 2018)**. Foods or beverages with low ethanol content are beneficial, especially in a country with religious restrictions regarding foods and beverages containing alcohol. The lactose fermentation of kefir generated ethanol, which contributed to the kefir's characteristic aroma **(Ertekin and Guzel-Seydim, 2010)**. Generally, the level of ethanol in kefir is influenced by yeast metabolism and heterofermentative bacteria that produce ethanol **(Farnworth, 2008)**.

## Antioxidant activity

The antioxidant activity of kefir-spirulina in this study was determined based on DPPH radical scavenging activity and presented as half-maximal inhibitory concentration $IC_{50}$. The average total antioxidant activity of kefir-spirulina showed relatively strong activity, with $IC_{50}$ of 43.65 ppm and 42.00 ppm, for formulas A and B, respectively (Table 2). An antioxidant is considered to be very strong if the $IC_{50}$ is less than 50 ppm, strong if $IC_{50}$ is 50 ppm – 100 ppm, weak if $IC_{50}$ is 150 ppm – 200 ppm, and very weak if $IC_{50}$ is more than 200 ppm **(Molyneux, 2004; Badarinath et al., 2010)**.

Kefir has been known to contain various antioxidant compounds capable of binding free radicals, decreasing the malondialdehyde concentration, and increasing glutathione peroxidase, thus regulating the level of oxidative stress **(Rosa et al., 2017)**. Antioxidant activity in kefir-spirulina increased during the fermentation process. The proton of the acids produced by lactic acid bacteria during fermentation can be donated to the free radicals, increasing the primary antioxidant capacity **(Waris and Ahsan, 2006)**.

## Bodyweight of experimental rats

The rats' body weight during the whole experiment (before and after treatment) is shown in Table 3. After 7 days of acclimatization, all rats except for the normal control (K1) were induced by STZ-NA. After 3 days, all diabetic rats received treatments for 28 days, except for the negative control (K2). Rats in diabetic control groups (K2) experienced a decrease in their body weight ($p < 0.05$). This result is not surprising since STZ-NA induction may cause physiological changes and alteration in energy metabolism, affecting body weight. STZ induction may damage the pancreas. The pancreas' function is to synthesize and secrete insulin, which is vital in glucose utilization and, eventually, energy production. A disruption in insulin production causes a disturbance in energy homeostasis. As compensation, the body will shift towards fat and protein metabolism and may cause weight loss **(Szkudelski, 2001)**. When diabetic rats were treated with the diabetic drug (K3) or kefir-spirulina (P1 and P2), body weight was compensated. We found no significant body weight changes ($p > 0.05$) in diabetic rats treated with kefir-spirulina (P1 and P2) or with diabetic drugs (K3). Kefir has an immunomodulatory effect capable of improving intestinal microflora and absorption, therefore improving body weight **(Judiono, Djokomoeljanto and Hadisaputro, 2010)**.

## Fasting and postprandial glucose analysis

Table 4 compares the changes in fasting plasma glucose levels in the five groups. The rats' blood glucose level in the normal control (K1) remained relatively stable at a normal level. In contrast, diabetic rats with no treatment (K2) suffered from consistently high glucose levels. The effect of 28 days of administration of kefir-spirulina (P1and P2) or the diabetic drug pioglitazone (K3) on the diabetic rats significantly decreased the glucose level ($p < 0.001$). Pioglitazone reduced blood glucose by improving glucose uptake in peripheral tissue and eventually enhanced insulin sensitivity **(Waugh et al., 2006)**. On the other hand, kefir's ability to control plasma glucose levels has been shown through several studies **(Nurliyani, Harmayani and Sunarti, 2015; Bellikci-Koyu et al., 2019)**. Kefir contains bioactive components such as peptides, short-chain fatty acids (SCFA), and exopolysaccharides (EPSs) that could affect the plasma glucose level. EPSs can increase cAMP concentration on Langerhans' islands so that the pancreas can increase insulin secretion **(Al-Shemmari, Kassim Altaee and Hassan, 2018)**. It also activates glucagon-like peptide 1 (GLP 1) and gastric inhibitory peptide (GIP), which subsequently enhances insulin secretion **(Al-Shemmari, Kassim Altaee and Hassan, 2018; Hadisaputro et al., 2012; Judiono, Djokomoeljanto and Hadisaputro, 2011; El-Bashiti, Zabut and Abu Safia, 2019)**. Furthermore, EPSs have also been proved to have antioxidant properties through free radical scavenging activity **(Mao et al., 2014)**, which gives protection from oxidative damage that occurred on pancreatic cells. The high protein content in the kefir has an indirect role in regulating glucose levels through the regeneration and maintenance of pancreatic β cells **(Hulston, Churnside and Venables, 2015)**. SCFAs, on the other hand, are produced by the activities of probiotic microorganisms present in the kefir grain **(Simova et al., 2002)**. SCFA were thought capable of protecting pancreatic β-cells, increasing enzymatic antioxidant activity, and improving insulin resistance through regulating the expression of tumor necrosis factor-alpha (alpha TNFα) and nuclear factor-kappa B (NF-kB) **(Wang et al., 2017)**.

As expected, combining spirulina and kefir improved the ability to control glucose levels. Increasing the spirulina composition by a factor of two (formula B on group P2) improved the ability of kefir-spirulina to lower the glucose level (glucose changes of P1 vs P2 were 112.47 $mg.dL^{-1}$ vs 154.12 $mg.dL^{-1}$; post-hoc test ($p < 0.05$). This result indicated the synergistic effect of bioactive compounds present in the kefir-spirulina formulation. The synergistic effect of modified kefir has also been shown in several other studies **(Nurliyani, Harmayani and Sunarti, 2015; Judiono, Djokomoeljanto and Hadisaputro, 2011)**. Spirulina consists of several bioactive compounds such as phycocyanin, carotenoids, vitamin E, chlorophyll, flavonoids, saponins, and phenolic compounds **(Okechukwu et al., 2019)**. The C-phycocyanin (C-PC) peptide activates the insulin signaling pathway, regulates

glucokinase expression in the pancreas and liver, and protects pancreatic β cells **(Li et al., 2020)**. Previous studies showed that spirulina consumption could reduce blood glucose levels **(Huang et al., 2018; Aissaoui et al., 2017; Jarouliya et al., 2015)**. Spirulina reduced blood glucose levels by stimulating β cell activity and increasing glucose transport to peripheral tissues **(Layam and Reddy, 2007)**. Moreover, it has high fiber content, which slows down macronutrients' digestive process **(Hernández-Alonso et al., 2017)**, thus maintaining the plasma glucose level.

We also analyzed postprandial glucose levels. Compared with fasting glucose, postprandial glucose level was thought to be better associated with the risk factor of diabetes and the level of HbA1c **(Haddadinezhad and Ghazaleh, 2010)**, which reflects the glycemic control in diabetes patients over a certain period. According to the American Diabetes Association (ADA), the recommended postprandial glucose level for diabetes patients is below 180 $mg.dL^{-1}$ **(ADA, 2018)**. The postprandial glucose levels of the five groups are presented in Table 5. In general, the changes in postprandial glucose of all groups are in agreement with the results obtained in fasting glucose levels. As can be seen in Table 5, treatment with pioglitazone (K3) and kefir-spirulina (P1 and P2) successfully reduced ($p$ <0.001) the postprandial glucose concentration to less than 180 $mg.dL^{-1}$ (K3 = 110 $mg.dL^{-1}$, P1 = 170 $mg.dL^{-1}$, and P2 = 126 $mg.dL^{-1}$). The ability of kefir to reduce postprandial glucose has also been shown in other studies **(Alsayadi et al., 2014)**. The biggest changes in postprandial glucose levels in this study were observed in group P2, which was treated with kefir combined with 2% spirulina (formula B).

## SOD Activity

Table 6 shows the changes in the SOD activity of the experimental groups. Administration of kefir-spirulina in groups P1 and P2 significantly increased SOD levels ($p$ <0.001). The increase in SOD activity was 48.47 $U.mL^{-1}$, 41.86 $U.mL^{-1}$, and 25.86 $U.mL^{-1}$ for groups K3, P2, and P1, respectively. There is some background increase (6.39 $U.mL^{-1}$) in the negative control group (K2), while the normal control group (K1) showed a relatively constant SOD activity ($p$ >0.05). A study by **Omayma et al. (2013)** showed that fermented soy could normalize the SOD and glutathione peroxidase (GPx) activities in rat tumor tissue. Kefir has a better ability to donate protons than non-fermented milk. Therefore, kefir can give protection against free radicals and thus increase SOD activity **(Niccolai et al., 2019; Bellikci-Koyu et al., 2019; Zhang, Wu and Fei, 2016)**.

Oxidative stress is closely related to various diseases, including diabetes. The body will naturally develop enzymatic and nonenzymatic antioxidant defenses to balance oxidative stress. However, this endogenous protection might fail to prevent the overpopulated free radicals that are generated as the disease progresses. Therefore, exogenous antioxidant-rich foods such as kefir and spirulina are beneficial to back up the role of endogenous antioxidant systems.

## CONCLUSION

The present study has demonstrated that kefir-spirulina showed high nutrient content, strong antioxidant capacity, relatively low alcohol concentration, and an ability to control glycemic status and SOD activity of a diabetic rat model. Based on the properties exhibited by kefir-spirulina in this study, kefir-spirulina has the potential for use in the dietary approach to the management of diabetes.

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# IMPROVEMENT OF THE SUNFLOWER OIL STABILITY BY BLENDING WITH MORINGA OR SESAME OILS

*Amina Aly, Hoda Ali, Mohamed Abdeldaiem*

## ABSTRACT

Vegetable oils can be adjusted by different methods to enhance their commercial applications and to increase their pure quality nutrition. One of the most leisurely ways of creating new innovative products with desirable textured and oxidative properties is the mixing of vegetable fats/oils of various properties. Pure sunflower (SFO) blended with pure moringa (MOO) and sesame (SEO) oils in the ratio of 95:5 and 90:10 w/w. The outcomes showed that the highest acid value was observed at SFO + SEO 10% (0.782). Sunflower oil stabilized when blended with MOO and the levels of conjugated dienes (CD) and trienes (CT) were depressed compared to control. The TPC value was higher when the sunflower blended with moringa oil. The highest scavenging activity percentage was observed in SFO + MOO 5% and SFO + SEO 10% respectively. Meanwhile, the highest induction period at 100 °C was 11.45 hours in the treatment of SFO + MOO 5%. The master fatty acids in the sunflower and moringa oils are combined (SFO:MOO, 90:10), oleic, and palmitic acids. It seems from the current findings that suitable mixing of high linoleic oils with MOO will result in oil mixtures that can satisfy nutritional wants with expanded balance for home cooking and deep-frying.

**Keywords:** antioxidant; moringa oil; oxidative stability; sesame oil; sunflower oil

## INTRODUCTION

External conditions, such as air, light, and temperature, speed up oxidative reactions which may afford up in the development of off-flavors and odors related to volatiles low molecular weight, discoloration. Lipid oxidation is one of the culprits of degradation in fat and oils, contributing to the improvement of off-flavors' and bad flavors, resulting in decreased shelf-life. Oxidative oxidation is a significant financial problem in the food industry meals industry due to the fact it influences many first-class traits such as taste, color, appearance, and nutritional value of foods. It also yields highly harmful compounds **(Sikwese and Duodu, 2007)**. Hydroperoxides are the principal metabolites of lipid oxidation and are commonly referred to as peroxides. A part from that, the dedication of the peroxide amount can also be seen as an oxidative measure of lipid oxidation. Likewise, following the development of peroxides is an extension in UV absorbance at a rate of around 232 nm, which is typical of the conjugated diene systems. Oxidative rancidity is a key factor influencing the quality of refined and packed vegetable oils during storage **(Gulla and Waghray, 2012)**.

The comparatively low shelf-life of most commercially accessible vegetable oils restricts their utility in different applications. Autoxidation is considered to be the foremost route of degradation of edible oil, which causes unpleasant odors and flavors **(Merrill et al., 2008)**. Mixing vegetable oils with different properties is the easiest physical and economical method for modifying the composition of fatty acids. Production of bioactive ingredients and natural antioxidants, and making something new at a low-cost price **(Hashempour-Baltork et al., 2018)**. Sunflower oil has a significant source of monounsaturated fatty acids for dietary goods and due to its high oxidation resistance. This also has a high degree of natural antioxidants such as tocopherols, rendering this superior to other vegetable oils. Sunflower oil includes high concentrations of unsaturated fatty acids concentrations (77 – 82%) such as linoleic acid (59 – 67.5%) and oleic acid (14.0 – 18.1%), respectively.

*Moringa olefera* oil has bright yellow with a sweet, peanut-like nutty flavor. It is oil usually used without any pre-processing (refining, bleaching, and deodorization) due to good nutty flavor, lower peroxide level, which is necessary for most commercial vegetable oils. It can be considered a healthy diet. Other Investigations have also shown that C18:1 rich diets can reduce plasma cholesterol and decrease the risk of cardiovascular disorders. Oils high in monounsaturated fatty acids are attracting more support from the food industry because they have safe properties and greater oxidative stability **(Nadeem and Imran, 2016)**. In the same concern, **Lapčíková et al. (2018)** cited that free fatty aids increase thermal oxidation of oils, and their unsaturation rather than chain length leads to significant effects upon thermo-oxidative degeneration of oils. The oxidation rate of frying oil increases as the content of unsaturated fatty acids of the oil increases. The content of

linolenic acid is critical to the frying performance, the stability of the oil, and the flavor quality of fried food. Sesame oil is a healthy source of ω6 fatty acids and has a significant amount of sesamin and sesamolin lignans that have various effects on bioactivity and health safety. Despite having 85 percent of unsaturated fatty acids, sesame oil is one of the most stable oxidation vegetable oils. Sesame oil, however, with favorable nutritional and safety effects, is low in ω3 fatty acids and has limited use in the food industry due to its excessive price **(Hashempour-Baltork et al., 2018)**. Also, tocopherol equivalents have an antiinflammatory function and antiproliferative effects on cancer cells **(Dravie et al., 2020)**. Oils stability can be improved via the mixing of oleic acid oils with high contents **(Anwar et al., 2007)**. Oleic acid is greater resistant to oxidation compared with PUFA, both in normal storage and at high temperatures. In addition to compromising food consistency, at some stage in this process, it may additionally generate workable toxic compounds through the action of free and reactive oxygen species (ROS) which are hazardous to human health and are involved in degenerative diseases such as cancer and early aging **(Krishnaiah, Sarbatly and Nithyanandam, 2011)**. With the consideration of the harmful effects of lipid oxidation on food degradation and human health, there is a want to improve the exogenous antioxidants to avoid not just the assumed deleterious outcomes of free radical in the human body, but additionally, the degradation of fats and other components of meals stuffs **(Nyam et al., 2013)**. It is worthy to know that, the fatty acid content of new vegetable eatable oil may offer an intention for its probability uses for eatable or industrial use **(Ahamd et al., 2015)**. As a consequence, the blending of sunflower oil with oil rich in both oleic acid and natural antioxidants like moringa or sesame oils to improve its oxidation stability was the target of the present investigation. The major goal of the current study was to assess the consequences of the fatty acid content of sunflower, moringa, and sesame oils and the combos of them on the alteration in the peroxide value, acid value, thiobarbituric acid value, phenolic compounds, and antioxidant activity, as well as the fatty acid compositions and their oxidative constancy.

### Scientific hypothesis

Assess the outcomes of the fatty acid content of sunflower, Moringa, and sesame oils and the combos of them on the alteration in the peroxide value, acid value, thiobarbituric acid value, phenolic compounds, and antioxidant activity, as considerably as the fatty acid compositions and their oxidative constancy.

## MATERIAL AND METHODOLOGY

The commercial varieties of sunflower oil (*Helianthus annuus* L.), moringa oil (*Moringa oleifera*), and sesame oil (*Sesamum indicum*) were obtained from Agriculture Research Center, Giza, Egypt. Pure SFO was blended with pure MOO and SEO oils in the proportion of 95:5 and 90:10 w/w. The oils were extensively stirred to create even mixtures and resulted in seven oil samples, namely; SFO, SFO:MOO (95:5), SFO:MOO (90:10), SFO:SEO (95:5), SFO:SEO (90:10), MOO, SEO stored in a brown bottle and kept in a freezer at -20°C until used for different analysis.

### Physico-chemical properties of the oils

Physico-chemical properties of the pure and mixed oils; color index, flow time, refractive index, acid value, peroxide value, and free fatty acids were measured using the standard method of **(AOCS, 1996)**.

### Conjugated dienes and trienes determination

A spectrophotometer (Jasco 530 UV-visible Japan) was engaged in measuring the conjugated fatty acids (conjugated dienes and trienes) of various oils. Optical density at 234 and 268 nm were, respectively used for determining conjugated dienes and trienes **(AOCS, 1985***)*. The findings are presented as the 232 and 268 nm absorbance values.

### Total phenolic compound (TPC)

The method of Folin Ciocalteu was used to evaluate the total phenols content of as **Singleton and Rossi (1965)** described. The absorbance using (Jasco 530 UV-visible spectrophotometer Japan) was measured at 765 nm. Phenolics content was calculated by a standardized curve gained from the calculation of the absorption of a specified gallic acid concentration and the results were presented as equal gm gallic acid (GAE) per 100 g oil.

### Thiobarbituric acid (TBA)

Thiobarbituric acid (TBA) value was evaluated using the method defined by **Guillén-Sans and Guzmán-Chozas (1998)**.

### Free radical scavenging activity (DPPH)

The antioxidant functions of the oil samples were calculated using the stable DPPH system as updated by **Güllüce et al. (2004)**. Absorbance was recorded at 517 nm utilizing a UV-visible spectrophotometer (Jasco V-530, Japan). Radical scavenging activity was defined as the inhibition percentage and estimated utilizing the equation below:

%DPPH = [Absorbance of Control - Absorbance of Sample/Absorbance of Control] x 100

### Oxidative stability measurement

Rancimat has been used to assess the oxidative stability of sunflower and blended oils using a 679 Rancimat (Metrohm, Herisau, Switzerland) at 100 ºC with an airflow rate at 20 L per 1 hour as defined by **Pablos-Méndez et al. (1997)**. The result of the Rancimat test was calculated by induction time measurement.

### The protective factor

The protective factor (PF) of the examined oils is extracted as a percentage extension of the induction period **(Abramovič and Abram, 2006)**, according to the following equation: PF = IP sample / IP control X 100.

### Fatty acid composition

Gas chromatographic analysis was employed to identify the composition of the fatty acid by preparing the methyl esters as reported by **Stahl (1969)**.

### Identification of the fatty acids methyl esters

The fatty acid methyl esters for different samples have been analyzed consistent with the **IUPAC (1987)** approach, using (HP 6890 gas chromatography instrument, Hamilton, USA) fitted with Innowax-crosslinked polyethylene glycol column 30 m; i.d. 0.32 nm; 0.5 µm film thickness. Fatty acid samples were recognized by comparing the fatty acid retention times peak regions with those of standard ones. These standards consist of, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), arachidic acid, oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0) and behenic acid (C20:1).

### Cox value

The oils' Cox value (oxidizability) was defined by the unsaturated C18 fatty acid percentage, using the formula proposed by **Fatemi and Hammond (1980)**:

Cox value = (1 [18:1%] + 10.3[18:2%] +21.6 [18:3]) / 100

The ODR and LDR are quantified by **Pleines and Friedt (1988)** and estimate the effectiveness of desaturation from oleic to linoleic (ODR) and from linoleic to linolenic acid (LDR) within the desaturation process. They were computed as follows:

ODR = %C18: 2 + %C18: 3/ %C18: 1+ %C18: 2+%C18: 3

LDR = %C18: 3 / %C18: 2 + %C18: 3

### Statistical analysis

Results were focused on statistical analysis by the variance method analysis and the means of three samples ± SD were evaluated by the least significant difference (LSD) at a frequency level of 0.05 according to (Duncan's Multiple Range Test).

## RESULTS AND DISCUSSION

One of the simplest procedures for making new specific products is the mixing of two or more oils with different characteristics. Blending different types of vegetable oils can not only adjust the profile of fatty acids but can also increase the degrees of bioactive lipids and natural antioxidants within the mixtures and provide higher great oils, in addition, to a progressed dietary fee at lower-priced **(Aladedunye and Przybylski, 2013)**.

### Physico-chemical properties of oils

***Colour***

Bearing on the changes in oil color, it was obvious that the blending caused a slight increase in color, as it increased from 0.099 in SFO to 0.111, 0.124, 0.107, and 0.120 for SFO + 5% MOO, SFO + 10% MOO, SFO + 5% SEO, SFO + 10% SEO, respectively (Figure 2, Table 1). The color of the oils is particularly due to the presence of the pigments that are collected along with the oil throughout refining and are effectively getting rid of during the bleaching processing of oil **(Basuny and Al-Marzouq, 2016)**. Vegetable oils with a low index of color are better fitted for both edible and domestic purposes. Moreover, **Padmavathy, Siddhu and Sundararaj (2001)** stated that sensory attributes of vegetable oils could color and taste might be enhanced by mixing, as the mixtures would fulfill the main objective of storage stability.

***Flow time***

With frying time and oil temperature of the oil changes considerably. It is viewable from (Table 1), the relative flow time (as an indicator for viscosity) of oil at 60 ºC was measured as a relative flow time and considered an indicator of oil viscosity. Data in (Table 1) show that the flow time value for sunflower was the highest (2.690 $g.cm^{-3}$) when compared with MOO (1.872 $g.cm^{-3}$) and SEO (0.953 $g.cm^{-3}$). So, when MOO and SEO were added to the SFO its flow time decreased. There is a relationship between the viscosity level and the degree of unsaturation oil. It means that blinding sunflower oil in different parts of MOO and SEO reduces the changes in viscosity values through the frying process. This means that a higher level of SEO (10%) mixed with SFO resulted in the greatest change in SFO viscosity (1.654 $g.cm^{-3}$). This can be explained by the fact that polyunsaturated fatty acids appear to be oxidized easily and form polymer compounds **(Bracco, Dieffenbacher and Kolarovic, 1981)**.

***Refractive index***

From the obtained results as shown in Table 1 that, there are no substantial differences between pure or blended oils. The variation in refractive indices could be due to specific gravity, molecular weight, increase in saturation, and linearly autoxidation stage. The refractive index of the used oils in the present investigation is in the agreement with the findings of **Mishra and Sharma (2011)** cited that no significant modifications were noted in the refractive index of, the refined rice bran oil and blended with refined sunflower oil. Otherwise, **Adel, Shaker and Mounir (2015)** studied whether mixing palm oil with different quantities of sunflower or soybean oil resulted in the significant refractive index values decrease significantly. These reductions may be ascribable to the increase of monounsaturated fatty acids of palm oil in the blended oils.

***Acid value***

Low acidity oils are considered acceptable for edible application and have to be explored for economic, nutritional, and health reasons. The acid value developments for sunflower, oil without, and with the addition of MOO and SEO shown in Table 1 it could be noticed that the acid values of sunflower before blending and when added to Moringa and sesame oils were 0.501, 0.421, 0.393, 0.670, and 0.782 for sunflower oil, sunflower oil +5% Moringa oil, sunflower oil +10% Moringa, sunflower oil +5% sesame, and sunflower oil 10% sesame, respectively.

The acid value was relatively low for sunflower oil and 10% Moringa oil. Mixing sunflower oil with several parts of Moringa oil significantly decreases the acid values. This reduction was increased by bringing up the mixing of Moringa ratio oil. Compared to sunflower oil Moringa oil, has higher oxidative stability as a result of the higher content of oleic acid (monounsaturated) and lower content of polyunsaturated fatty acid substance.

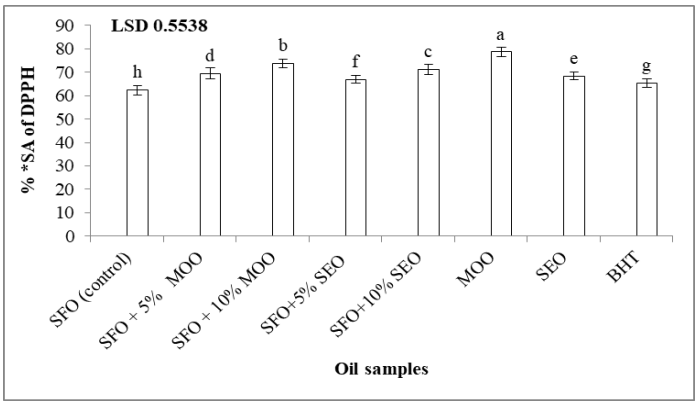


**Figure 1** Percentage of DPPH radical sunflower oil scavenging activity and its blends with moringa or sesame oils. Note: SA = Scavenging activity, SFO = Sunflower oil, MOO = *Moringa olefera* oil and SEO = Sesame oil, BHT = Butylated hydroxyl toluene. Means with different letters above bars varied considerably at $p$ <0.05. Bars ± standard deviation (n = 3).

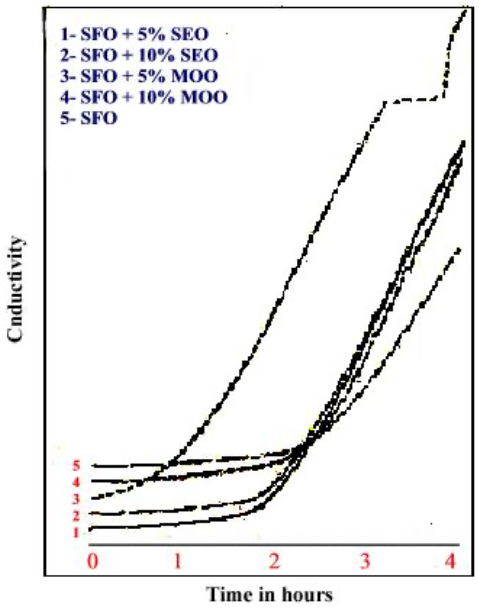


**Figure 2** Effect of blending of sesame oil (5 and 10%) and moringa (5 and 10%) on the oxidative stability of sunflower at 100 °C. Note: SEO = sesame oil, MOO = *M. olefera*, and SFO = Sunflower oil.

An increment in the number of acidities was partly due to the hydrolysis and further oxidation of the formed byproducts **(Wanasundara, Wanasundara, and Shahidi, 2001)**. The low acid number of Moringa oil measures up to sunflower oil point out a potential low free composition of fatty acid that means less vulnerability to rancidity.

## Peroxide value

Peroxide value (PV) is a measure of the content of the acid composition of peroxides and hydroperoxides suggesting less sensitivity to physicochemical and antioxidant possessions that require rancidity. The data in (Table 1) resulted in a substantial difference in PV ($p$ >0.05), this was observed between control and SFO samples containing PVs reduced MOO and SEO. Sunflower oil significantly ($p$ >0.05) had the highest peroxide value (0.720 meq.$O_2$ per kg of oil), though the most reduced value (0.615 and 0.530 meq.$O_2$ per kg of oil) was found in sunflower oil blend in Moringa oil were enlisted at level 5 and 10 percent, respectively. Blowing up the MOO proportion within the mixes brought an increase in the antioxidant strength and subsequently delayed the rise in PV. These findings are consistent with the level of unsaturation oil, easily unsaturated fatty acids respond with oxygen to form peroxides **(Marina et al., 2009)**. Similarly, **Mariod et al. (2005)** tested the impacts of various mixes of sunflower kernel oil and *Sclerocarya birrea* oil on the progress of PV and detailed that the mixes of 10 percent. Berry oil with sunflower kernel oil showed a significant progression in oxidative stability compared to pure sunflower kernel oil.

The decline in PV, as established by mixing, was largely due to the decrease of unsaturated C18:2 at the disbursement of C18:1 **(Reynhout, 1991)**. The increase of oleic acid content of Moringa oil coupled with its high unsaturated and little peroxide may meet the requirements of Moringa oil for use in industries, such as food, biofuel generation, Agrochemicals, paint, and varnish manufacture **(Aly, Maraei and Ali, 2016)**.

## Conjugated dienes and conjugated trienes

Quantity of CD and CT may be a sound test for determining the oxidative stability of the oils. Lipids with dienes show strong absorption at 232 nm, as absorbed by conjugated trienes at 270 nm. The increment in CD and CT substances is proportional to the oxygen absorption. The low CD and CT levels will increment the oxidative strength of the oil's absorptivity at 232 and 270 nm in SFO and oil mixes, due to the appearance of primary and secondary items of oxidation. Arrangement of aldehydes, ketones (rancid off-flavor compounds), and other oxidation items taken after by an increment of at 270 nm in absorptiveness (Table 2). Referable to the formation of CT as well as unsaturated ketones and aldehydes, the variation of absorptivity at 270 nm provided and pattern similar to that of absorptivity at 232 nm displayed. The elevated substance of conjugated oxidative items in SFO can be ascribed to its high content of linoleic acid (60% of total fatty acids), samples of SFO oil stabilized when combined with MOO, and reduced the CD and CT levels compared to control samples as displayed in Table 2, moreover, demonstrated that MOO is superior to SEO. Estimation of CD and CT may be is a good parameter for determining the oils oxidative stability. The formation of high contents of CD may be related to the presence of higher contents of polyunsaturated fatty acids. **Siddiq et al. (2005)** explored the antioxidant effectiveness of methanolic extract of *Moringa oleifera* leaves for stabilizing SFO under rapid aging using the capacity of CD and CT contents. **Iqbal and Bhanger (2007)** also, identified the antioxidant action of garlic extracts in SFO, evaluated under speeded up situations, using CD and CT as oxidative degradation indicators. **Barakat and Ghazal (2016)** saw a promising potential in the nutrition aspects of moringa seeds oil. The increased content of unsaturated fatty acids may have a healthy effect on human nutrition from moringa seeds oil.

## Phenolic content of pure and blend oils

Total phenolic contents (TPC) of various oil tests were assessed as phenolics may act as cancer prevention agents and secure lipids from peroxidation. Oils mixtures differing in TPC values, presenting 26.17, 31.28, 22.17, and 24.27 mg Gallic acid equivalents (GAE) per 100 grams of MOO 5%, MOO 10%, SEO 5%, and SEO 10%, respectively (Table 2). The TPC was superior when sunflower blended with Moringa oil than with sesame oil. These results recommend that moringa oil contains noteworthy amounts of phenolic components that under the accelerated oxidative conditions may mostly contribute to the oil stability. The constancy of the oil is not only associated with the overall volume of phenolics but moreover with the concentration of choosing phenols. Without a doubt, the concentration of phenolic content in oilseed is a critical calculate whereas assessment of the oil quality because these compounds have been connected with oil color and the shelf-life, and especially its oxidation resistance **(Cheikh-Rouhou et al., 2006)**. Since of the ability of phenolic compounds to give hydrogen molecule or an electron to create steady radical intermediates that can serve as vital antioxidants agents, consequently, and they can prevent deterioration by quenching the radical reactions caused by lipid oxidation **(Abdelazim, Mahmoud and Ramadan-Hassanien, 2013; Koski et al., 2003)**. Phenolic compounds have much influence on stability and may prevent deterioration through quenching of radical reactions responsible for lipid oxidation **(Koski et al., 2003)**. Moreover, it has been found that the natural antioxidants of the blends give better results concerning the oxidative stability and quality parameters. The mixing not only stabilizes the eatable oils but also assists to promote and strengthen oil's nutritional and functional properties, integrating the positive features of the two oils into one, thus increasing the consumer viabilities.

## Thiobarbituric acid (TBA)

The thiobarbituric acid corrosive test is typically carried out to regulate the secondary oxidation substances that are mindful of the off-flavor. The TBA test refers to the aldehyde level shown within the oil by the response of malonaldehyde with TBA. The changes within the TBA levels (absorbance at 530 nm) of sunflower oil mixed with separate amounts of moringa and sesame oils are indicated in (Table 2). The most noteworthy ($p$ >0.05) TBA level was reported for sunflower, while, sunflower oil and its blind with MOO at level 10% (v/v) had ($p$ >0.05) significantly the most reduced values was 0.097 as absorbance at 532 nm. The TBA value is considered a strong chemical consistency parameter for assessing the oxidative state of new eatable oils and fats for calculating the degree of oxidation that occurred **(Lalas and Tsaknis, 2002)**.

## Free radical scavenging activity (DPPH)

The DPPH radicals have been utilized to prove the potential of SFO and its mixes to resist lipid peroxidation. The provided data listed in (Figure 1) can be indicated that mixing SFO with MOO and SEO expanded the scavenging activity percentage of SFO. The scavenging activity of the oil blends increased with increasing the ratio of MOO and SEO oils added. The highest scavenging activity percentage was observed in SFO + MOO 5% and SFO + SEO 10% (73.71 and 71.21%) respectively. Moringa oil is wealthy with oleic acid monounsaturated (~79%) and very low in PUFA **(Ogunsina et al., 2014)**, and this is following our results, this oil contains an abundance of antiradical molecules such as tocopherols, phenolics, and carotenoids to avoid the peroxidation of its PUFA substance and extinguishing free radicals within the human body. Moreover, sesame oil has the prevalent oxidative constancy and the useful physiological impacts are linked to tocopherols and lignans, particularly sesamin and sesamolin **(Hashempour-Baltork et al., 2018)**.

**Table 1** Physico-chemical possessions of sunflower oil and blending with moringa and sesame oil.

| Oil samples | Physico-chemical properties | | | | |
|---|---|---|---|---|---|
| | Color Index Abs at 430 nm | Flow time g.cm$^{-3}$ | Refractive index | Acid value mg KOH.g$^{-1}$ oil | Peroxide value meq.$O_2$.kg$^{-1}$ oil |
| SFO (control) | 0.099$^{d}$ ±0.03 | 2.690$^{a}$ ±0.08 | 1.472$^{a}$ ±0.06 | 0.501$^{d}$ ±0.08 | 0.720$^{a}$ ±0.03 |
| SFO + 5% MOO | 0.111$^{c}$ ±0.06 | 2.011$^{c}$ ±0.07 | 1.470$^{a}$ ±0.08 | 0.421$^{e}$ ±0.06 | 0.615$^{b}$ ±0.07 |
| SFO + 10% MOO | 0.124$^{b}$ ±0.05 | 2.270$^{b}$ ±0.09 | 1.470$^{a}$ ±0.04 | 0.393$^{e}$ ±0.04 | 0.530$^{c}$ ±0.05 |
| SFO + 5% SEO | 0.107$^{c}$ ±0.02 | 1.952$^{d}$ ±0.07 | 1.469$^{a}$ ±0.03 | 0.670$^{c}$ ±0.02 | 0.672$^{b}$ ±0.04 |
| SFO + 10% SEO | 0.120$^{b}$ ±0.05 | 1.654$^{e}$±0.02 | 1.464$^{a}$ ±0.01 | 0.782$^{b}$ ±0.06 | 0.561$^{c}$ ±0.03 |
| MOO | 0.149$^{a}$ ±0.02 | 1.872$^{d}$ ±0.02 | 1.468$^{a}$ ±0.05 | 0.270$^{f}$±0.01 | 0.260$^{e}$ ±0.02 |
| SEO | 0.146$^{a}$ ±0.04 | 0.953$^{f}$ ±0.01 | 1.471$^{a}$ ±0.03 | 0.930$^{a}$ ±0.07 | 0.341$^{d}$ ±0.04 |

Note: Values are mean of three replication ± standard deviation. Letters after the values denote within-column significant differences ($p$ <0.05). Abbreviations: SFO = Sunflower oil, MOO = *M. olefera* oil and SEO = Sesame oil.

**Table 2** Oxidation products of sunflower oil and blending with moringa and sesame oil.

| Oil samples | Products of oxidation assays | | | |
|---|---|---|---|---|
| | CD Abs at 232 nm | CT Abs at 270 nm | Phenolic content gm.100gm$^{-1}$ oil | TBA Abs at 530 nm |
| SFO (control) | 2.414$^{c}$ ±0.43 | 1.207$^{b}$ ±0.68 | 21.15$^{d}$ ±0.25 | 0.136$^{a}$ ±0.18 |
| SFO + 5% MOO | 2.188$^{c}$ ±0.12 | 1.719$^{a}$ ±0.17 | 26.165$^{c}$ ±0.15 | 0.117$^{c}$ ±0.56 |
| SFO + 10% MOO | 1.951$^{d}$ ±0.81 | 1.379$^{b}$ ±0.71 | 31.276$^{b}$ ±0.17 | 0.097$^{d}$ ±0.26 |
| SFO + 5% SEO | 3.137$^{a}$ ±0.56 | 1.804$^{a}$ ±0.53 | 22.174$^{d}$ ±0.46 | 0.129$^{b}$ ±0.47 |
| SFO + 10% SEO | 2.836$^{b}$ ±0.19 | 1.820$^{a}$ ±0.12 | 24.268$^{c}$ ±0.26 | 0.121$^{b}$ ±0.28 |
| MOO | 1.683$^{d}$ ±0.42 | 0.820$^{c}$ ±0.27 | 44.183$^{a}$ ±0.53 | 0.045$^{d}$ ±0.54 |
| SEO | 2.314$^{c}$ ±0.57 | 1.461$^{b}$ ±0.28 | 24.890$^{c}$ ±0.19 | 0.112$^{b}$ ±0.21 |

Note: Values are sum of 3 replicates ± standard deviation. Letters after the values denote within-column significant differences ($p$ <0.05). Abbreviations: CD = Conjugated diene, CT = Conjugated triene, T.B.A = Thiobarbituric acid, Scavenging activity = SA, SFO = Sunflower oil, MOO = *Moringa olefera* oil and SEO = Sesame oil.

**Table 3** Effect of blending of sesame or moringa oils (5 and 10%) on oxidative stability of sunflower oil at 100 ºC.

| Oxidative Stability (OS) | Sunflower oil | Sesame oil | | Moringa oil | |
|---|---|---|---|---|---|
| | control | 5% | 10% | 5% | 10% |
| *IP (hrs) | 4.33 | 8.01 | 9.32 | 11.45 | 10.35 |
| **PF % | - | 1.85 | 2.15 | 2.64 | 2.39 |

Note: IP = Induction period determined by Rancimat method, **PF = Protection factor.

## Impact of mixing of sesame or moringa oils (5 and 10%) on the constancy of sunflower oil at 100 °C

Confidence of the oxidative steadiness (OS) record by Rancimat is the most commonly used procedure to determine the susceptibility of consuming fats and oils under accelerated conditions. Oxidative steadiness, classified under specified conditions as oxidation resistance is one of the most imperative measures of eatable oils' consistency, quality of eatable oils' consistency **(Vidrih, Vidakovič, and Abramovič, 2010)**. The stability of oxidation was due to the presence of natural antioxidants in seeds which indicated resistance to oxidation. Findings of the induction period at 100 °C and the obtained results are shown in Table 3 and graphically represented in (Figure 1). The induction period at 100 ºC indicating remarkable stability of Moringa oil when mixed with sunflower oil at a concentration of 5% 11.45 h. This resistance to oxidative rancidity may be credited to the presence of endogenous phenolic antioxidants and a high considerable concentration of tocopherols. Studies report great oxidative soundness of seed fat of diverse species of Moringaceae, which incorporate *M. Oleifera* **(Anwar and Bhanger, 2003; Lalas and Tsaknis, 2002)**. In this concern, **Padmavathy, Siddhu and Sundararaj (2001)** detailed that mixing vegetable oils not only enhances the sensual consistency, moreover improves the capacity soundness of the eatable oils. In the expansion, oil gotten from the combination of chia and sesame oils has a superior proportion ratio of ω-3:ω-6 fatty acids, by precocious oxidative stability fluctuating among 6 and 8 hours at 110 ºC and storability at 25 ºC involving 80 and 123 days **(Rodríguez et al., 2020)**.

## The protective factor (PF)

Represents the oxidation resistance of oils as rate expansion of the IP. The most noteworthy PF% was obtained in the case of the blending of 5% and 10% of MOO. In the case of the PF, the value is more prominent than one, heighten the sample's stabilizing power. Without a doubt, the probability of utilizing the oils assisting or combining with other oils could be a rule of *M. oleifera* devastates valorization **(Barakat and Ghazal, 2016)**.

**Table 4** Change of the fatty acids composition (relative content, %) of sunflower and oil blends.

| Fatty acids | SFO | SFO + 5% MOO | SFO + 10% MOO | SFO + 5% SEO | SFO 10% SEO | MOO | SEO |
|---|---|---|---|---|---|---|---|
| $C_{14:0}$ Myristic acid | ND | ND | ND | ND | ND | ND | 0.08 ±0.03 |
| $C_{16:0}$ Palmitic acids | 4.35 ±0.27 | 4.42 ±0.39 | 4.68 ±0.81 | 5.25 ±0.3 | 6.80 ±0.62 | 4.90 ±0.31 | 8.90 ±0.31 |
| $C_{16:1}$ Palmitoleic acid | 3.1 ±0.64 | 3.01 ±0.31 | 3.08 ±0.73 | 2.91 ±0.21 | 2.78 ±0.83 | 2.90 ±0.23 | 0.26 ±0.61 |
| $C_{18:0}$ Stearic acid | 3.18 ±0.09 | 3.61 ±0.21 | 4.34 ±0.18 | 3.91 ±0.91 | 4.82 ±0.65 | 4.50 ±0.38 | 5.21 ±0.69 |
| $C_{18:1}$ Oleic acid | 23.41 ±0.32 | 29.83 ±0.42 | 37.32 ±0.5 | 25.31 ±0.72 | 28.90 ±0.81 | 74.60 ±0.43 | 35.86 ±0.15 |
| $C_{18:2}$ Linoleic acid | 60.30 ±0.85 | 55.81 ±0.02 | 51.34 ±0.43 | 55.8 ±0.17 | 52.90 ±0.19 | 5.01 ±0.19 | 41.20 ±0.27 |
| $C_{18:3}$ Linolenic acid | 0.13 ±0.11 | 0.18 ±0.04 | 0.27 ±0.26 | 0.96 ±0.43 | 1.64 ±0.21 | 1.20 ±0.24 | 4.9 ±0.07 |
| $C_{20:0}$ Arachidic acid | 1.90 ±0.59 | 2.15 ±0.21 | 2.31 ±0.04 | 1.40 ±0.12 | 0.94 ±0.19 | 2.70 ±0.06 | 0.51 ±0.54 |
| $C_{22:0}$ Behenic acid | 0.71 ±0.76 | 0.73 ±0.21 | 0.77 ±0.1 | 0.68 ±0.21 | 0.63 ±0.63 | 1.30 ±0.23 | 0.43 ±0.03 |
| SFA | 10.14 | 10.91 | 12.10 | 11.24 | 13.19 | 13.4 | 15.13 |
| MUFA | 26.51 | 32.84 | 40.40 | 28.22 | 31.68 | 77.50 | 36.12 |
| PUFA | 60.43 | 55.18 | 51.61 | 56.76 | 54.54 | 6.21 | 46.10 |
| PUFA/SFA | 5.96 | 5.06 | 4.27 | 5.05 | 4.13 | 0.46 | 3.05 |
| Cox value | 6.47 | 6.09 | 5.72 | 6.20 | 6.10 | 1.52 | 5.66 |
| ODR | 0.72 | 0.65 | 0.60 | 0.69 | 0.65 | 0.08 | 0.46 |
| LDR | 0.002 | 0.003 | 0.005 | 0.02 | 0.03 | 0.20 | 0.11 |

Note: Results are given as the average of triplicate determinations ± standard deviation. SFO = Sunflower oil, MOO = Moringa olefera oil and SEO = Sesame oil, SFA = Saturated fatty acids. MUFA = Monounsaturated fatty acids, UFA = Polyunsaturated fatty acids, USFA = Unsaturated fatty acids, ODR = oleic desaturation ratio, LDR linoleic desaturation ratio. N.D. = not detected.

## Composition of fatty acids of the sunflower oil and moringa or sesame oil blends

The unrefined vegetable oils are typically more oxidatively steady than the comparing refined and prepared oils. Mixing of vegetable oils and fats has risen as a conservative way of altering the physicochemical properties of vegetable oils, other than improvement in oil stability **(Ramadan and Wahdan, 2012)**. Concerning their oxidative stability and the presence of minor contents of the fatty acid consents, such as carotenoids, tocopherols, metal particles, polar lipids, and the original sum of hydroperoxides. The composition of SFO fatty acids and oil mixes is presented in (Table 4). Mixing sunflower oil with MOO and SEO modified the major fatty acids in the mixtures. Whereas significant improvements were noted in the contents of oleic acid (C18:1) and linoleic acid (C18:2) of mixed oils. The most elevated rate of linoleic acid 60.3 % (C18:2) was noted in sunflower oil comparing to MOO and SEO. The key fatty acids in the mixtures of sunflower oil and Moringa oil (SFO: MOO, 90:10), oleic and linoleic acids with 37.32% and 51.34% respectively. Blending SFO achieved a substantial rise from 26.51% to 32.84% and 40.40% in the MUFA substances of the SFO:MOO mixtures 95:5 and 90:10, respectively. On the other hand, the PUFA substances were diminished from 60.43 to 55.18 and 51.61% for SFO:MOO blends 95:5 and 90:10, respectively. Qualifying in the saturated fatty acids (SFA) is more remarkable in the sunflower oil when blended with sesame oil than Moringa oil. The relation between the content polyunsaturated and saturated FA is stated as the P/S index. This assessment is a critical factor in determining the nutritional value of definite oil. Oils and fats with a greater number of P/S index than one are regarded to have a nutritious rate **(Zambiazi et al., 2007)**. Sunflower oil had the most noteworthy polyunsaturated fatty acids rate, whereas Moringa oil had the greatest rate of monounsaturated and most reduced polyunsaturated. Which makes it attractive in terms of quality steadiness cooking and frying. The increased consumption of healthy fats (monounsaturated/oleic) is also associated with a diminished chance of the incidence of coronary heart diseases **(Ahamd et al., 2015)**. Additionally, high-oleic oils contain low levels of saturated fatty acids. High-oleic oils can therefore be seen as alternative health to somewhat hydrogenated vegetable oils **(Abdulkarim et al., 2005)**. Within the same regard **Allam (2001)**, examined the oxidative stability of (SFO), mixed with nine oils, and characterized by their corrosive oleic acid substance. **Ogunsina et al. (2014)** cited that combining *Moringa oleifera* oil with sunflower oil and soya bean oil in various amounts may lead to an increment in oleic acid and a decreased in linoleic acid. Other authors appear a wide spectrum of important fatty acids in the normal sunflower oil **(Vidrih, Vidakovič and Abramovič, 2010)**. The oleic desaturation ratio (ODR) and linoleic desaturation ratio (LDR) of values showing, the productivity of the

desaturation frameworks from C18:1 to C18:2 and from C18:2 to 18:3, respectively, are appeared in Table 4. The major sum of ODR in the blends oil (0.69) was found in sunflower with sesame oil 5% to be moderately high relative to that of LDR (0.2). Such quantities justify clarify the watched high substance of C18:2 and low content of C18:3 observed in the present work. Moderately higher normal means of ODR and LDR clarify the increment of C18:2 substances. The high ODR values suggest that the biosynthesis pathway was effective within the forming of PUFA (C18:2 and C18:3) from the desaturation of MUFA (C18:1). Nonetheless, the low LDR sums indicate that this process was not so effective in the forming of C18:3 from the desaturation of C18:2. Consequently, C18:3 substance was decreased and C18:2 expanded to reach a concentration greater than C18:1.t of C18:1. Besides, oleic and linoleic acids are the main constituents of samples. The combination of vegetable oils and fats has developed as a temperate method of altering the physicochemical properties of vegetable oils, in addition to improving oxidative stability **(Anwar et al., 2007)**. Without a doubt, the probability of utilizing the *M. oleifera* oils assisting or combining with other oils could be a rule of *M. oleifera* devastate valorization **(Barakat and Ghazal, 2016)**.

## CONCLUSION

The obtained results from this investigation revealed that the highest acid value was observed at SFO + 10% SEO. Sunflower oil alleviated when blended with MOO and the levels of conjugated dienes (CD) and trienes (CT) were depressed relative to control. The TPC value was greater when the sunflower blended with moringa oil. The highest percentage of scavenging activity was found in SFO + MOO 5% and SFO + SEO 10% respectively. Meanwhile, the longest induction period at 100 °C was 11.45 hours in the treatment of SFO + MOO 5%. When sunflower and moringa oils are combined (SFO:MOO, 90:10), the master fatty acids are oleic and palmitic. The natural antioxidants of the mixed oils have been found to provide superior results concerning oxidative stability and quality parameters. The mixing oils do not only stabilize the eatable oils but, furthermore provides the progress and upgrades the dietary and useful qualities of the oils by integrating the positive characteristics of the two oils into one, hence thus enhancing their economic possibility. The current study illustrated that *M. olifera* oil found in Egypt contains an excellent potential for eatable and commercial application. Overall, mixing vegetable oils allows the food industry more freedom to have useful properties or required dietary requirements.

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# THE NUTRITIONAL CHARACTERISTICS AND ACCEPTABILITY OF BAOBAB (*ADANSONIA DIGITATA* L) PULP AS NUTRIENT CONCENTRATE SUBSTITUTE IN CUSTARD POWDER

*Kazeem Olatoye, Oluwatobi Olusanya, Abiola Olaniran*

## ABSTRACT
Micronutrient deficiency in the human diet is of serious concern in developing nations. The utilization potential of Baobab pulp (BP) as a micronutrient source in custard formulation was evaluated. Custard powder was formulated from corn-starch, salt, flavor, colorant, and a commercial nutrient concentrate (NC) using a standard recipe, as a control sample. The nutrient concentrate was substituted by 20 – 50% of Baobab pulp in custard formulation. Custard powder was characterized by chemical, physicochemical, pasting, and sensory properties, using standard procedures. Substitution of Baobab pulp for nutrient concentrate in custard formulation resulted in a significant ($p \leq 0.05$) increase in the crude protein (2.23 – 3.67%), fat (1.49 – 2.40%), fiber (2.95 – 4.85%), and ash (2.21 – 3.63%) contents of custard powder. The vitamins A, C, and Beta-carotene contents of Baobab pulp-fortified custard varied ($p \leq 0.05$) significantly. Substitution of Baobab pulp for nutrient concentrate increased the mineral composition (Calcium (1.16 – 5.75 ppm), Magnesium (1.09 – 1.83 ppm), Potassium (0.81 – 2.68 ppm), and Iron (0.12 – 0.39 ppm)) of custard samples significantly ($p \leq 0.05$). The addition of Baobab pulp in the custard powder formulation enhanced its chemical composition and sensory attributes. The enhancement level increases with an increase in the proportion of baobab pulp.

**Keywords:** Baobab pulp; nutrient concentrate; micronutrient deficiency; custard; fortification

## INTRODUCTION
Vitamins and minerals are essential micronutrients for human growth and well-being and should therefore be adequately provided in the human diet. Alleviation of micronutrient deficiencies has been receiving attention for decades through different interventions **(Alimi, Workneh and Oke, 2016; Olaniran et al., 2020a)**. Studies revealed that over 2 billion people are vitamin A, zinc, iron, and iodine deficient which are crucial vitamins and minerals, in the world **(Das et al., 2013)**. Growing children are particularly prone to this menace **(De-Regil et al., 2013; Olaniran et al., 2020b)**. These nutritional concerns can be efficiently managed courtesy of food fortification. This is a cost-effective strategy towards micronutrient delivery into a large population segment, with no alterations of food consumption patterns **(Bhagwat et al., 2014)**. In communities with a high economically poor population segment, the use of synthetic chemical ingredients for food fortification is challenging to sustain. Most developing nations are dependent on energy-given foods that are deficient in micronutrients **(Affonfere, 2018; Olaniran and Abiose, 2019)**. Custard powder is a high caloric, cornstarch-based diet commonly formulated with the inclusion of synthetic micronutrients. Conventionally materials such as flavors, salt, colorants, protein sources, concentrates are added, to enhance its sensory and nutritional qualities **(Okoye, Nkwocha and Agbo, 2008; Awoyale et al., 2015)**. However, both health and cost implications of commercial concentrate inclusion make it highly imperative to hunt-out for cheap and natural micronutrient-rich food materials as a substitute, particularly for low-income earners. Hence, a trial can be giving to baobab pulp, based on its reported micronutrient profile. Baobab pulp is being reported to contain trace elements that have the potentials to alleviate micronutrient deficiency if incorporated into the human diet **(Adejuyitan et al., 2012)**. It's a reservoir of riboflavin, ascorbic acid, pectin niacin, malic, citric, succinic acids, vitamins A, D, and E **(Donkor et al., 2014)**. Many food materials of African origin had been tested with huge successes, as food fortificant. For instance, baobab fruit pulp fortified yogurt **(Abdullahi et al., 2014)**. However, literature reports on the substitution of baobab fruit pulp for synthetic nutrient concentrate as a means of improving the micronutrient profile of starch-based custard is sparse. Outcomes of the study may result in the creation of novel custard varieties with higher safety propensity and also encourage the utilization of natural food material. Therefore, substitution

potentials of baobab pulp for commercial nutrient concentrate in custard powder formulation were evaluated.

### Scientific hypothesis

Baobab pulp can serve as a better and cheaper substitute for nutrient concentrate in custard.

The addition of Baobab pulp will increase micronutrients in custard **(Muthai et al., 2017)**.

## MATERIAL AND METHODOLOGY

### Sources of materials

The Baobab fruit was obtained from Kwara State University, Malete. Corn starch obtained from Shoprite, Ilorin; salt, flavor, color, and nutrient concentrate were purchased from the Mandate market, Ilorin.

### Extraction of Baobab Fruit Pulp

Baobab fruits were split into halves using a mortar and pestle and the pulp (Figure 1) was scraped out from the seeds with a plastic spoon. The pulp was pulverized, screened through a 2 mm sieve, and packed in polyethylene bags, sealed, and kept before use **(Dendegh, Ukeyima and Dendegh, 2019)**.

### Custard Formulation

The modified method of **Odimegwu et al. (2019)** was employed in the custard formulation. All ingredients were kept constant with exception of nutrient concentrate, which was replaced with 0%, 20%, 30%, 40%, 50% and 100% of Baobab pulp and samples coded as BB0, BB20, BB30, BB40, BB50 and BB100 respectively (Table 1). Samples were homogenized with a sterilized blender, (7-IN-1 ETKAL-868 blender TUV, Rheinland) cooled and packaged in polyethylene bags, sealed-up, and then kept at room temperature pending analysis (Figure 2).

### Determination of Proximate Composition of the formulated custard powder

The proximate composition, including crude protein, fat, moisture, fiber, ash, and carbohydrate (by difference) contents of the formulated custard samples were determined according to **Horwitz and Latimer (2010)**.

### Determination *o*f Micronutrient Content of the formulated custard powder

***Determination of β-Carotene content***

Petroleum ether (40 mL) and acetone (30 mL) were added to five grams of custard sample for extraction. Extracts were placed in separating funnels and distilled water was slowly added along the neck of the separating funnel without shaking, to avoid emulsion formation. The two phases were left to separate, and the lower aqueous layer was discarded. To remove residual acetone, each sample was washed 3 – 4 times with 200 mL distilled water. In the last phase, washing was done by ensuring that no amount of the upper phase was discarded. Then, the upper layer was collected into a 50 mL flask using anhydrous sodium sulfate (ACS reagent, ≥99%, Redi-Dri™ Sigma-Aldrich, Canada) filter arrangement to remove residual water as described by **Cortés-Herrera et al. (2019)**. The absorbance was read at 450 nm using a UV-visible spectrophotometer (Shimadzu-UV 1800). The concentration of beta carotene was calculated under the standard curve.

***Determination of Vitamin A content.***

The method described by **Okwu (2004)** was used to determine vitamin A. Sample (1 g) was weighed into a beaker and macerated with a 10 mL mixture of acetone and n-hexane (1:1) and filtered. Thereafter, 10 mL of 50% $(NH_4)_2SO_4$ solution was added, vigorously shaken, and allowed to settle. The upper layer was collected and the absorbance read in Spectrophotometer (Spectro21D, Pec Medicals, USA) at 450 nm against hexane as blank beta-carotene served as standard. Retinol equivalent was obtained by the conversion factor: 1 mg retinol equal to 12 mg of dietary beta-carotene.



**Figure 1** Dried Baobab pulp.



**Figure 2** Babobab flour.

***Determination of Vitamin C concentration***

Vitamin C concentration was determined according to the method described by **Vikram, Ramesh and Prapulla (2005)**. Precisely 1 g of the sample was mixed with meta-phosphoric acid and centrifuged at 10,000 rpm for 10 min. at 4 °C in a refrigerated centrifuge (Model H-2000C). The supernatant was sieved through Whatman No 4 filter paper. The filtrate was diluted with 1 mL of 0.8% meta-phosphoric acid and filtered with a 0.450 Millipore filter and 20 µL of the sample injected into the HPLC **(**Shimadzu 20 A-Series, with column -NH2 -LUNA-100A (250X4.6mm), Diameter – 5 uL and Refractive Index Detector.

***Determination of Mineral Content***

Three macros (Ca, Mg, K,) and one micro (Fe) mineral contents in each custard sample were determined by digesting 2.0 g of the sample with the aids of the Atomic Absorption Spectrophotometer (Buck 205 model; Buck Scientific Inc., USA). The outlined procedure of the Association of Official Analytical Chemists **(Horwitz and Latimer, 2010)**, approved method (968.08) was employed.

## Determination of Anti- nutrient contents

The method of **Olatoye and Arueya (2019)** was employed to determine the phytate content. Determination of oxalate was carried out according to **Day and Underwood (1986)** while the Folin-Denis colorimetric method described by **Kirk, Sawyer and Frait (1998)** was employed to determined tannin content.

## Determination of Physicochemical properties

The pH of the formulated custard samples was measured using a pH 211 microprocessor pH meter (Hanna, Woonsocket, RI, USA). Abbe refractometer was used to determine the total soluble solids content (Brix) while titratable acidity was determined according to **Olaniran et al. (2020c)**.

## Determination of Pasting Properties

Pasting properties of the custard powder were characterized using the Rapid Visco Analyzer (RVA Model 3c, Newport Scientific PTY ltd, Sydney) as described by **Olaniran, Abiose and Gbadamosi, (2019)**. The pasting parameters recorded were pasting time, pasting temperature, peak viscosity, through viscosity, breakdown viscosity, setback viscosity, and final viscosity.

## Sensory Analysis

Custard gruel prepared from custard powder samples were subjected to sensory evaluation using a scoring test. Fifty (50) semi-trained panelists, selected from the students of Kwara State University, Malate, Nigeria, who are all familiar with custard products were trained on the use of sensory evaluation procedures. Custard samples were evaluated and scored for appearance, flavor, taste, mouthfeel, and general acceptability using a 9-point hedonic scale, where "9" represented "like extremely" and "1" represented "dislike extremely" **(Olaniran, Abiose and Gbadamosi, 2019)**.

## Statistical analysis

All data obtained (in triplicate) were statistically analyzed using a one-way analysis of variance (ANOVA). Separation of means was done by Duncan's Multiple Range Test (DMRT), using the (SPSS) Statistical package for social science, IBM VERSION 21.0 package (Armonk, New York). Significance was accepted at the 0.05 probability level ($p \leq 0.05$).

# RESULTS AND DISCUSSION

The proximate composition of Baobab pulp-fortified custard powder is present in Table 2.

Substitution of Baobab pulp for nutrient concentrate in custard formulation resulted in a significant ($p \leq 0.05$) increase in the crude protein, fat, fiber, and ash contents of custard powder and ranged between (2.23 – 3.67%), (1.49 – 2.40%), (2.95 – 4.85%) and (2.21 – 3.63%) respectively, (Table 2).

The starch content of Baobab pulp-fortified custard powder is present in Figure 3.

Although moisture content also increases with an increase in the addition of Baobab pulp and ranges between (9.30 and 11.53%). This range was similar to 10% moisture earlier postulated for a good shelf life of powdery products **(Ensminger et al., 1995)**. The low moisture content of food materials usually enhances their stability and adaptability for subsequent use. Improved protein content might be as a result of the fairly high protein content of baobab pulp, as a report by **Osman (2004)**. Protein is known for its importance for the growth and repair or replacement of worn-out tissues in humans. Lack of or insufficient protein usually leads to kwashiorkor in children. Although, products with high-fat content can be susceptible to rancidity, with probable off-flavor and odor development. The low range of fat (1.49 – 2.40%) obtained in this product is desirable as a carrier of fat-soluble vitamins (A, D, E, and K) and a dietary source of essential fatty acids. Fiber is considered an efficient protective agent for a wide variety of illnesses, including cardiovascular disease, colon cancer, and constipation **(Marlett, McBurney and Slavin, 2002)**. The increase of fiber content in this product is therefore a plus. The baobab pulp was reportedly high in pectin and fiber contents **(Kamatou, Vermaak and Viljoen, 2011)** and could have contributed to the increase in fiber contents. Higher ash content in fortified custard samples than the control (BB0) also indicates the possibility of a better mineral profile. Baobab pulp was reported to be a rich source of the essential mineral element, need by the human body **(Adejuyitan et al., 2012)**. The carbohydrate content (73.88 – 81.83%) reduces with the increased addition of Baobab pulp and with the control sample being the highest. The high carbohydrate content of the custard samples showed that they are a good source of energy for the body.

**Table 1** Custard formulation with Baobab pulp as a replacement for commercial nutrient concentrate.

| Samples | NC/BP | Nutrient concentrate (g) | Baobab (g) | Corn starch(g) | Salt (g) | Flavor (g) | Colouring (g) |
|---|---|---|---|---|---|---|---|
| $BB_0$ | 100:0 | 10.0 | 0 | 35.0 | 1.0 | 1.5 | 2.5 |
| $BB_{20}$ | 80:20 | 8.0 | 2.0 | 35.0 | 1.0 | 1.5 | 2.5 |
| $BB_{30}$ | 70:30 | 7.0 | 3.0 | 35.0 | 1.0 | 1.5 | 2.5 |
| $BB_{40}$ | 60:40 | 6.0 | 4.0 | 35.0 | 1.0 | 1.5 | 2.5 |
| $BB_{50}$ | 50:50 | 5.0 | 5.0 | 35.0 | 1.0 | 1.5 | 2.5 |
| $BB_{100}$ | 0:100 | 0 | 10.0 | 35.0 | 1.0 | 1.5 | 2.5 |

Note: *(NC: Nutrient concentrate and BP: Baobab pulp) **$BB_0$**: 100% Nutrient concentrate and 0% Baobab pulp; **$BB_{20}$**: 80% Nutrient concentrate and 20% Baobab pulp; **$BB_{30}$**: 70% Nutrient concentrate and 30% Baobab pulp; $BB_{40}$: 60% Nutrient concentrate and 40% Baobab pulp; **$BB_{50}$**: 50% Nutrient concentrate and 50% Baobab pulp; **$BB_{100}$**: 0% Nutrient concentrate and100% Baobab pulp.

**Table 2** Proximate composition of Baobab pulp-fortified custard powder.

| Sample | NC/BP | Moisture (%) | Protein (%) | Fat (%) | Fibre (%) | Ash (%) | Carbohydrate (%) |
|---|---|---|---|---|---|---|---|
| $BB_0$ | 100:0 | 9.30 ±0.36 $^{e}$ | 2.23 ±0.51 $^{e}$ | 1.49 ±0.02 $^{e}$ | 2.95 ±0.05 $^{e}$ | 2.21 ±0.04 $^{e}$ | 81.83 ±0.50 $^{a}$ |
| $BB_{20}$ | 80:20 | 9.77 ±0.55 $^{d}$ | 3.31 ±0.04 $^{d}$ | 2.21 ±0.03 $^{d}$ | 4.38 ±0.06 $^{d}$ | 3.28 ±0.04 $^{d}$ | 77.06 ±0.39 $^{b}$ |
| $BB_{30}$ | 70:30 | 10.43 ±0.35 $^{c}$ | 3.49 ±0.10 $^{c}$ | 2.33 ±0.07 $^{c}$ | 4.61 ±0.13 $^{c}$ | 3.45 ±0.10 $^{c}$ | 75.69 ±0.36 $^{c}$ |
| $BB_{40}$ | 60:40 | 10.47 ±0.61 $^{c}$ | 3.58 ±0.04 $^{b}$ | 2.38 ±0.02 $^{b}$ | 4.73 ±0.05 $^{b}$ | 3.54 ±0.04 $^{b}$ | 75.30 ±0.70 $^{c}$ |
| $BB_{50}$ | 50:50 | 10.63 ±0.35 $^{b}$ | 3.61 ±0.03 $^{b}$ | 2.40 ±0.02 $^{b}$ | 4.77 ±0.04 $^{b}$ | 3.57 ±0.03 $^{b}$ | 75.01 ±0.26 $^{d}$ |
| $BB_{100}$ | 0:100 | 11.53 ±0.46 $^{a}$ | 3.67 ±0.06 $^{a}$ | 2.44 ±0.04 $^{a}$ | 4.85 ±0.08 $^{a}$ | 3.63 ±0.06 $^{a}$ | 73.88 ±0.64 $^{e}$ |

Note: Means ± standard deviation of triplicate samples with different superscripts along the columns differ significantly at $p \leq 0.05$. Key: NC (nutrient concentrate), (BP) baobab pulp.

**Table 3** Vitamin content of Baobab pulp fortified-custard powder.

| Sample | NC/BP | Vitamin C (mg.100g$^{-1}$) | Vitamin A (mg.100g$^{-1}$) | Beta-Carotene (mg.100g$^{-1}$) |
|---|---|---|---|---|
| $BB_0$ | 100:0 | 238.00 ±0.00 $^{a}$ | 0.34 ±0.00 $^{c}$ | 2.00 ±0.00 $^{e}$ |
| $BB_{20}$ | 80:20 | 238.25 ±0.66 $^{a}$ | 0.34 ±0.00 $^{c}$ | 2.06 ±0.00 $^{d}$ |
| $BB_{30}$ | 70:30 | 236.50 ±1.39 $^{b}$ | 0.34 ±0.00 $^{c}$ | 2.08 ±0.00 $^{c}$ |
| $BB_{40}$ | 60:40 | 237.00 ±0.25 $^{b}$ | 0.34 ±0.00 $^{c}$ | 2.10 ±0.00 $^{b}$ |
| $BB_{50}$ | 50:50 | 235.50 ±0.75 $^{c}$ | 0.53 ±0.23 $^{b}$ | 2.10 ±0.00 $^{b}$ |
| $BB_{100}$ | 0:100 | 156.50 ±9.55 $^{d}$ | 0.66 ±0.23 $^{a}$ | 3.49 ±1.21 $^{a}$ |

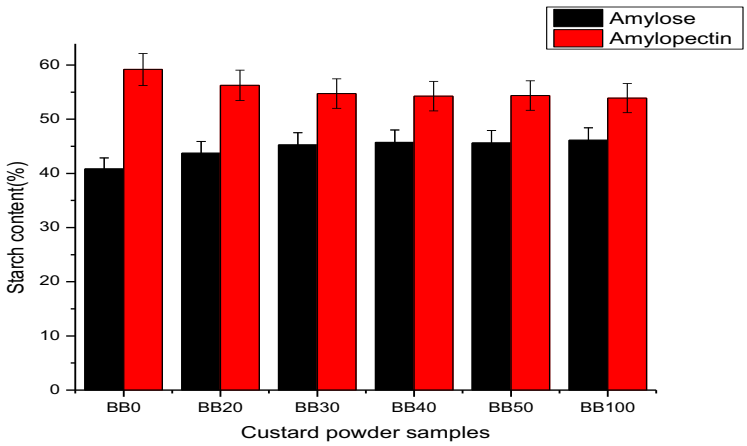


**Figure 3** Starch content of Baobab pulp-fortified custard powder.

The vitamins A, C, and Beta-carotene content of Baobab pulp-fortified custard varied ($p \leq 0.05$) significantly (Table 3). The vitamin C content ranged from 156.50 to 238.25 mg.100g$^{-1}$ with the highest value recorded for the control sample (BB0) and lowest value for sample BB100. These values were higher than the ranged (34 – 200 mg.100g$^{-1}$) reported by **Sidibe and Williams (2002)**, but lower than 300mg.100g$^{-1}$ reported by **Gebauer, El-Siddig and Ebert (2002)** in similar studies. The variations can be associated with differences in growth conditions, genetic factors and geographical locations of the baobab tree used, together with the analytical procedure employed **(Olatoye and Arueya, 2019)**. The recommended daily intake (RDI) of ascorbic acid is about 30 mg per day for adults and 17 mg per day for children **(Othman, Kihel and Amara, 2019)**. Consequently, this needed concentration of ascorbic acid can be easily obtained from the consumption of this custard powder. Ascorbic acid is well known as a good antioxidant against free radical damage **(Chambial et al., 2013)**. The vitamin A and beta carotene contents significantly increased in custard powder with an increase in the addition of Baobab pulp. Perhaps as a result of high contents of both vitamins A and its precursor (beta carotene) in the baobab pulp used. Baobab pulp was earlier reported as a reservoir of riboflavin, ascorbic acid, pectin niacin, malic, citric, succinic acids, vitamins A, D, and E **(Donkor et al., 2014)**. The beta carotene content ranged between 2.00 and 3.49 mg.100g$^{-1}$, with Sample BB100 being the highest. Carotenoids are photosynthesized pigments that cannot be produced by the human body. These pigments, which can be transformed into vitamin A should be provided through dietary intake **(van den Berg, Dentener and Lelieveld, 2000)**. The Vitamin A contents ranged from 0.34 to 0.66 mg.100g$^{-1}$ with Sample BB100 being the highest. Consumption of Baobab fruit pulp-fortified custard powder, therefore, constitutes a rich source of carotene and vitamins A.

The mineral composition of custard samples significantly ($p \leq 0.05$) increases with an increase in substitution levels of Baobab pulp for nutrient concentrate (Figure 4). Ca, Mg, P and Fe ranged between (1.16 – 5.75 ppm), (1.09 – 1.83 ppm), (0.81 – 2.68 ppm) and (0.12 – 0.39 ppm) respectively. Baobab pulp, being a rich source of mineral might have responsible for this trend. Magnesium and calcium are essential in human and animal health, especially for children, pregnant and lactating mothers, and the elderly. Inadequate dietary supply and a high risk of calcium deficiency in sub-Sahara African have been previously reported, based on food balance sheets (FBSs) **(Broadley et al., 2012; Joy et al., 2014)**. Adequate consumption of Potassium was closely linked to lower blood pressure in individuals with raised blood pressure **(He and MacGregor, 2008)**. Potassium also plays a prominent role in the synthesis of amino acids and proteins. Iron is an essential constituent of hemoglobin found in blood and contributes to the combat of anemia **(Blanco-Rojo and Vaquero, 2019)**. Therefore, the results of this study suggest that custard powder enriched with baobab fruit pulp may be a good source of macro and micro minerals need for body functions. The findings can also support the recommendation of fruit pulp as a possible strategy to tackle the problem of macro and microelements deficiencies through its inclusion in food consumption.

Anti-nutrient (phytate, tannin and oxalate) contents of the custard powder ranged from (0.01 to 0.02 mg.g$^{-1}$), (0.06 to 2.12 mg.g$^{-1}$) and (0.35 to 36 mg.g$^{-1}$) respectively and increases, with higher substitution level of Baobab pulp for nutrient concentrate (Figure 5). Phytate and oxalate could adversely affect mineral bioavailability **(Bhandari, Kasai and Kawabata, 2003)**. Tannins, also inhibit the absorption of minerals like iron and zinc but their ranges in the product were within the level that the human body can tolerate as safe **(Ojinnaka, Okudu and Uzosike, 2017; Olatoye and Arueya, 2019)**. Health benefits of tannin had been reported **(Ugwu and Oranye, 2006)**. These include reductions in the pathogenesis of cancer development and damage to the intestinal tract **(Makkar and Becker, 1996; Ugwu and Oranye, 2006)**. Therefore, the presence of low concentrations of tannin in the formulated custard powder is an added value. Titratable Acidity (TTA) and pH values of custard powder samples ranged between (0.15 – 0.96 mg.100g$^{-1}$) and (2.84 – 3.43) respectively as shown in Table 4. Both are significantly varied among samples. pH decreases with increase addition of Baobab pulp, the reverse was the case with TTA. Sample BB0 (without Baobab pulp) had the highest pH (3.34) and lowest TTA (0.15 mg lactic acid per 100g) values, while sample BB100 (with 100% Baobab pulp) had the lowest pH (2.84) and highest (0.96 mg lactic acid per 100g) TTA values. This implies that the product becomes more acidic with the inclusion of Baobab pulp, probably due to the presence of organic acids citric, tartaric, malic, succinic, and ascorbic **(Besco et al., 2007; Donkor et al., 2014)**. This acidity can bring about self-sanitation of this product during storage. More so, high Titratable Acidity (TTA) and very low pH recorded is a contributing factor to the fact that; the taste of the Baobab fruit pulp is tart and acidic and can improve the flavor of the custard. However, the Brix value, (probably a reflection of soluble sugar, particularly sucrose) was low in all samples. This may be desirable for a product of this kind, where consumers are used to the addition of excess sucrose before consumption. High sucrose consumption on consumer's health, has been closely linked with certain implications, such as incidences of obesity, diabetes, and dental decay **(Ajieroh, 2010)**.

Peak viscosity values ranged from 238.79 to 261.46 RVU with samples BB0 as the lowest and BB100 the highest (Table 5). The peak viscosity is defined as the maximum viscosity attained by gelatinized starch during heating in water **(Ojo, Ariahu and Chinma, 2017)**. The peak viscosity is closely associated with the degree of starch damage and its high value may indicate higher starch granule damage and starch binding capacity **(Ribotta et al., 2007)**. It is also an indication of the solubility of the blends for products with high gel strength and elasticity requirements. It can be inferred that the solubility and the degree of starch damage in this product could increase with the addition of Baobab pulp. The values of breakdown viscosity ranged from 71.33 to 116.25 RVU. Sample BB0 had the lowest and BB100 the highest values.

**Table 4** Physicochemical properties of Baobab pulp-fortified custard powder.

| SAMPLE | NC/BP | TTA (mg.100g$^{-1}$) | pH | Brix |
|---|---|---|---|---|
| $BB_0$ | 100:0 | 0.15 ±0.02 $^{e}$ | 3.43 ±0.02 $^{a}$ | 2.00 ±0.00 $^{a}$ |
| $BB_{20}$ | 80:20 | 0.36 ±0.00 $^{d}$ | 3.23 ±0.02 $^{b}$ | 2.00 ±0.00 $^{a}$ |
| $BB_{30}$ | 70:30 | 0.42 ±0.00 $^{c}$ | 3.14 ±0.02 $^{c}$ | 2.00 ±0.00 $^{a}$ |
| $BB_{40}$ | 60:40 | 0.49 ±0.02 $^{c}$ | 3.09 ±0.02 $^{d}$ | 2.00 ±0.00 $^{a}$ |
| $BB_{50}$ | 50:50 | 0.60 ±0.03 $^{b}$ | 3.02 ±0.04 $^{e}$ | 2.00 ±0.00 $^{a}$ |
| $BB_{100}$ | 0:100 | 0.96 ±0.02 $^{a}$ | 2.84 ±0.02 $^{f}$ | 2.00 ±0.00 $^{a}$ |

Note: Means ± standard deviation of triplicate samples with different superscripts along the columns differ significantly at $p \leq 0.05$. Key: (NC) nutrient concentrate, (BP) baobab pulp.

**Table 5** Pasting properties of Baobab pulp-fortified custard powderSAMPLE.

| SAMPLE | NC/BP | Peak (RVU) | Trough (RVU) | Breakdown (RVU) | Final viscosity (RVU) | Setback (RVU) | Peak Time (sec) | Pasting Temperature(°C) |
|---|---|---|---|---|---|---|---|---|
| BB0 | 100:0 | 238.79 ±36.06 $^{d}$ | 122.54 ±48.79$^{f}$ | 71.33 ±41.01$^{f}$ | 163.50 ±15.56 $^{f}$ | 40.96 ±33.23 $^{e}$ | 4.97 ±0.05 $^{d}$ | 79.45 ±0.57 $^{c}$ |
| BB20 | 80:20 | 149.83 ±22.63 $^{e}$ | 149.83 ±22.63 $^{e}$ | 78.88 ±119.50 $^{e}$ | 195.29 ±6.36 $^{e}$ | 45.46 ±16.26 $^{d}$ | 5.07 ±0.00 $^{c}$ | 80.33 ±0.60 $^{a}$ |
| BB30 | 70:30 | 161.21 ±6.36 $^{d}$ | 161.21 ±6.36 $^{d}$ | 90.54 ±60.10 $^{c}$ | 207.33 ±19.80 $^{d}$ | 46.13 ±13.44 $^{c}$ | 5.17 ±0.05 $^{ab}$ | 80.28 ±0.60 $^{ab}$ |
| BB40 | 60:40 | 170.92 ±33.94 $^{b}$ | 170.92 ±33.94 $^{d}$ | 86.75 ±15.56 $^{d}$ | 222.29 ±37.48 $^{b}$ | 51.38 ±71.42 $^{a}$ | 5.10 ±0.14 $^{ab}$ | 79.88 ±0.04 $^{b}$ |
| BB50 | 50:50 | 166.25 ±56.57 $^{c}$ | 166.25 ±56.57 $^{c}$ | 96.46 ±36.06$^{b}$ | 213.00 ±155.56 $^{c}$ | 46.75 ±98.99 $^{b}$ | 5.03 ±0.05 $^{c}$ | 79.88 ±0.04 $^{b}$ |
| BB100 | 100:00 | 180.5 ±67.88 $^{a}$ | 180.50 ±67.88 $^{a}$ | 116.25 ±84.85$^{a}$ | 231.88 ±36.06 $^{a}$ | 51.38 ±31.82 $^{a}$ | 5.23 ±0.14 $^{a}$ | 80.38 ±0.60 $^{a}$ |

Note:Means ± standard deviation of double samples with different superscripts along the columns differ significantly at $p \leq 0.05$. Key: (NC) nutrient concentrate, (BP) baobab pulp fruit.

**Table 6** Sensory acceptability of Baobab pulp-fortified custard powder.

| SAMPLE | NC/BP | Overall Acceptance | Appearance | Taste | Mouthfeel | Flavor |
|---|---|---|---|---|---|---|
| $BB_0$ | 100:0 | 7.73 ±0.70 $^{a}$ | 7.73 ±0.70 $^{a}$ | 7.00 ±1.11 $^{b}$ | 6.47 ±0.97 $^{d}$ | 6.87 ±0.94 $^{e}$ |
| $BB_{20}$ | 80:20 | 6.67 ±0.61$^{b}$ | 6.90 ±0.76 $^{b}$ | 6.03 ±1.22 $^{d}$ | 6.20 ±1.10$^{e}$ | 6.87 ±0.78 $^{e}$ |
| $BB_{30}$ | 70:30 | 6.63 ±1.00 $^{b}$ | 6.67 ±0.84 $^{c}$ | 7.03 ±1.07 $^{b}$ | 6.83 ±0.99 $^{c}$ | 6.93 ±1.11 $^{d}$ |
| $BB_{40}$ | 60:40 | 6.53 ±0.51 $^{c}$ | 6.67 ±0.96 $^{c}$ | 7.10 ±1.24 $^{b}$ | 7.17 ±1.02 $^{b}$ | 7.11 ±0.88 $^{c}$ |
| $BB_{50}$ | 50:50 | 6.43 ±0.50 $^{d}$ | 6.33 ±0.80 $^{d}$ | 6.30 ±0.80 $^{c}$ | 6.50 ±1.11 $^{d}$ | 7.27 ±0.64 $^{b}$ |
| $BB_{100}$ | 0:100 | 6.20 ±0.93 $^{e}$ | 6.23 ±0.90 $^{e}$ | 7.73 ±0.91 $^{a}$ | 8.10 ±0.76 $^{a}$ | 7.37 ±0.72 $^{a}$ |

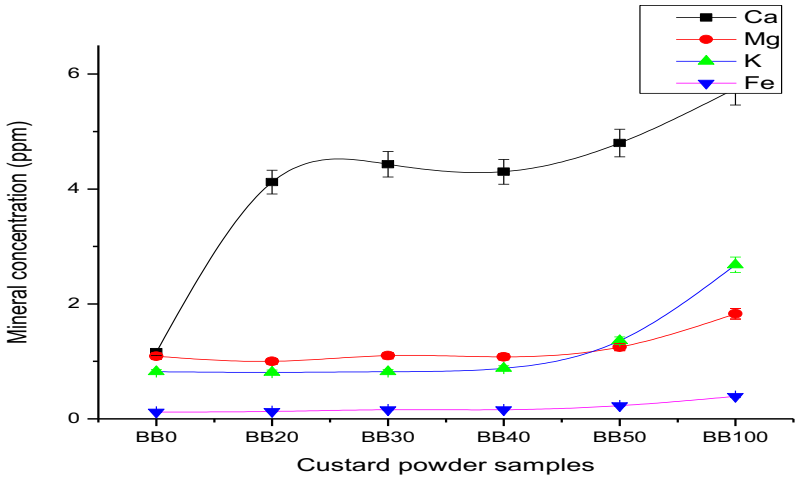


**Figure 4** Mineral content of Baobab pulp-fortified custard powder.

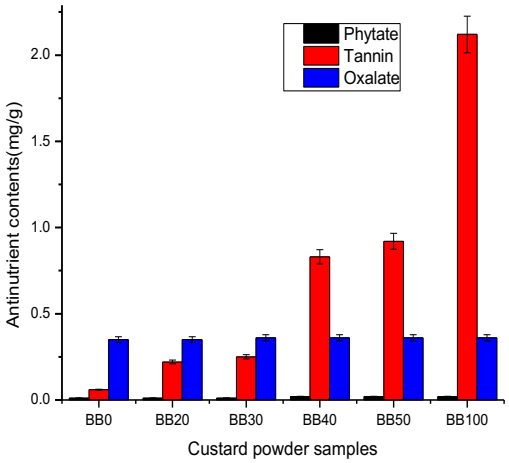


**Figure 5** Anti-nutritional properties of Baobab pulp-fortified custard powder.

The recorded high values supported the fact that high peak viscosities are associated with higher breakdown viscosity **(Shimelis, Meaza and Rakshit, 2006)**. This is in agreement with **Adebowale, Afolabi and Olu-Owolabi (2006)** who reported that the higher the breakdown viscosity, the lower the ability of the sample to withstand heating and shear stress during cooking. Hence, resistance to heating and shear stress becomes lower with the addition of Baobab pulp to the formulated custard. Final viscosity ranged from 163.5 RVU to 231.88 RVU with samples BB0 as the lowest and BB100 as the highest. Starch molecules (especially amylose), usually re-associate during cooling, leading to the formation of a gel structure, and thereafter, the viscosity will increase to a final viscosity **(Ragaee and Abdel-Aal, 2006)**. Setback viscosity ranged from 40.96 RVU to 51.38 RVU with sample BB0 as the lowest and sample BB100 as the highest. Higher setbacks viscosity results in lower retro-gradation during the cooling of products **(Ikegwu, Okechukwu and Ekumankana, 2010)**. Starch retro-gradation is usually characterized by a series of physical changes such as increased viscosity and turbidity of pastes, gel formation, exudation of water **(Hoover, 2001)**. Starch retro-gradation is the phase where the mixture's association between the starch's molecules (during cooling) occurs to a greater or lesser degree and a measure of the stability of a paste after cooking. Pasting time ranged from 4.97 to 5.23 s with sample BB0 as the lowest and sample BB100 as the highest. Pasting time is the measure of the cooking time **(Adebowale, Afolabi and Olu-Owolabi, 2006)**; pasting temperature ranged from 79.45 °C to 80.38 °C with sample BB0 as the lowest and the highest was sample BB100. The pasting temperature gives an idea of the minimum temperature required to cook a sample. It indicates the temperature required to cook the flour beyond its gelatinization points **(Alake et al., 2016)**.

Sensory evaluation is the expression of an individual likes or dislikes for a product as a result of biological variation in man. It is a subjective source of product information. Sensory properties respond to people to products in terms of appearance, aroma, taste, texture, and after taste, without the benefit of the label, pricing, or other imagery **(Olaniran, Abiose and Gbadamosi, 2019)**. There were significant differences ($p \leq 0.05$) in organoleptic qualities of custard powder fortified with Baobab pulp (Table 6). Overall acceptability values ranged between (6.20 and 7.73) while the appearance ranged from (7.73 to 6.23) and both reduces with the substitution of Baobab pulp for commercial nutrient concentrate. This could be associated with the creaminess (color) of Baobab pulp. However, no rejection of any sample was recorded as all values were greater than 5 on a 9-point hedonic scale.

This implied that all custard samples were generally acceptable for all evaluated parameters as none scored below the minimum acceptable rating of five on a 9-point hedonic. However, taste, mouthfeel, and flavor improved with an increase in the proportion of baobab pulp as adjudged by the panelists. Samples BB0 and BB100 had the lowest and highest value in each case. Therefore, the involvement of Baobab pulp in custard powder formulation enhanced its sensory attributes.

## CONCLUSION

Substitution of Baobab pulp for nutrient concentrate in custard formulation increased the proximate composition, carotene, and vitamin A contents of the formulated custard powder. Therefore, consumption of Baobab fruit pulp-fortified custard powder may have health benefits since the formulated custard is rich in macro and micronutrients. Further studies on the impact of baobab pulp substitution for synthetic concentrate on the bioavailability of trace elements and storage properties of custard powder are recommended.

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# CONSUMER PREFERENCES ON THE MARKET OF PLANT-BASED MILK ANALOGUES

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## ABSTRACT

The consumption of plant-based food products instead of animal-based products tends to increase worldwide which can be caused by the global expansion of alternative food products. The article's objective is to investigate the perception of plant-based milk analogues by real and potential consumers and determine the prospects of increasing their assortment. The assortment of plant-based analogues of milk and products made thereof is analyzed. A projection of the almond milk sales was made as one of the most effective product categories. Questioning of consumers conducted to investigate consumer treatment of plant-based milk analogues. The survey covered 436 respondents, of which 37.7% consume plant-based milk regularly and 47.7% of respondents consume this product occasionally. About 15% of respondents have never consumed it. The results show that experimenting with diets and health concerns more often young people (under 30 years of old). Plant-based milk was consumed by men (47.6%) and women (52.4%). Prices for plant-based milk analogues are too high, and its reduction will increase the sales of this product according to respondents. The taste is one of the crucial factors in choosing plant-based milk analogues by respondents. By taste-based preference, the largest shares are with almond milk (20.4%) and oat milk (15.3%). At the same time, 16.2% of the respondents have no preference regarding plant-based milk analogues. It was found that using the titles of traditional milk products for naming plant-based milk analogues is not troublesome for most of the respondents. The derived figures give evidence that consumers, not being sufficiently aware of the origin of plant-based milk analogues, do not understand the differences between traditional products and their plant-based analogues.

**Keywords:** consumer properties; plant-based milk analogues; fenugreek; consumer preferences; market

## INTRODUCTION

Balanced nutrition is an integral part of a healthy way of life. Currently, there is a worldwide trend that the consumption of plant-based food products substitutes the consumption of animal-based products. According to some nutritionists, the reason is that such food products can be better digested and do not contain harmful hormones and antibiotics that may be found in animal-based food products **(Bestfoodfacts, 2017)**. **Haas et al. (2019)** claim that plant milk consumers evaluated plant milk significantly better; they considered plant milk much better digestible and allergy-free.

Furthermore, the share of consumers with intolerance of lactose and milk allergy, which replace the products with lactose or milk proteins from the diet and replace them with plant proteins, has recently grown. From this point of view, the assortment of food products has been widening in the world by including food products oriented on special population groups, alternative food products in particular: plant-based analogues of milk and dairy products, meat, etc. Another reason for the rapidly growing popularity of plant-based alternatives is that their manufacturing is more environmentally friendly than the manufacturing of animal-based food products.

The usefulness of such products results from the absence of lactose, milk proteins, cholesterol, and lower caloricity compared to traditional milk. Since utility characteristics of plant-based analogues of milk also depend on the raw material from which they are made, they are used as the basis to produce food products for medical purposes **(Stannard, 2018; Vanga and Raghavan, 2018; Sethi, Tyagi and Anurag, 2016)**. For example, soymilk isoflavones contribute to the prevention of osteoporosis, atherosclerosis, cancer, and cardiovascular diseases, inhibit the aging process, etc. **(Omoni and Aluko, 2005)**. Phytosterols of rice milk exhibit anti-diabetic, anti-inflammatory, and antioxidant properties; contribute to lowering cholesterol and high blood pressure **(Biswas et al., 2011)**. Milk from almond contains arabinose and alpha-tocopherol, making it antioxidant and prebiotic **(Burton and Ingold, 1989; Mandalari et al., 2008)**.

The rapid growth of the number of people with diets including only plant-based food products is reported in EU countries as follow: 8 million in Germany, 800 thousand in Belgium, 3.3 million in France, 200 thousand in Greece,

800 thousand in the Netherlands, etc. **(The Vegan Society, 2018)**. The ever-growing consumption of mostly plant-based food products are observed in Eastern European countries. Thus, according to expert data, 2 million people in Ukraine abandon meat and stick to diet systems like vegetarianism, veganism, with raw food diets gaining in popularity **(Zik, 2017)**. However, consumers often complain that plant-based food product offer is limited in the market. That also refers to specific categories of population (people with certain illnesses, allergies, etc.), for which accessible food products are mostly produced abroad. There is a need to supply the market with food products made of raw materials of domestic origin. Therefore, research of the trends in the global market of plant-based analogues of milk products is important. The research focused on the market tendencies and outlooks in the field of plant-based alternatives is episodic and systematic analytical information is currently missing. An important aspect of research represents the analysis and systematization of data on the current assortment of this product category and its relevance to consumer needs and expectations.

Investigation of the consumer behavior focused on their motivation and perception of a given product has great significance in forming the assortment of plant-based milk analogues.

As plant-based analogues of milk and other plant-based food products are rather new product groups on the consumer market, analyses of their assortment and dynamics of their market have been out of research focus. Research devoted to the performance of the food industry in the overall or market tendencies in traditional milk products is shown in works of many scientists, such as **Burke et al. (2018)**; **Haghighat, Thimmarayappa and Niavand (2015)**; **Popescu (2015)**; **Tycha and Stetsenko (2017)**; **Karpii, Khtei and Shul (2013)**; **Havaza (2015)**; **Ilchuk and Radko (2013)**; **Fedulova (2018)**; **Vlasenko (2013)** and others. These research works are confined to market analysis and areas of assortment expansion for certain categories of milk and dairy products consumers, with plant-based analogues left out of focus.

Regarding the market of plant-based analogues of food products and particularly milk and dairy products analogues, researchers such as **Lagally, Clayton and Specht (2017)**; **Wild et al. (2014)**; **Doris (2018)** investigated issues of market dynamics and technological specifics of product manufacturing. There is a lack of summed up and systematized approaches to the analysis of the current performance of the market of plant-based analogues of milk products. Issues related to factors determining the assortment of this product group and considering consumer needs, potentials for expansion of the resource base for their manufacturing remain unsolved.

It should be noted that the term "plant-based milk" is not correct, as this drink takes origin from the extract of seeds, nuts, or cereals mixed with water in a certain proportion. The most widespread notions today are "plant-based substitute of milk", "alternative milk" (according to literature sources), and, accordingly, "alternative milk products", as well as "plant-based analogue of milk". In our opinion, the correct term for the name of the studied products is plant-based milk analogues. Using other terms can mislead consumers because their meaning implies the identity of the nutritional and biological value of traditional milk and its plant-based analogues **(Koshelnyk, Motuzka and Babiy, 2020)**.

### Scientific hypothesis

The article's objective is to investigate existing and potential consumers' preferences for plant-based milk analogues and to determine the prospects for increasing their assortment.

According to the objective, the scientific hypotheses were set:

Hypothesis 1: There is a difference between consumer perceptions of the nutritional value of plant-based milk and traditional milk.

Hypothesis 2: The demand for plant-based milk analogues may be increased throughout attracting potential consumers.

Hypothesis 3: The assortment of plant-based analogues of milk and dairy products should be increased by launching new products into the market.

Hypothesis 4: The majority of consumers consider prices of plant-based milk analogues to be too high.

## MATERIAL AND METHODOLOGY

This research was conducted by the use and processing of primary and secondary data. The information base of the research is official statistical reports of the State Statistics Service of Ukraine, data from international organizations (FAO, UN, etc.), scientific literature (data of domestic and foreign scientists), and Internet resources. This research is conducted using the methods of statistical and system analysis, generalization, comparison, etc. The primary data was obtained by the questioning of consumers, intended to determine key factors behind the consumption of plant-based milk analogues in Ukraine. The questioning was conducted in 2019 – 2020 online, using the Google Forms platform. The questioning covered 436 respondents, of which 37.7% consume plant-based milk on regular basis, 47.7% consume it occasionally, and a 14.9% never consume it. According to the age structure of those who consume plant-based milk analogues, the largest group consists of respondents aged 25 to 30 years (43.2%), then – aged 18 to 24 years (28.4%), the smallest group consists of respondents older than 50 years. The results show that young audiences respondents (under the age of 30) are more inclined to experiment with nutrition and they take care of their health (namely the consumption of non-traditional milk). In general, both men (47.6%) and women (52.4%) consume plant-based milk analogues approximately equally.

### Statistical analysis

A comparison of plant-based milk analogues between those who consume them and those who do not consume them was based on a statistical assessment of similarities between the shares of responses.

Large scopes of experimental data demonstrate that the test to check the significance of the paired correlation coefficients is applicable for assessing the similarities in the structure of variables. For the experiment presented in the article, the hypothesis $H_0$ implies that there are no significant variations between those who consume

plant-based milk and those who do not consume it, i. e. the structures of responses are similar. In this case, $p$ <0.05, which confirms the significance of the criterion. In the other case ($p$ >0.05), there are large variations between the shares of responses.

The criterion was computed by the software for statistical analysis of data IBM SPSS Statistics v.21 in the subsection "T-criterion" for the paired samples of the section "Comparison of averages". To make parametric criteria applicable, the data were checked for the normality of distribution by the Kolmogorov-Smirnov criterion in the section "Non-parametric criteria". This criterion enables us to confirm the normality of the data because all the computed $p$-values >0.05. This demonstrates that the values of the variables follow the normal distribution well enough so that the parametric tests can be applied.

The regression analysis, used for projecting the sales of almond milk, is realized in MS Excel 2016 software package using the application? "Data analysis", "Regression" module. The computation algorithm for the annual projected values of the indicator was the use of linear regression **(Yashchenko, 2016)**. The computation algorithm for projected values of sales in almond milk using linear regression includes the following four phases: 1) Setting of the parameters and computation of the trend, 2) Assessment of the trend quality, 3) Determining the trend coefficients, 4) Deriving the estimates and the projection using the trend coefficients and the values of the time parameter. A detailed description of the project phases is given below.

Phase 1. Annual projected values of the indicator are computed by the equation:

$$\hat{Y} = a_0 + a_1 \cdot t \ , \qquad (1)$$

where $\hat{Y}$ – annual estimates of the indicator;

$a_0$ and $a_1$ – equation coefficients;

$t$ – time parameter ($t$ = 1, …, $n$, $n$ – number of years).

The equation (1) is built based on linear regression, which involves the construction of a straight line with the values of indicators located on it maximally approximated to the actual ones.

Phase 2. The equation assessment is made by the coefficient of determination and the significance test criteria:

1. The coefficient of determination.

The share of dispersion that is explained by the linear relationship in the overall dispersion of the effective feature $Y$ is characterized by the coefficient of determination.

The nearer is the value of the coefficient of determination to 1, the more accurate is the choice of the equation. When its value is higher than 0.8, the equation will be considered accurate, and when it is lower than 0.5, the equation will require refinement either through selecting other factors or by increasing the period for the available data.

2. The equation significance test: the hypothesis on the existence of the linear relationship between independent and dependent variables is checked by Fisher's ratio test.

Two hypotheses are checked for this purpose: $H_o$, which does not imply a relationship between the values t and Y, and $H_1$, which implies a relationship between the variables. When the value of $p$ is higher than 0.05, then the hypothesis $H_o$ will be accepted, with the equation referred to as insignificant or inadequate. And when the value of $p$ is lower than 0.05, then the hypothesis $H_1$ will be accepted, with the equation referred to as significant or adequate, i. e. applicable for further computations.

3. The significance of equation coefficients is assessed by Student's t-test.

Two hypotheses are also checked for this purpose: $H_o$, which implies that the variable t does not have a heavy influence on the variable Y, and $H_1$, which implies that the influence is heavy. When the value of $p$ is higher than 0.05, then the hypothesis $H_o$ will be accepted, with the coefficient at the variable t referred to as insignificant. And when the value of $p$ is lower than 0.05, then the hypothesis $H_1$ will be accepted, with the coefficient at the variable t referred to as significant, meaning that it can be relied upon in making conclusions about the relationship between the variables.

In our case, the derived coefficient of determination and the test significance criteria confirm the applicability of the equation for further projections.

Phase 3. The equation coefficients are computed by the method of least squares. This method minimizes the sum of the squares of deviations of actual values from estimated ones:

$$\sum \left( Y_{ij} - \hat{Y}_{ij} \right)^2 \rightarrow \min$$

It allows us to derive $a_0$ and $a_1$ – the coefficients of the equation (1).

Phase 4. To compute the annual projected values of the indicator, we have to insert the derived coefficients and the time parameter value in equation (1):

($p$ = 0002, i. e. $p$ <0.05)

## RESULTS AND DISCUSSION

The global market of plant-based food products has been undergoing rapid development. The year 2016 was declared the Year of the Pulse by the United Nations Organization due to the rapidly growing demand for plant proteins and beans, lentils, peas, chick-peas, etc. in particular **(FAO, 2016)**. The research company Mintel refers to this global trend in nutrition as "power of plants". According to the outlooks of American analytical companies, the share of plant-based food products will exceed the animal-based ones by 20% in 25 years. The report of Nielsen Holdings PLC from September 2018 shows that compared with 2017 the market of plant-based food products grew by 20% and exceeded 3.3 billion USD **(Zegler, 2017)**.

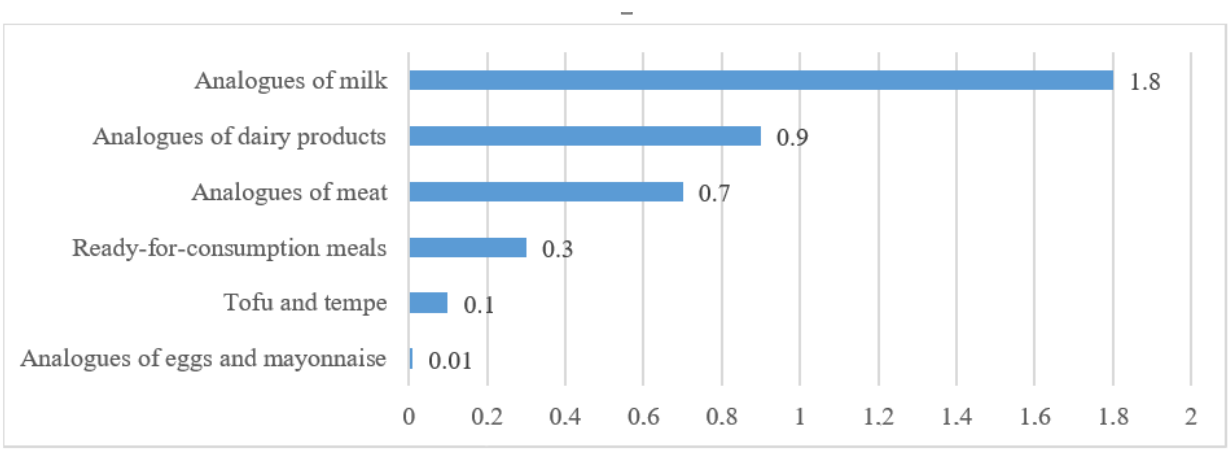


**Figure 1** The retail market of plant-based analogues of food products in billion USD.

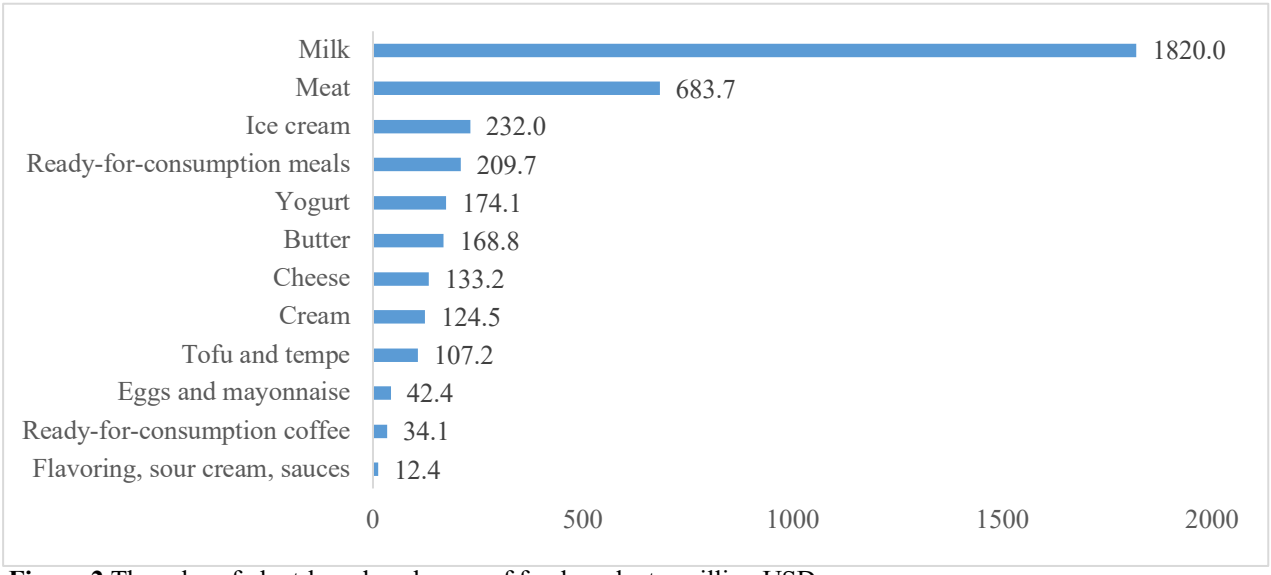


**Figure 2** The sales of plant-based analogues of food products, million USD.

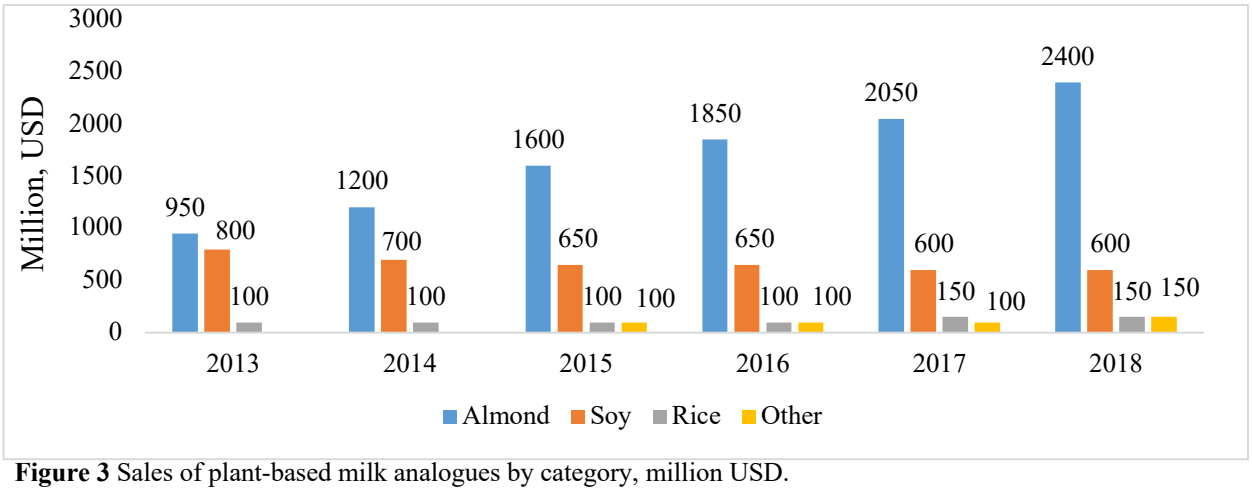


**Figure 3** Sales of plant-based milk analogues by category, million USD.

The assortment of plant-based food products is quite extensive and represented by confectionery and baked products, sauces, plant-based substitutes of meat and meat products. In November 2018, the total sales of food analogues reached 3700 million USD **(Crosser et al., 2019)**. **Kubicová and Habánová (2012)** claim that the consumption of milk (excluding cheese, cottage cheese, sour milk products, and butter) decreased in the past 17 years. For example, milk demand and its consumption in Slovakia decreased annually by up to 1.88 kg per capita that is largely caused by an increase in milk prices and an increase in the range of high-quality plant-based milk analogues and their pleasant organoleptic characteristics. **Krivošíková et al. (2019)** also claim that milk consumption in the Slovak Republic is insufficient. The retail market of plant-based analogues of food products is shown in Figure 1.

The largest market share belongs to plant-based analogues of milk. Their sales on the alternative food market are worth 1.8 billion USD. The second rank holds analogues of dairy products: yogurts, creams, ice creams, etc. (0.9 billion USD). Plant-based analogues of meat also have a considerable share (0.7 billion USD). Other food products are being launched to the market, and their share is relatively small (0.3 billion USD for ready-to-consumption meals; 0.1 billion USD – tofu and tempeh; 0.01 billion USD – analogues of eggs and mayonnaise) **(Future Market Insights, 2019)**.

The sales of plant-based analogues of food products are shown in Figure 2.

It shows that the largest sales reached plant-based analogues of milk (1820 million USD), followed by analogues of meat (683.7 million USD). Other popular milk analogues are ice cream, yogurts, butter, cheese, cream, etc. It should be noted that a special commodity category constitutes ready-to-consumption meals made of plant ingredients and imitating traditional meals cooked by the use of animal-based components by appearance and taste.

Companies leading the global market of plant-based analogues of milk are «The Hain Celestial Group», «Koninklijke Wessanen», «Organic Valley Cropp Cooperative», «SunOpta», «OATLY», «Califia Farms», «Eden Foods», «Danone», «Earth's Own Food Company», «Blue Diamond Growers» and others.

There are many kinds of vegetable raw materials, which are used to produce drinks. Since it is a relatively new product category, a classification of plant-based analogues of milk is missing in the literature. However, an attempt of the overall classification of these products is made in some scientific works, with outlining five categories of drinks made of raw materials as follow **(Sethi, Tyagi and Anurag, 2016; Vanga and Raghavan, 2018)**:

-cereals: oat, rice and/or corn milk, and milk made of spelt;
-soybeans: soy milk, milk made of peanut, lupine, Chinese black-eyed peas;
-nuts: almond, cocoa, and/or pistachio milk, milk made of hazelnut and walnut;
-seeds: sesame milk, milk made of sunflower, flax, cannabis, and other seeds;
-pseudo-cereals: milk made of quinoa, amaranth.

The most widespread plant analogues of milk are made of soybeans, almonds, rice, and cocoa; drinks made of quinoa, sesame, and spelt can also be found.

Manufacturers can expand the assortment of drinks by adding cocoa, vanilla fruit fillers, etc.

The largest consumption of plant-based drinks is recorded in the U.S. and Canada. While sales of alternative milk grew by 61% in 2012 – 2017, sales of cow milk fell by 15%. Company **Future Market Insights (2019)** expects that the market of plant-based substitutes of animal milk will grow up to reach 9.5 billion USD by the end of 2022, with an annual increase of 7.1%. As estimated by «Euromonitor», since the beginning of 2014 sales of alternative milk drinks grew by 31% in the U.S., by 24% in Europe, by 14% in the Asian-Pacific area, by 17% in Latin America. According to «Innova Market Insights», the global market of "plant-based milk" exceeded 16 billion USD in 2018 **(Innova Market Insights, 2017)**. According to data in Figure 3, the sales of soymilk are lower in 2018 compared to the previous years **(Proconsulting, 2019; Agroreview, 2018)**. As rice milk is popular mostly in Asia, its sales are considerably lower than the other categories of milk. The sales of other categories of milk are quite the same, but data from 2013 and 2014 are missing. This may be caused by the fact that many kinds of alternative drinks were launched on the market in recent years. The largest demand is reported for almond milk and we expect that its sales will grow.

Data in Figure 3 in the text demonstrate that the sales of almond drinks are the most effective of all the plant-based milk analogues. The regression equation (Figure 4) enables us to reveal that from 2013 to 2018 the average annual growth in sales of almond milk made 287.14 million USD. According to our projections, sales can reach 3254 million USD in 2021. Considering the popularity of a vegetarian diet and the intensifying movement for animal wellbeing, these figures are expected to be increasing. This is confirmed by cases of milk farms in the U.S., which exchange animal breeding for plant growing, and European farms that switch from the traditional production of milk to plant growing only. The most significant reasons encouraging people to stick to plant-based diets are as follows: care about animals, improvement of health, the negative impact of meat and dairy production on the environment; safety of these food products, price, distrust to the labeling of animal-based food products, etc. **(Stannard, 2018)**. The strongest motivating factor for people to consume plant-based food products is the ethical treatment of animals, striving to improve health, and belief that the manufacturing of meat and dairy products has negative environmental effects. Furthermore, there are several media reports on the high consumption of natural resources for the production of animal-based food products and its contribution to the greenhouse effect, entailing global warming.

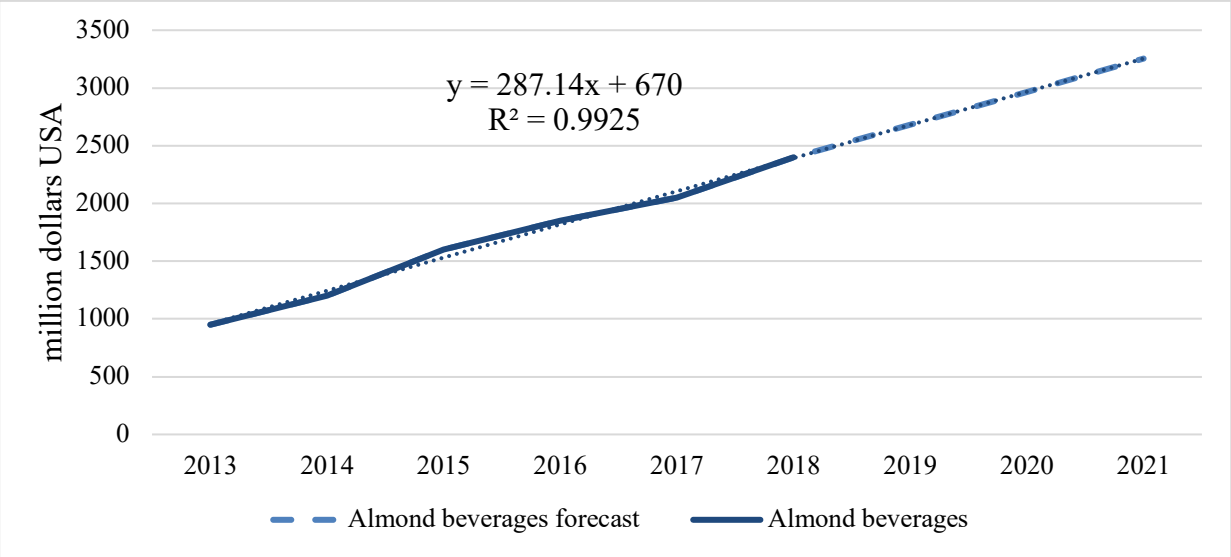


**Figure 4** The reported and projected sales dynamics for almond beverage.

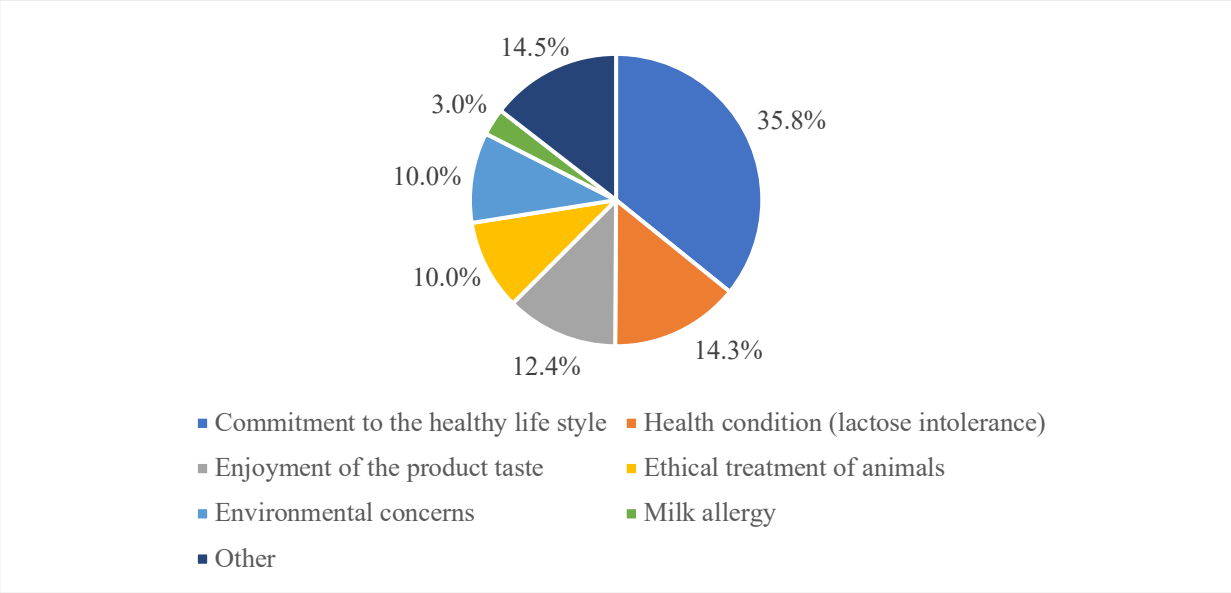


**Figure 5** The reasons driving the respondents to consume plant-based milk analogues.

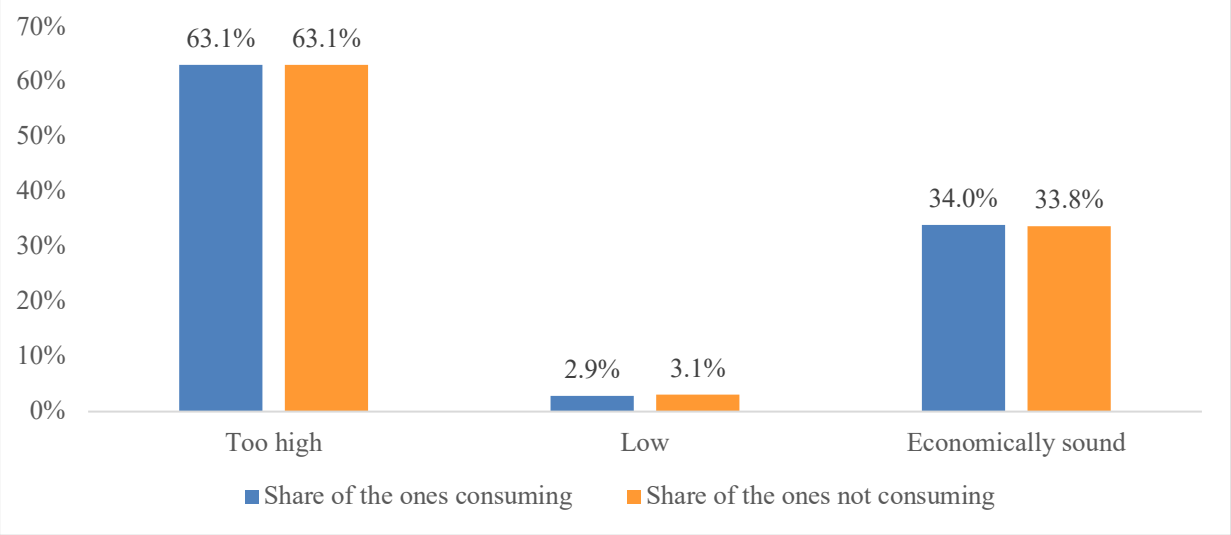


**Figure 6** The assessment of price for plant-based milk analogues.

Striving to improve one's health is associated with numerous data alleging that consumption of large amounts of red meat and products made thereof is fraught with cancer diseases. The price of food products matters for a part of consumers, but this data may be not relevant for the majority of countries because the price for alternative plant-based food products in Ukraine is much higher than the price for conventional animal-based food products. At the same time, the safety of foods and distrust of the labeling of animal-based food products are subjects of concern because such products may contain residues of hormones and antibiotics used for animal breeding which are not indicated on the labels or package. A minority of consumers choose a plant-based diet as an alternative to traditional food products. This is especially important for those who have problems with health (such as intolerance of lactose) but do not want to abandon all the conventional food products. Surprisingly, only 2% of consumers reported a plant-based diet to be a component of a healthy lifestyle. Of the essential reasons driving the respondents to consume plant-based milk analogues, commitment to a healthy lifestyle, health condition (the existence of lactose intolerance), and enjoyment of the product taste should be emphasized (Figure 5). The data in Figure 5 show that the principal reason for the plant-based milk analogues consumption is a commitment to a healthy lifestyle (35.8% of the respondents). 14.3% of the consumers suffer from specific metabolism diseases such as lactose intolerance. On the other hand, the allergy affects only 3% of the consumers, because the milk allergy quite rarely occurs in the adult age.

12.4% of the consumers give preference to plant-based milk analogues because they enjoy the product taste. We also found out that only 10% of the consumers prefer plant-based milk analogues in the daily diet because of environmental concerns and ethical treatment of animals. These results confirm the findings and data on the reasons for the consumption of plant-based food products published previously **(Stannard, 2018)**.

It should be noted that the majority of the reasons are largely explained by the impact of media sources on the marketing strategies of manufacturers. Food products made of plant-based raw materials are undoubtedly useful for health and should be included in a diet although they can harm the human body and the environment. Plant-based food products may contain residues of pesticides and chemical fertilizers; their manufacturing may affect the environment, especially in cases when food products are made from genetically modified soybeans. Therefore, a consumer should not abandon animal-based food products and should switch to a plant-based diet based on the doctor's recommendations. It is also important to take into account that many consumers consider the nutritional value of plant-based milk analogues to be the same as cow's milk, or even more nutritious **(Food Insight, 2018; National Dairy Council, 2018)**. The content of chemicals in milk and its plant-based analogues is significantly different in particular vitamins content and protein digestibility **(Mäkinen et al., 2016; Jeske, Zannini and Arendt, 2017; Carvalho et al., 2001; Haas et al., 2019)**. Based on the mentioned facts, we can identify special categories of consumers of alternative food products as follow: solvent consumer segment that wants to taste unusual foods, fans of a healthy diet and environmental activists – protectors of animals and fighters for the clean environment. In Ukraine young people (younger than 30 years old) experiment with diets and consume plant-based milk analogues more often than in the world where plant-based analogues of milk are mostly consumed by women aged 30 – 34 and 55 – 64, and men aged 55 – 64 and elder than 64 **(Crosser et al., 2019)**.

As we have already mentioned, plant-based analogues of food products are preferred by the well-off population with a rather high-income level. Results show that there are 12.9% of consumers of plant-based analogues of milk and 12.0% of consumers of plant-based analogues of meat with annual income 50 – 59,999 USD and 60 - 69,999 USD; 35.0% of consumers of plant-based analogues of milk and 37% of consumers of plant-based analogues of meat with the income of 100,000 USD or more **(Crosser et al., 2019)**. Regarding Ukraine, the majority of respondents consider prices for plant-based milk analogues as too high (Figure 6).

Data in Figure 6 confirm that prices for plant-based milk analogues are too high for 63.1% of consumers of these products, and for 63.1% of the consumers that do not consume plant-based milk analogues. It is statistically tested that there is no significant difference between the assessment of price for plant-based milk analogues by the consumers who consume plant-based milk and consumers who do not consume them ($p$ = 0.004, i.e. $p$ <0.05). A review of literature sources confirms that the majority of consumers around the world, which stick with the principles of a healthy diet, prefer plant-based substitutes of milk **(Schmidt, 2018; Mascaraque, 2020)**.

According to the questioning of consumers who live healthily and prefer natural and organic food products, the majority of them prefer plant-based analogues of milk. This results in consumers with a healthy lifestyle. For the majority of respondents, the consumption of plant-based milk analogues is associated with a healthy lifestyle (48.5% of the ones consuming this product, and 47.7% of the ones not consuming it) (Figure 7).

Regarding the taste preferences, the largest share of consumers (20.4%) prefer almond milk, and 15.3% of consumers oat milk. 16.2% of the respondents have no preference regarding plant-based milk (Figure 8).

Results in Figure 9 show, that the most favorite plant-based milk analogues trademarks are (Alpro) (25.0%) and (EcoMil) (14.3%). Only 3.1% of the respondents have no trademark preferences.

The consumer choice of plant-based milk analogues is influenced by many factors such as price, quality, taste characteristics, product composition, and producer (Figure 10) **(McCarthy et al., 2017)**. The most important factor of purchasing decisions is taste characteristics (reported by 38.1% of the ones consuming this product, and 38.5% of the ones not consuming it), followed by price (reported by 21.2% and 20.0%). It is demonstrated that no significant variations occur in the criteria of choice of plant-based milk analogues ($p$ = 0.000, i.e. $p$ <0.05).

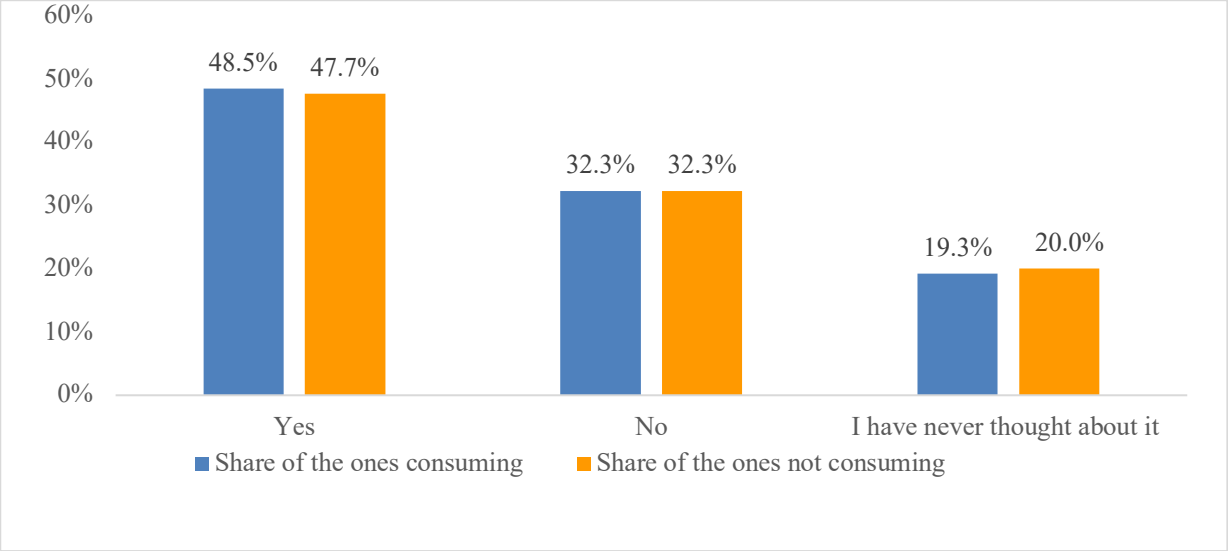


**Figure 7** An analysis of associating consumption of plant-based milk analogues with the healthy lifestyle.

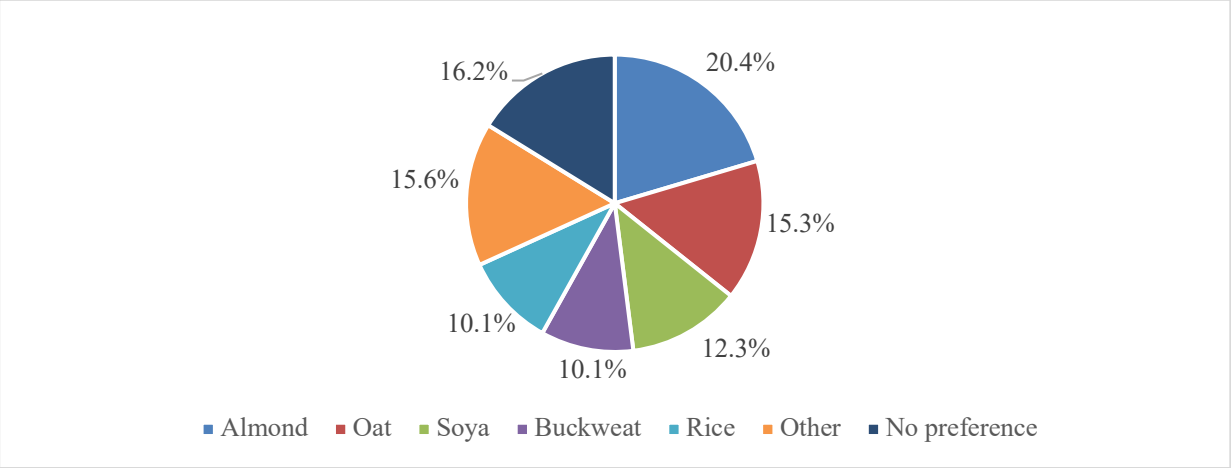


**Figure 8** Preferences of plant-based milk analogues.

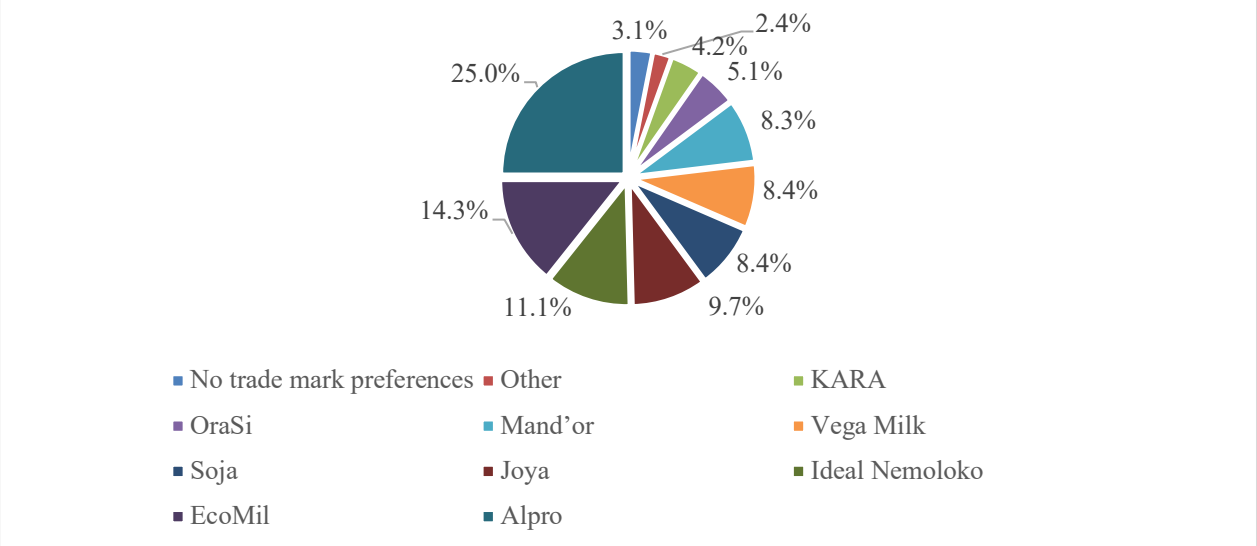


**Figure 9** Trademark preferences of plant-based milk analogues.

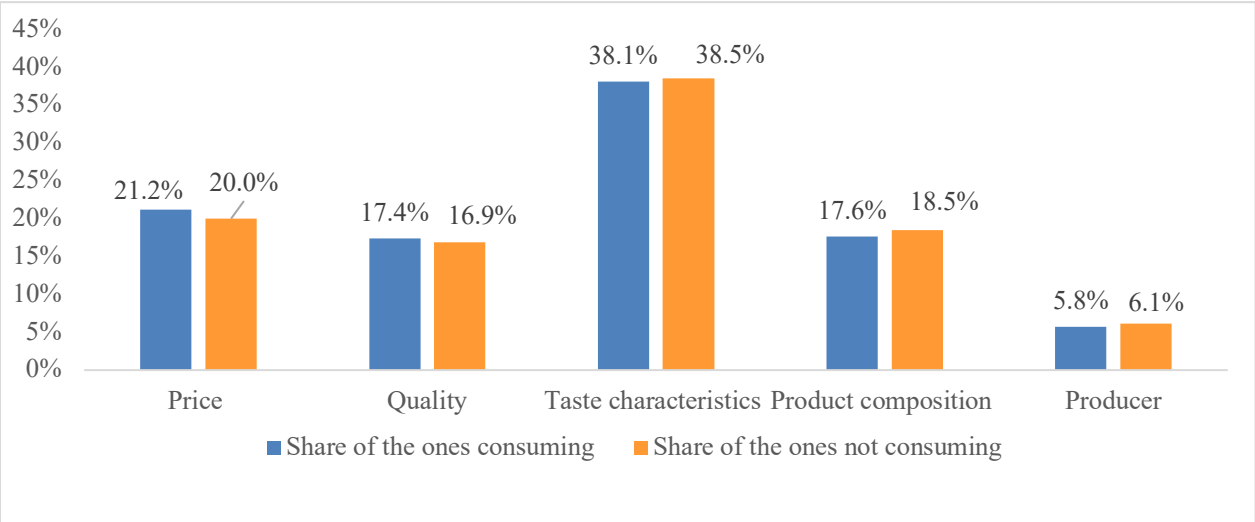


**Figure 10** Criteria of choice of plant-based milk analogues.

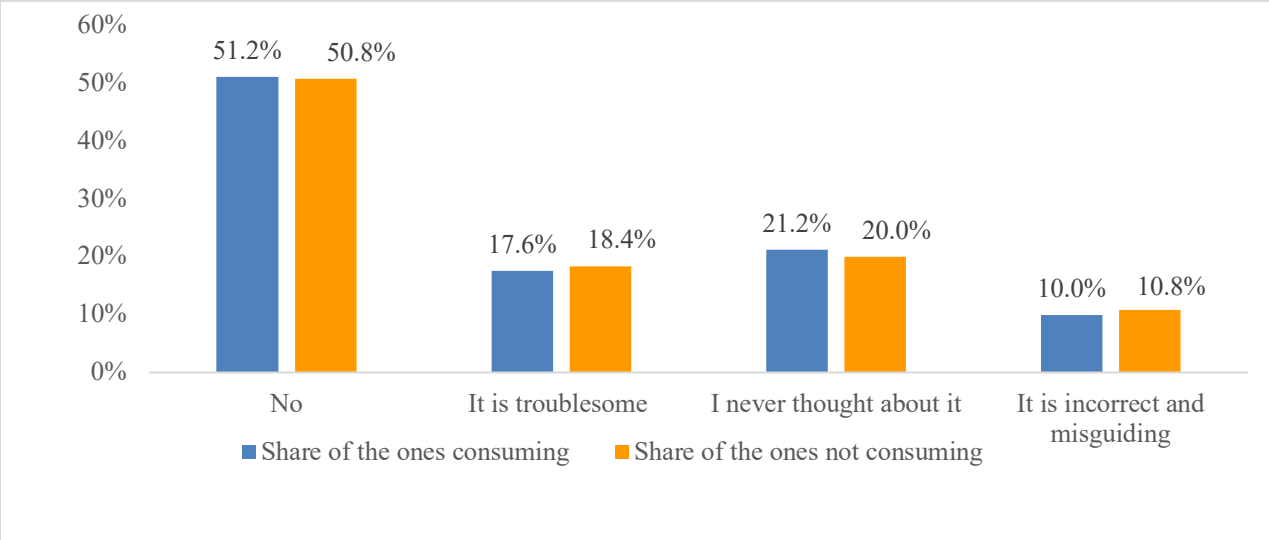


**Figure 11** Consumer attitudes to using the titles of traditional milk products for naming plant-based milk analogues.

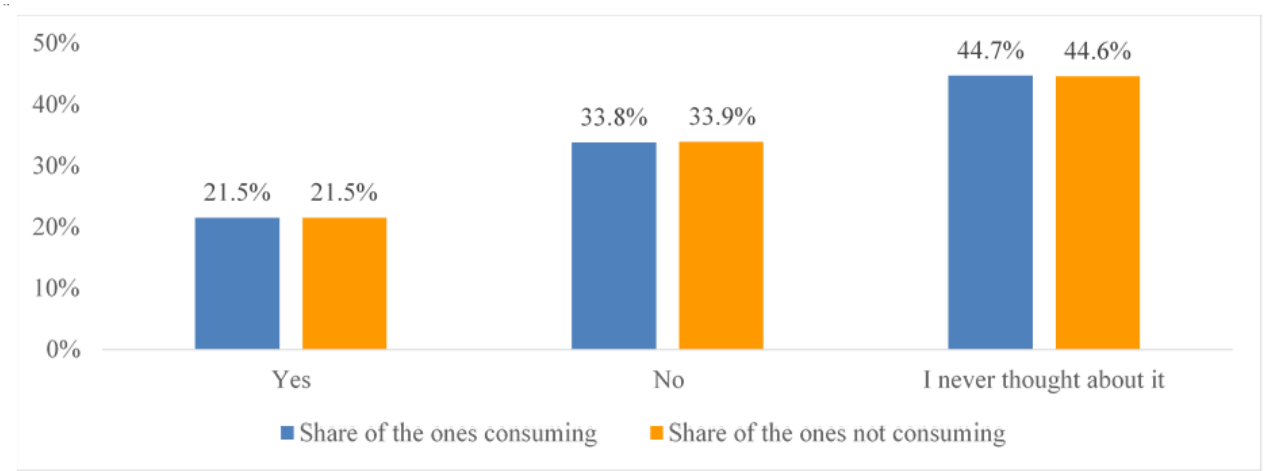


**Figure 12** Consumers´ awareness of the differences between traditional milk and its plant-based analogues in terms of understanding the differences between traditional products and their plant-based analogues.

We consider the term plant-based analogue of milk to be more correct than the others because an analogue means something with similarities or likenesses to another object or phenomenon. These food products tend to be called "milk" due to their similarity in appearance and contents with animal milk. Another important factor is that such extracts are a favorable environment for microflora, thus enabling their use for making yogurts, ice cream, cheese, etc.

Plant-based milk analogue is a drink made by milling a vegetable raw material and mixing it with water, further homogenization of a liquid resulting in the distribution of particles by size within the limit of 20 μm. The final product imitates cow milk by appearance and contents. However, the majority of consumers agree that the name of traditional milk products can be easily used to label their plant-based analogues (Figure 11).

The research results confirm that 51.2% of consumers accept that the name of traditional milk products (such as "milk") is used for plant-based milk analogues labeling. At the same time, only almost 10% of the respondents think that it would be incorrect and misleading. It is demonstrated that no significant variation occurs in the response to the question "Do you consider using the name of traditional milk products ("milk") for labeling of plant-based milk analogues to be a problem?" ($p$ = 0.001, i.e. $p$ <0.05).

The results also reveal that consumers, who are not sufficiently informed about the origin of plant-based milk analogues, do not understand the differences between traditional products and their plant-based analogues (Figure 12).

The majority 44.7% of respondents have never thought about whether plant-based milk analogues have similar nutritional or biological value as milk. However, one of the three respondents thinks that plant-based drinks are not similar to cow milk by nutritional or biological value. It is statistically tested that no significant variation occurs in the responses to this question given by the ones consuming and not consuming plant-based milk ($p$ = 0.005, i.e. $p$ <0.05).

Since almost one-half of our respondents have never thought about the identity of plant-based milk analogues and traditional milk by nutritional or biological value, it indicates their inadequate awareness. At the same time, it may harm public health because the chemical composition, nutritional and biological value of the traditional milk and its plant-based analogues are essentially different; in particular, by composition and biological accessibility of protein and calcium, which deficit may cause certain health problems.

## CONCLUSION

The market of plant-based milk analogues has been rapidly expanding. There is an upward tendency in the consumer demand for plant-based milk analogues, although this food segment has not yet been popular for consumers. This opens up opportunities for further development and new product launching at the market. The research results (covering 436 respondents) show that the sample, being diverse enough, could demonstrate consumer treatment of this product group, market supply, prices, and tendencies.

Using z-criterion, the following findings could be made and confirmed statistically:

1. Plant-based milk analogues have certain differences in the majority of real and potential consumers compared with traditional milk. They are for the most part associated with the healthy lifestyle, ethical treatment of animals, environmental concerns, and problems with one's health (which proves hypothesis 1).
2. Research results do not demonstrate a strong differences between the consumers that consume plant-based milk analogues and the ones that do not consume them (the significance of z-criterion is much less than 0.05). Therefore we can conclude that appropriately chosen promotional tools can affect potential consumers of plant-based milk analogues and thus increase the demand (which proves the hypothesis 2).
3. Respondents mostly make a choice of plant-based milk analogues based on the product taste. It implies that producers should expand the assortment line of their products by experimenting with various tastes to attract potential consumers. Especially since the main consumers of plant-based milk analogues are people younger than 30 years old, i.e. the consumers seek for innovations and experiments in the diet (which proves hypothesis 3).
4. Producers of plant-based milk analogues should carefully take the production costs and producer prices into consideration. As the research results show, price reduction increases the sales of this product (which proves hypothesis 4).

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# CHARACTERIZATION OF *LACTOBACILLUS* SPECIES PROPOSED AS PROBIOTICS

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## ABSTRACT

An isolated *Lactobacillus* from several various sources were identified depending on morphological, microscopical, and biochemical tests in vitro analysis of probiotic properties that included: an ability to tolerate in different concentrations of bile salt, survival in acidic conditions, their antimicrobial activity, and S-layer characterizations were carried out. It was noticed that isolates of *Lactobacillus rhamnosus* and *L. delbrueckii* have a broad activity of antimicrobial and found the isolate *L. rhamnosus* represented with a survival percentage of 6.9% at pH 4.5 and 5.1% at pH 2.0) also *L. rhamnosus* (5.7% at pH 4.5 and 4.9% at pH 2.0) tolerated acidic media, *Lactobacillus* spp. has antimicrobial activity against all gram-positive and negative tested isolates. 70 kDa of S-layer protein bands were detected with whole-cell SDS-PAGE analysis, and it's predominant in cells of isolates grown in MRS broth anaerobically. It was noticed that the collected *Lactobacillus* isolates could be used as pprobiotics

**Keywords:** *Lactobacillus* spp.; acidic tolerance; bile tolerance; S-Layer.

## INTRODUCTION

All types of bacteria in the gastrointestinal tract (GIT) are influenced by the most important conditions; physiologic, e.g. (acidity, enzymes, and bile salt), and the existence of normal inhabitant bacteria of GIT. Since gastric juice is composed of acids, salts, and enzymes, it provides a chemical environment in the stomach that devastates the microorganisms found naturally there **(Falk et al., 1998; Sghir et al., 2000)**. Lactic acid bacteria were found in a minor proportion with a hundred species of bacteria that were included in the complex microbial community of GIT. *Lactobacillus* species are non-pathogenic bacteria, positively react to Gram stain, and represent necessary microbiota in the lumen of the intestinal tract. The occurrences of *Lactobacillus* have been widely studied concerning their health-promoting effects and their role in maintain normally GIT habitat microbial balance and protects the intestine from infection. Moreover, the role of lactobacilli in reliefs of lactose intolerance improves food nutritional value, supports nonspecific immunity, and reduces serum cholesterol levels **(Hooper et al., 1999; Perdigon et al., 2000; Suvarna and Boby, 2005; Kim et al., 2008)**. The benefits of *Lactobacillus* bacteria that are classified as probiotic in the Generally Recognized as Safe list (GRAS) to the host body have been extensively discussed in the literature. Their probiotic's beneficial properties have been proposed including their ability to produce the antimicrobial substance, their antagonism activity, their role to improve non-specific immunity, and their influence on gut metabolic activities **(Marteau and Rambaud, 2002)**. The majority of *Lactobacillus* spp. have been characterized as probiotics ability which has been isolated from humans and animals. These isolated species are considered to be a starter culture for fermented foods, particularly milk or milk-derived products **(Caplice and Fitzgerald, 1999)**. Recently, different kinds of plants such as vegetables, fruits, medicinal plants, grains, and cereals have been produced and marketed in the form of lactic acid fermented plant beverages (FPBs) or foods for health-promoting in Thailand **(Duangjitcharoen et al., 2009)**. Currently, *Lactobacillus* strains are predominantly administered as probiotic remedies in pharmaceutical forms of capsules or powders as well sold out as enriched yogurt or milk in the dairy and food markets **(Sanders and Klaenhammer, 2001; De Roos, 2000)**. Bacterial strains of probiotic colonized GIT are dependent on their ability to adhere to intestinal epithelial cells through adherent factors. The process is mediated either non-specifically via physic-chemical factors such as hydrophobicity or specifically by adhesive bacterial surface molecules and epithelial receptor molecules such as S-layer, fibronectin, and mucin binding proteins, which is essential for adherent and colonization **(Altermann et al., 2004; Holzapfel et al., 1998)**. S-layers monomolecular crystalline arrays and recognized in many different species of Bacteria and Archaea as an outermost structure of the cell envelope **(Sára and Sleyrtr, 2000)**. A few pathogenic bacteria including *Clostridium difficile*, and *Bacillus anthracis* possess S-layer protein consisting of two types of S-layer subunits **(Takeoka et al., 1991; Mesnage et al., 1997)**. Whereas among the lactic bacteria spp., S-

layers have been found in most species of the genus *Lactobacillus* **(Masuda, 1992; Yasui, Yoda and Kamiya, 1995)**. The S-layer represents a typical surface structure in a lot of Lactobacilli spp. and has been demonstrated probiotic properties for human and animal consumers **(Åvall-Jääskeläinen and Palva, 2005; Bernardeau et al., 2000)**. Thus, S-layers will be targeted in the future for their great potential roles in applications of Nanobio technologies.

### Scientific hypothesis

The hypothesis of this work is to select qualified *Lactobacillus spp*. bacteria to propose as probiotic, and spotlight on particular properties such as S-layer and other properties that make *Lactobacillus* suitable candidates for probiotics.

## MATERIAL AND METHODOLOGY

The study was done in laboratories of the Food Contamination Research Center/Iraq Ministry of Science and Technology. Fifty food samples (Dairy milk and cheese) were collected from local markets in Baghdad. The process was sampling and it lasted from February 2019 to September 2019.

### Lactobacillus isolation

*Lactobacillus* isolation from food sources was performed following **(Perelmuter et al., 2008)**; one g of food sample was mixed in 10mL of sterile normal saline then, stirred vigorously and let the suspension settle for 10 min at room temperature. One hundred µL of the suspension was spread on previously prepared and sterile MRS medium (Oxoid LTD, limited, Hamshire, UK), all inoculated plates were incubated at 37 °C under aerobic conditions for 24 – 48 h. The growth colonies were purified and picked up for further analysis.

### *Lactobacillus* Biochemical characterization

*Lactobacillus* isolates were tested to be based on utilizing various carbohydrates according to **(Kandler and Weiss, 1986)**. The biochemical identification of the obtained isolates at the genus/species level was performed according to established phenotypic criteria, and for acid and gas production from glucose, arginine hydrolysis, and fermentation of L-arabinose, lactose, mannitol, melibiose, raffinose, ribose, sucrose, sorbitol, xylose, and salicin (Merk) as described by **(Samells et al., 1995)**.

### Bile salt and acidic tolerance

The minimal inhibitory concentration (MIC) of bile salt was evaluated for each of the *Lactobacillus* isolates, Then 20 µL of bacterial growth, 1 x $10^8$ (cfu.mL$^{-1}$), of each collected isolates were transferred to 980 µL of newly prepared sterile MRS broth containing 3% bile salts and incubated at 37 °C for 2 h.

For bacteria survival rate examination under the acidic condition at 37 °C, all isolates were tested following **(Perdigon et al., 2002)**; about 20 µL of 1 x $10^8$ (cfu.mL$^{-1}$) of each fresh isolates were moved to 980 µL of an acidic buffer consisting of 3.5 g of D-glucose, 2.05 g from NaCl and 0.6 g of $KH_2PO_4$, 0.11 g of $CaCl_2$ and 0.37 g of KCl per liter, with adjusted pH to 2.0 and 4.5. Then, isolates were examined for tolerance to bile salt and acid when samples were taken at 0 min and 2 h and plated on freshly prepared sterile MRS agar plates after making of serial dilution, and plates were incubated at 37 °C for 48 h, the plates were enumerated by choosing a couple of plates (30 – 300 colony) for each dilution.

### Antimicrobial Activity

*Lactobacillus* antimicrobial activity was determined through the agar spot method, following **(Casey et al., 2004; Baccigalupi et al., 2005)**, with minor modifications; briefly, about 5 µL of (1 x $10^8$ cfu.mL$^{-1}$) of freshly prepared growth to each obtained isolate was plated into agar spots made previously on the MRS agar plate. All inoculated plates were incubated at 37 °C for the development of spots. Each of the indicator bacteria (bacteria spp. used is mentioned below) was inoculated on Luria broth and incubated overnight at 37 °C with shaking. Then, the bacteria were mixed with 15 mL of Luria soft agar (0.7% agar, w/v) and dispensed onto MRS agar plates that were full of grown colonies of *Lactobacillus*. All plates were incubated overnight at 37 °C and the inhibition zones were subjected to measure and record **(Bauer et al., 1996)**. The indicators were *Shigella dysentery*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli*.

### Antibiotics Susceptibility of *Lactobacillus* isolates

Anti-Bacterial sensitivity of *lactobacilli* in vitro was studied via the disc diffusion method according to the guidelines of the National Committee for Clinical Laboratory Standards **(Wayne, 2015)**. It was studied against eight antibiotics: Ampicillin 25 µg, Chloramphenicol 50 µg, Gentamycin 10 µg Carbenicillin 100 µg, Nalidixic acid 30 µg, Methenamine Mandelate 3 µg, and Cortimoxazole 25 µg.

### Protein extraction and SDS-PAGE

For extraction of S-layer, *Lactobacillus* spp. was cultivated anaerobically in MRS broth at 37 °C. In general, 100 mL of pre-warmed MRS medium was inoculated 1: 100 (v/v) with an overnight culture and cultivated until the optical density at 600 nm reached 0.4 and 0.7. Cells were harvested by centrifugation at 12000 ×g for 15 min at 4 °C. The cells were washed twice with 100 mL of ice-cold water. The cell pellet was extracted with 0.1 volume of 4 M guanidine hydrochloride (pH 7) for one hour at 37 °C and centrifuged at 12000 ×g for 15 min. The supernatant, containing S-layer protein monomers, was dialyzed against water at 4 °C for 16 – 24 h **(Boot et al., 1993)**. The dialyzed extracts were analyzed by SDS-PAGE **(Smit et al., 2001)**. SDS-PAGE of protein samples was carried out using Precision Plus Protein Standard (low molecular weight marker (10 – 250 kDa) – Hi-media). The samples were run on 12% polyacrylamide gel at 100 V. Protein bands were visualized by Coomassie blue staining. Protein concentration was determined according to Bradford's method **(Bradford, 1976)**. For normalization of the measured absorption values, BSA (Hi-media) was used. Finally, all samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following Laemmli method on a vertical Slab gel

**(Laemmli, 1970)**; Acrylamide gel in form of resolving 12% and stacking gel 4% was prepared and the samples were run at 100 V. Protein bands were seen after gel stained with Coomassie Brilliant Blue stain (Merck) R-250 **(Dunn, 1993)**.

### Statistical Analysis

The percentage of survival rate and the mean cell forming units were implemented by using EXEL 2010 software.

## RESULTS AND DISCUSSION

### Isolation and identification of *Lactobacillus*

Based on the isolation of *Lactobacillus spp*., the bacteria were positive to grow on MRS selective media, and when examined under the light microscope, all isolates acquired blue-purple stain hence they react positively when stained with Gram. Also, based on biochemical tests displayed in Table 1, all isolates were negatively reacting with Catalase. Results of gas production from glucose to all inoculated test tubes after observation for 2 – 3 days showed that all isolates were positive for gas production from glucose fermentation. Findings of Carbohydrates fermentation analysis were listed in positive and negative reactions. Outcomes of testing the ability of isolates to grow at different temperatures showed the ability of all isolates to grow at 45 ºC whereas no growth was seen at 10 °C and 15 °C.

### Bile and acid tolerance

From the different concentrations of bile salts taken, 3% bile salts in MRS broth was the minimal inhibitory concentration of bile from most *Lactobacillus* isolates as shown in Table 2 and Figure 1, hence the survival of *lactobacillus* post 2 h. Incubation in MRS containing 3% bile salts was analyzed and the results are shown in Table 2, isolate of *L. delbrueckii* showed an excellent survival of 89%, LGG showed the ratio of survival 91%. Also, *lactobacillus* survival rates in acidic buffer with pH 4.5 and 2.0 were examined by the difference in viable cell counts following 0 min 2 h of incubation as shown in Table 3 and Figure 2. The isolated *L. rhamnosus* 6.90% at pH 4.5, 5.12% at pH 2.0, showed the highest survival in acidic pH, immediately followed by *L. plantarum, L. delbrueckii,* and *L. fermentum*5.70, 5.40 and 4.03% at pH 4.5, 4.90, 4.31 and 2.33% at pH 2.0 respectively.

**Table 1** Biochemicalte stresults of collected *Lactobacillus* isolates.

| Biochemical test | Collected *Lactobacilli* isolates | | | |
|---|---|---|---|---|
| | ***L. delbrueckii*** | ***L. fermentum*** | ***L. plantarum*** | ***L. rhamnosus*** |
| Catalase | - | - | - | - |
| Gas from glucose | + | + | + | + |
| Ammonia from Arginine | - | + | + | - |
| Growth at 10°C | - | - | - | - |
| Growth at 15 °C | - | - | - | - |
| Growth at 45 °C | + | + | + | + |
| Glucose | + | + | + | + |
| Xylose | + | D | + | + |
| Ribose | + | + | + | + |
| Melezitose | - | - | - | - |
| Arabinose | + | D | + | + |
| Mannitol | ND | - | - | - |
| Trehalose | d | d | d | d |
| Melibiose | + | + | + | + |
| Raffinose | + | + | + | + |
| Galactose | + | + | + | + |
| Salicin | - | - | - | + |
| Maltose | + | + | + | + |
| Sucrose | + | + | + | + |
| Mannose | D | W | - | + |
| Fructose | + | + | + | + |
| Lactose | + | + | + | + |
| Rhamnose | - | - | - | - |
| Sorbitol | ND | ND | - | - |

Note: Symbols: w – weak positive reaction; ND – no data available; d – different.

**Table 2** Concentration and survival rate of *Lactobacillus* isolates in the presence of 3% bile slats.

| Isolates | Pre-incubation | | Bile tolerance | |
|---|---|---|---|---|
| | **Mean * 10 $^{8}$cfu.mL$^{-1}$** | **Survival rate %** | **Mean * 10 $^{8}$cfu.mL$^{-1}$** | **Survival rate %** |
| LGG | 6.57 | 100 | 6.30 | 91% |
| *L. delbrueckii* | 6.43 | 100 | 6.11 | 89% |
| *L. fermentum* | 5.93 | 100 | 4.85 | 82% |
| *L. plantrum* | 6.18 | 100 | 5.1 | 82% |
| *L. rhamnosus* | 6.19 | 100 | 4.98 | 80% |

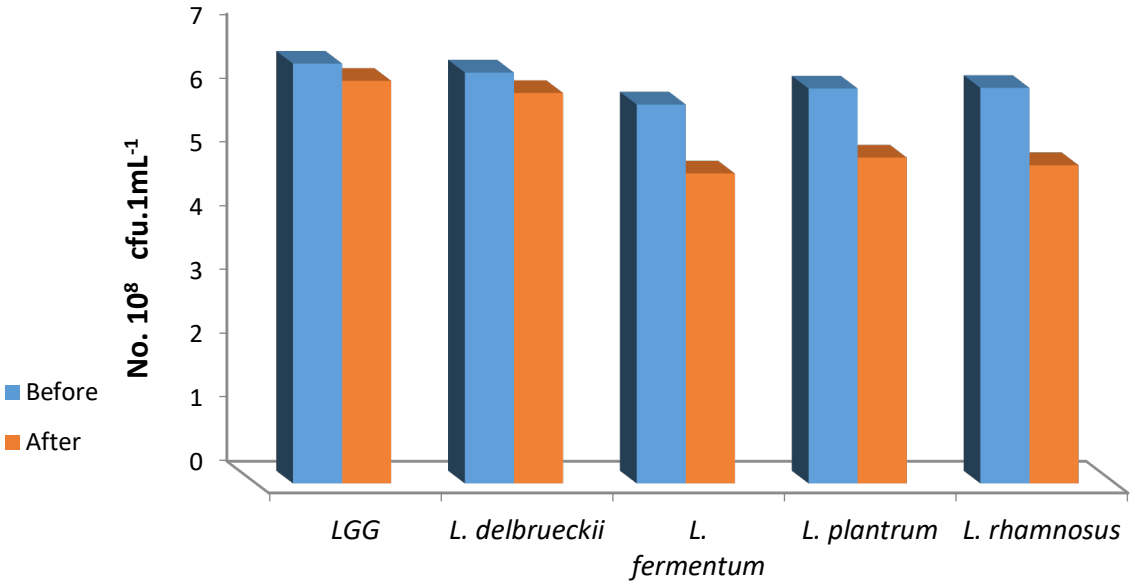


**Figure1** Concentration and survival rate of *Lactobacillus* isolates rate in the presence of 3% bile salts.

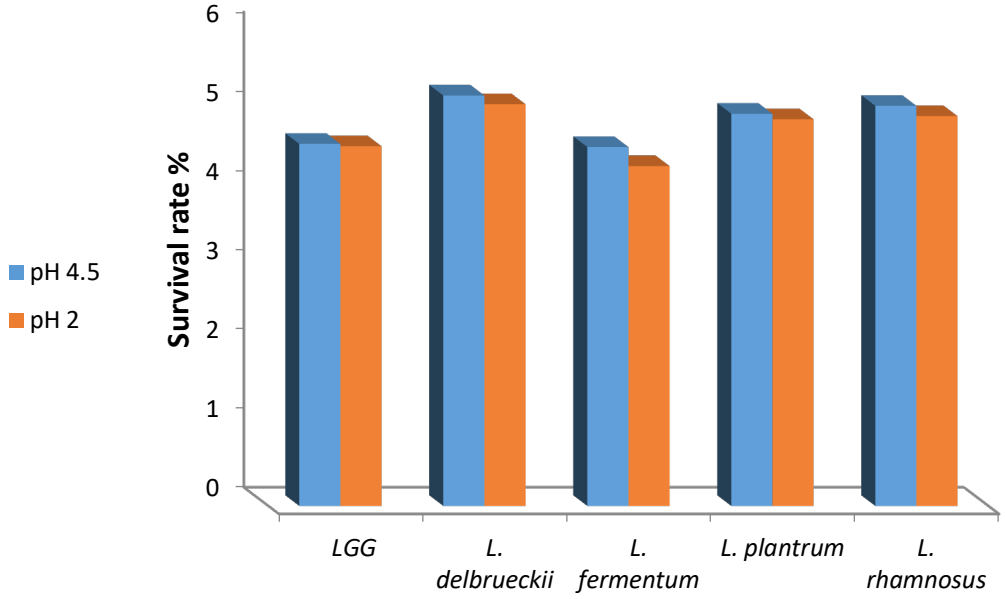


**Figure 2** Concentration and survival rate of *Lactobacillus* isolates at the acidic condition.

**Table 3** Concentration and survival rate of *Lactobacillus* isolates at the acidic condition.

| Isolates | pH 4.5 | | pH 2.0 | |
|---|---|---|---|---|
| | **Mean * $10^7$cfu.mL$^{-1}$** | **Survival rate %** | **Mean * $10^7$cfu.mL$^{-1}$** | **Survival rate %** |
| LGG | 4.57 | 1.0 | 4.54 | 0.91 |
| *L. delbrueckii* | 5.18 | 5.40 | 5.07 | 4.31 |
| *L. fermentum* | 4.53 | 4.03 | 4.29 | 2.33 |
| *L. plantarum* | 4.95 | 5.70 | 4.88 | 4.90 |
| *L. rhamnosus* | 5.05 | 6.90 | 4.92 | 5.12 |

### Tolerance to acid and alkaline conditions

Based on bile salt and acid tolerance: different concentrations of bile salt in MRS broth were prepared and tested on lactobacillus isolates to determine the minimal inhibitory concentration of bile salt tolerance as shown in Table 2 and Figure 1. Results showed that MIC was 3%. The vitality of *lactobacillus* post 2 h, incubation in MRS containing 3% bile salts that *L. delbrueckii* showed an excellent survival ratio of 89%, LGG showed a ratio of survival 91%. Also, lactobacilli survival rates in acidic buffer with pH 4.5 and 2.0 were examined by the difference in viable cell counts following 0 min 2 h, incubation as shown in Table 3 and Figure 2.

The isolated *L. rhamnosus* showed the highest survival in acidic pH; 6.90% at pH 4.5, 5.12% at pH 2.0, immediately followed by *L. plantarum, L. delbrueckii,* and *L. fermentum* showed; 5.70, 5.40 and 4.03 % at pH 4.5, 4.90, 4.31 and 2.33% at pH 2.0, respectively. *Lactobacillus* bacteria colonized the hindgut region in the human host and should be able to survive with the acid condition and bile salt before reaching the target tissue.

Therefore, tolerance of *Lactobacillus* to acid and bile salt equally is an important issue to select *Lactobacillus* bacteria as a probiotic.

**Table 4** Antimicrobial substances activity exhibited by *Lactobacillus* isolates.

| Strains | *E. coli* | *Salmonella typhi* | *Pseudo. areuginosa* | *Staph. aureus* | *Shigella dysenteriae* |
|---|---|---|---|---|---|
| LGG | ++ | + | + | +++ | +++ |
| *L. delbrueckii* | ++ | ++ | ++ | +++ | +++ |
| *L. fermentum* | +++ | + | -/+ | +++ | +++ |
| *L. plantarum* | +++ | ++ | -/+ | +++ | +++ |
| *L. rhamnosus* | +++ | ++ | ++ | +++ | +++ |

**Table 5** The antibiotic suscptibility test of *Lactobacillus* isolates.

| **Antibiotics [µg]** | ***L. delbrueckii*** | ***L. fermentum*** | ***L. plantarum*** | ***L. rhamnosus*** |
|---|---|---|---|---|
| Ampicillin 5 | - | - | - | - |
| Chloramphenicol 50 | + | + | + | + |
| Nitrofurantion50 | - | - | - | - |
| Gentamicin10 | - | - | - | - |
| Carbenicillin 100 | - | - | - | - |
| Nalidixic Acid 30 | - | - | - | - |
| Cotrimoxazole 25 | - | - | - | - |
| MethenamineMandelate 3 mg | - | - | - | - |

Note: + Resistance, - Sensitive.

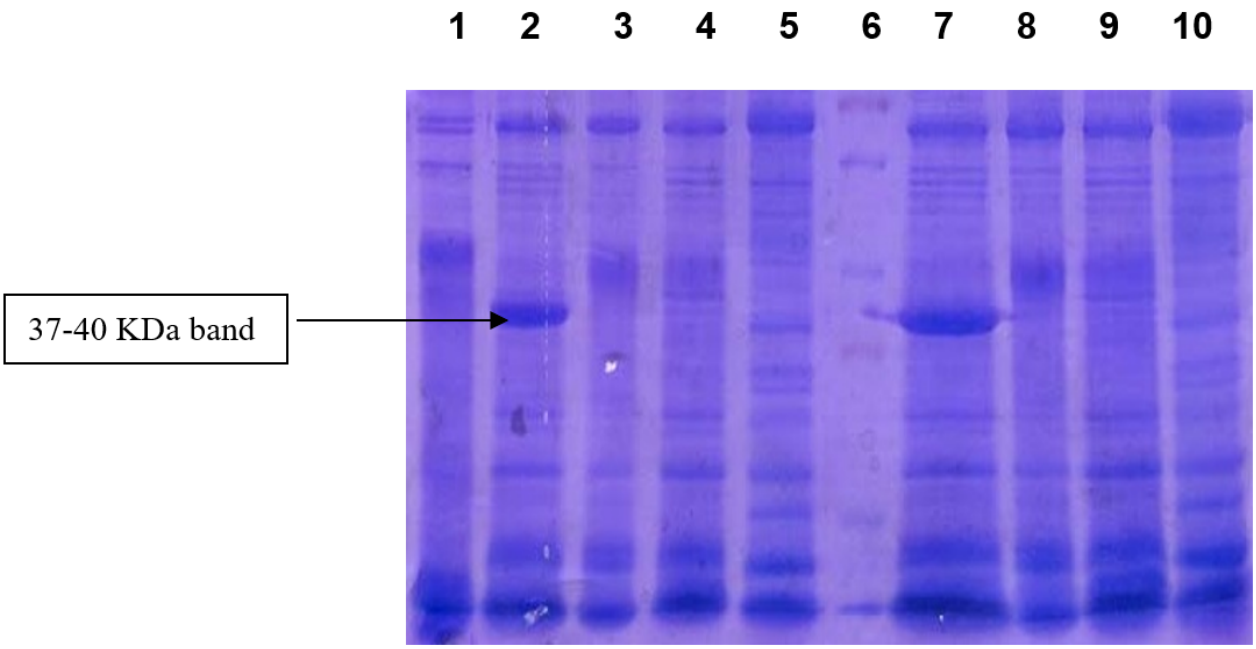


**Figure 3** SDS-PAGE protein profiles of *Lactobacillus* isolates and the reference molecular protein marker. Note: lane 6: reference protein marker; lane 1: *L. rhamnosus;* lane 2: *L. fermentum;* lane 4: *L. delbrueckii.*

Our result of this study agreed with the outcomes of other researches, they reported a survival rate of *Lactobacillus* isolates in pH 2.5 **(Kim et al., 2007; Perelmuter et al., 2008)**. Nevertheless, the survival rate of *Lactobacillus* has not been more than 0.2%.

### Antimicrobial test

The Findings of antimicrobial activity are presented in Table 4; all collected *Lactobacillus* isolates showed antagonistic activity in various degrees when examined against gram-negative bacteria and gram-positive bacteria.

The standard strain LGG and the *Lactobacillus* isolates showed the highest inhibitory activity against *Staphylococcus aureus,* and *Shigella dysenteriae* whereas, only *L. fermentum, L. fermentum,* and *L. rhamnosus* displayed the highest inhibitory activity against *E. coli*. Strain LGG and the collected isolate *L. rhamnosus* belong to the same species, but they showed a difference in their antimicrobial activity since antimicrobial characteristics should be of a specific strain. Once they colonize the hindgut of the human intestine, they loss their antimicrobial activity in the acidic environment **(Perelmuter et al., 2008; Enan et al., 2013)**. They are isolated, identified, and characterized seven small peptides by LGG that have a mass of about 1 kDa and that exert a bactericidal action against both Gram-positive and Gram-negative bacteria **(Lu et al., 2009)**. While *Lactobacillus* spp. suppose to be getting their opportunity to display their antimicrobial activity due to production elements as; lactic acid, $H_2O_2$, and bacteriocins other antibacterial molecules **(Eschenbach et al., 1989; Kučerová et al., 2007)**.

The result of this study agrees with that recorded by **(Perelmuter et al., 2008; Casy et al., 2004)**, they had been observed a range of lactobacilli antimicrobial responses towards *E. coli* and bacteria besides, they approved the inhibitory activity of *Lactobacillus* spp. affected by the acidic environment. These results are following those reported by **(Rodes et al., 2013)**.

### Antibiotics Susceptibility Patterns:

Outcomes of antibacterial sensitivity of *Lactobacillus* isolates were revealed in Table 5: all collected isolates of food source were resistant to Chloramphenicol 50 µg whereas, all of them showed high sensitivity to; Ampicillin 5 µg, Nitrofurantoin 50 µg, Gentamicin 10 µg, Carbenicillin 100 µg, Nalidixic Acid 30 µg, Cotrimoxazole 25 µg and Methenamine Mandelate 3 mg. The safety of *Lactobacillus* isolates must be carefully evaluated. One safety aspect is the transfer or acquisition of antibiotic resistance. Because nonpathogenic enteric bacteria may also be a source for resistance genes that may spread to potential pathogens. Therefore, investigation activities to obtain *Lactobacillus* isolates for probiotic uses should include nonpathogenic as well as pathogenic bacteria **(Katla et al., 2001)**. *Lactobacillus* isolates were collected in this research qualified to use as probiotics because they exhibited minor antibiotic resistance. Thus, it is most important for the selection of probiotic strains without any highly transferable antibiotic resistance or especially virulence mechanisms **(Klein et al., 2000)**. Results obtained in this study agree with the findings reported by **(Katla et al., 2001; Klein et al., 2000)**.

### The S-layer protein profile:

The result of the detection S-layer protein profile through SDS-PAGE has demonstrated in Figure 3: SDS-PAGE protein of *Lactobacillus* isolates was quite similar except the *L. rhamnosus* produced patterns containing band about 37 – 40 kDa., according to the previous studies, one dominant band of 43 – 46 KDa, which is known as the S-protein **(Boot et al., 1993; Smit et al., 2001)**. In our study, S-layer extraction was carried out according to Boot's method **(Boot et al., 1993)**. The S-layer proteins characterized from the genus *Lactobacillus* range from 25 to 71 kDa in size **(Sára and Sleyrtr, 2000)**. The Putative S-layer protein was detected by whole-cell SDS-PAGE analysis. S-layer analysis revealed the presence of a unique and single S-layer with an oblique structure. The whole-cell protein patterns of the *Lactobacillus* isolates are fairly homogeneous with some variability, primarily localized in the molecular mass region estimated 30 – 70 kDa.

## CONCLUSION

This preliminary study was evaluated the presence of *Lactobacillus* spp. in foods and also it identified, and characterized some strains. Furthermore, it assessed collected isolates to determine their qualified characteristic to use as probiotics. The bacterial surface layer (S-layer) is an excellent candidate for *In vivo* and *In vitro* biotechnological applications. It had been shown to function as an adhesive, mediating binding of Lactobacilli to the host epithelial. The S-layer had been identified in several *Lactobacillus spp*. So, further investigations of *Lactobacillus* populations are necessary to obtain relevant data to be included in an important assessment that is related to food production.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# SURVEY OF SOMATIC CELL COUNTS AND MILK COMPOSITION IN BULK MILK OF EWES IN DAIRY PRACTICE

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## ABSTRACT

We evaluated milk quality during the sheep dairy period in the year 2018. The study was performed at fifteen dairy farms with differed breeds and crossbreds under Slovakian usual practical conditions (milking and pasture). At the first and seventh farm purebred Tsigai (TS) ewes were kept, at the eight to twelve farm there were purebred Lacaune ewes (LC) and the thirteen farm were kept crossbred Improved Valachian x Lacaune ewes (IV/LC, with a higher proportion of Improved Valachian), the fourteen farm crossbred Lacaune x East Friesian ewes and the last farm were ewes of the synthetic population of Slovak dairy ewe (SD). The milk yield recording and milk sampling were performed once a month during evening milking as a part of milk recording services. The basic milk composition was determined by MilkoScan FT120 (Foss, Hillerød, Denmark) and somatic cell count was determined using a Fossomatic 90 (Foss Electric, Hillerød, Denmark) after heat treatment at 40 °C for 15 min. We found the highest incidence of SCC on farm 14 with crossbred LC/EF 3.940 x 103 cells.mL$^{-1}$. Followed by farms 12 and 9 with purebred LC (SCC value of 3.318 and 2.489 x 103 cells.mL$^{-1}$). Farm 7 with purebred TS reached the lowest value (831 x 103 cells.mL$^{-1}$). The highest fat content was reached by the purebred TS, with gradual growth from March to July. Crossbreds and the synthetic population of Slovak dairy ewe (SD) had the lowest average fat content, which could be affected by feeding. Similar tendencies were found in protein content.

**Keywords:** SCC; fat; protein; lactose; sheep milk

## INTRODUCTION

Sheep's milk production accounts for about 3.6% of total world milk production. In the last year 2019, sheep milk production in Slovakia reached 13,524 tonnes, the highest level since entering the European Union. Ewe's milk is mainly used for making cheese in Slovakia. **Bianchi et al. (2004)** presented in their work that SCC was associated with various udder health statutes and lactational phases were evaluated to verify their role in milk quality with regards to its cheese-making properties.

Ewe's milk was much more concentrated with about twice as much fat and 40% more protein than cow and goat milk. That also found that sheep milk responded differently in the cheese-making procedure. It was more sensitive to rennet, coagulated faster, produced a firmer curd, and yielded more cheese per unit of milk than cow milk **(Wendorff and Haenlein, 2017)**.

Although the SCC is not considered a factor influencing the price of milk, it is also an important factor determining the yield and quality of the final product **(Oravcová et al., 2007)**.

Somatic cell count (SCC) in raw milk is widely used to differentiate between healthy and infected mammary glands in ruminants. In the US, Grade A Raw Milk Standards require that the SCC of raw sheep milk shall not exceed 750 000 cells.mL$^{-1}$ **(USPHS, 1999)**. In EU countries there are no standards considering the SCC of raw sheep milk. The quality of raw milk is regularly checked because milk is the ideal environment for developing microorganisms because of its high water and nutrient content. To avoid risks, and to ensure hygiene-sanitary quality and raw cows', sheep's and goats' milk safety, in its Regulations **(EC) No. 852/2004** and **(EC) No. 853/2004** European legislation lays down general food hygiene rules and specific ones for food of animal origin. It also sets out aspects relating to mandatory controls **(EC) No. 853/2004** on raw milk production on farms, and in dairy centres and laboratories. In dairy ewe's instantaneous physiological and pathological thresholds of SCC ranging from (0.25 to 1.0) × 106 cells.mL$^{-1}$ have been available since the early 1990s **(Ariznabarreta et al., 2002)**. **Berthelot et al. (2006)** recommends that a decision rule proposes to consider an udder as healthy if every SCC are lower than 0.500 × $10^6$ cells.mL$^{-1}$ and infected if at least two individual SCC are higher than 1 or 1.2 million cells.mL$^{-1}$. **Arias et al. (2012)** found in manchego sheep that milk yield was always higher for ewe with SCC ≤300 × $10^3$ cells.mL$^{-1}$ than for those with SCC > 300 × $10^3$ cells.mL$^{-1}$.

**Table 1** Months of test day, SCC*$10^3$ cells.mL$^{-1}$.

| Farms | March | April | May | June | July | August | Sept. | Decem. | Total |
|---|---|---|---|---|---|---|---|---|---|
| **1** | | 2747 | 1375 | | 2942 | | | | **2232** |
| **2** | | 1116 | 1309 | 702 | 1065 | | | | **1022** |
| **3** | | 1206 | 933 | 915 | 2002 | | | | **1198** |
| **4** | | | | | 881 | | | | **881** |
| **5** | | 2147 | | | 1049 | | | | **1501** |
| **6** | 1390 | | | | | | | | **1390** |
| **7** | | 776 | 840 | 1456 | 252 | | | | **699** |
| **8** | | 1266 | | | | 1095 | | 1105 | **1153** |
| **9** | | 2552 | 2307 | 3037 | 2059 | | | | **2463** |
| **10** | | 771 | 1307 | 663 | 1734 | | | | **1037** |
| **11** | | | 3262 | | | | 1667 | | **2332** |
| **12** | | 2530 | 4792 | 2676 | 3274 | | | | **3210** |
| **13** | | | | | 1521 | | | | **1521** |
| **14** | | 3627 | | 3266 | | | | 4926 | **3879** |
| **15** | | 2644 | 2145 | 2454 | 1766 | 2142 | | | **2209** |

Note: *SCC – somatic cell count.

Stress factors, for example, lamb separation, the start of machine milking, and sudden change in diet can increase the risk of infection **(Sinapis, 2007)**.

### Scientific hypothesis

The period of the year 2018 affects the milk quality of dairy ewes.

## MATERIAL AND METHODOLOGY

The study was performed at fifteen dairy farms with differed breeds and crossbreds under Slovakian usual practical conditions (milking and pasture). At the first and seventh farm purebred Tsigai (TS) ewes were kept, at the eight to twelve farm there were purebred Lacaune ewes (LC) and the thirteen farm were kept crossbred Improved Valachian x Lacaune ewes (IV/LC, with a higher proportion of Improved Valachian), the fourteen farm crossbred Lacaune x East Friesian ewes and the last farm were ewes of a synthetic population of Slovak dairy ewe (SD).

At all farms, the ewes were on pasture during the day and housed in the stable during the night. Twice a day the machine milking in the parlor was performed, whereat all animals received concentrates in amounts of 200 g per day.

The milking of ewes started shortly before Easter (suckling lambs were sold) and lambing of the most ewes was within 3 weeks (January/February).

The milk yield recording and milk sampling were performed once a month during evening milking as a part of milk recording services. Milk samples (50 mL) were collected from the whole milk yield into the recording jar (ICAR approved).

The basic milk composition was determined by MilkoScan FT120 (Foss, Hillerød, Denmark) and somatic cell count was determined using a Fossomatic 90 (Foss Electric, Hillerød, Denmark) after heat treatment at 40 °C for 15 min. Milk samples of the sheep were analyzed in the Central laboratory for milk analysis of Breeding Services of the Slovak Republic, s.e. by norm **ISO/IEC 17025 (2017)**. These instruments were calibrated monthly.

The calculation of SCC in the bulk milk tank was evaluated from individual samples by:

$$SCC_T = \frac{\sum_{j=1}^{n} SCC_j * MY_j}{\sum_{j=1}^{n} MY_j}$$

Where:
SCCT - in bulk; SCC1, SCC2, ... SCCj - individual SCC at sampling day; MY1, MY2, ... MYj - individual milk yield per milking at sampling day. We recalculated the basic milk components in the same way.

### Statistical analysis

The values were evaluated through mean and standard deviation by Microsoft Excel 2013.

## RESULTS AND DISCUSSION

In 2018 we found the highest incidence of SCC on farm 14 with crossbred LC/EF 3.940 x $10^3$ cells.mL$^{-1}$(Table 1). Followed by farms 12 and 9 with purebred LC (SCC value of 3.318 and 2.489 x $10^3$ cells.mL$^{-1}$). Farm 7 with purebred TS reached the lowest value (831 x $10^3$ cells.mL$^{-1}$).

For individual animals, the best approach has been provided by **Berthelot et al. (2006)**. The mentioned author suggested that values <0.5 × $10^6$ cells mL$^{-1}$ indicate a healthy mammary gland and values >1.0 × $10^6$ cells.mL$^{-1}$indicate a mammary gland with clinical or subclinical mastitis. Furthermore, there is no need to perform a simultaneous bacteriological examination of milk samples to confirm the problem. Values between 0.5 × $10^6$ and 1.0 × $10^6$ cells.mL$^{-1}$, according to those authors, indicate 'suspected disease'. There is a need for performing the bacteriological examination in milk. From a practical point of view, individual milk SCC is used, for subclinical mastitis control; "doubtful" ewes are grouped either with "healthy" (when farmers decide to cull "infected" females) or "infected" ewes (to implement a selective drying-off therapy). The lambs' mouths and milkers' hands are the sources of milk contamination **(Albenzio et al., 2003)**.

**Table 2** Months of test day, fat g.100g$^{-1}$.

| Farms | March | April | May | June | July | August | Sept. | Decem. | Total |
|---|---|---|---|---|---|---|---|---|---|
| **1** | | 7.98 | 7.69 | | 8.56 | | | | 8.08 |
| **2** | | 6.77 | 6.75 | 7.33 | 8.08 | | | | 7.24 |
| **3** | | 6.75 | 7.56 | 8.69 | 8.66 | | | | 7.92 |
| **4** | | | | | 9.58 | | | | 9.58 |
| **5** | | 7.44 | | | 9.25 | | | | 8.34 |
| **6** | 6.91 | | | | | | | | 6.91 |
| **7** | | 5.96 | 7.29 | 8.08 | 8.37 | | | | 7.43 |
| **8** | | 5.53 | | | | 4.83 | | 7.84 | 6.07 |
| **9** | | 5.03 | 4.25 | 4.64 | 6.01 | | | | 4.98 |
| **10** | | 5.67 | 5.74 | 6.12 | 7.75 | | | | 6.32 |
| **11** | | | 7.36 | | | | 7.47 | | 7.42 |
| **12** | | 7.64 | | | | | | | 7.64 |
| **13** | | | | | 6.08 | | | | 6.08 |
| **14** | | 6.00 | | 6.70 | | | | 8.20 | 6.97 |
| **15** | | 5.70 | 5.90 | 5.75 | 7.70 | 6.84 | | | 6.38 |

**Table 3** Months of test day, protein %.

| Farms | March | April | May | June | July | August | Sept. | Decem. | Total |
|---|---|---|---|---|---|---|---|---|---|
| **1** | | 5.59 | 5.71 | | 6.68 | | | | 6.00 |
| **2** | | 5.33 | 5.26 | 5.80 | 6.01 | | | | 5.60 |
| **3** | | 5.23 | 5.30 | 5.65 | 6.56 | | | | 5.69 |
| **4** | | | | | 6.40 | | | | 6.40 |
| **5** | | 5.30 | | | 6.37 | | | | 5.84 |
| **6** | 5.74 | | | | | | | | 5.74 |
| **7** | | 5.84 | 5.84 | 6.26 | 6.71 | | | | 6.16 |
| **8** | | 4.98 | | | | 5.35 | | 6.56 | 5.63 |
| **9** | | 5.30 | 5.57 | 5.53 | 5.72 | | | | 5.53 |
| **10** | | 5.04 | 5.28 | 5.56 | 6.16 | | | | 5.51 |
| **11** | | | 4.92 | | | | 7.08 | | 6.00 |
| **12** | | 5.86 | | | | | | | 5.86 |
| **13** | | | | | 6.20 | | | | 6.20 |
| **14** | | 5.31 | | 5.45 | | | | 6.41 | 5.72 |
| **15** | | 5.44 | 5.41 | 5.15 | 5.77 | 5.59 | | | 5.47 |

**Table 4** Months of test day, lactose %

| Farms | March | April | May | June | July | August | Sept. | Decem. | Total |
|---|---|---|---|---|---|---|---|---|---|
| **1** | | 4.73 | 4.76 | | 4.46 | | | | 4.65 |
| **2** | | 4.87 | 4.77 | 4.76 | 4.62 | | | | 4.75 |
| **3** | | 4.81 | 4.83 | 4.69 | 4.56 | | | | 4.72 |
| **4** | | | | | 4.54 | | | | 4.54 |
| **5** | | 4.80 | | | 4.58 | | | | 4.69 |
| **6** | 4.60 | | | | | | | | 4.60 |
| **7** | | 4.94 | 4.84 | 4.58 | 4.65 | | | | 4.75 |
| **8** | | 4.98 | | | | 5.00 | | 4.69 | 4.89 |
| **9** | | 4.90 | 4.95 | 4.82 | 4.74 | | | | 4.85 |
| **10** | | 4.95 | 4.99 | 4.81 | 4.66 | | | | 4.85 |
| **11** | | | 4.55 | | | | 3.95 | | 4.25 |
| **12** | | 4.81 | | | | | | | 4.81 |
| **13** | | | | | 4.68 | | | | 4.68 |
| **14** | | 4.86 | | 4.57 | | | | 4.57 | 4.66 |
| **15** | | 4.81 | 4.80 | 4.78 | 4.63 | 4.61 | | | 4.73 |

These authors have found that within 4 weeks lasting experiment there was higher SCC at machine milking of ewes when compared to suckled ones, as a consequence of higher bacterial positive samples at machine milking. **Tomáška et al. (2015)** evaluated the most farms with SCC values >1.0 × $10^6$ cells.mL$^{-1}$. **Pazzola et al. (2014)** stated values of SCC 1.251 ±2.991 x $10^3$ cells.mL$^{-1}$. They were characterized by a very large range of variation. In agreement with the review by **Riggio and Portolano (2015)**, a value of SCC >1.0 × $10^6$ cells.mL$^{-1}$ is a normal finding in milk from healthy ewes. **Kuchtík et al. (2017)** founded SCC <0.5 × $10^6$ cells mL$^{-1}$. **Vara Martínez et al. (2018)** evaluated 1.032 x $10^3$ cells.mL$^{-1}$.

The highest fat content was reached by the purebred TS, with gradual growth from March to July (Table 2). Crossbreds and the synthetic population of Slovak dairy ewe (SD) had the lowest average fat content, which could be affected by feeding. Similar tendencies were found in protein content (Table 3). These changes in the milk composition are mainly related and could be also explained by the stage of lactation.

A similar fat content, but higher protein content was observed as compared to the results of **Oravcová et al. (2007)**. From the available publications **Špánik et al. (1996)**, **Margetín et al. (1995)**, **Margetín et al. (1996)**, **Margetín, Hlavatý and Přibil (1998), Tomáška et al. (2014)** and **Oravcová et al. (2005)**, which researched the composition of Tsigai milk rearing in Slovakia, a positive trend of increasing milk production was observed.

These changes in the milk composition are mainly related and could be also explained by the stage of lactation.

It is known that the fat and protein content of milk is dependent on nutrition, and indirectly, nutrition will also affect the solids-non-fat (SNF) of milk. Table 2, Table 3 and Table 4 are presented the basic composition of milk during the milking period. We have found a gradual increase in milk components, except for lactose, which is related to the increasing number of somatic cells during the milking period and consequently the health of the milk udders.

Both fat and protein tend to increase throughout the lactation as well as **Kuchtík et al. (2017)**. This would typically result in higher cheese yields in late lactation milk **(Wendorff and Haenlein, 2017)**. As the SCC increases in the milk supply, the composition of milk also changes. As SCC increased, milkfat and the Casein/Total Protein ratio decreased. The protein recovery rate was lower in the high SCC milk while cheese yield was not significantly different.

**Bocquier and Caja (2004)** are reported that a high level of nutrition will reduce the level of milkfat but increase milk protein and casein. Conversely, a negative energy balance will decrease milk protein and increase milkfat. Milk protein will increase with an increased level of dietary protein. When feeding higher levels of concentrate in the diet, milkfat will be decreased and milk protein will be increased. The degree of impact from the nutrition of the ewe will be limited by the potential milk production capacity of the animal dictated by genetics. These trends are consistent with our results.

## CONCLUSION

We found a high SCC of over 1 million on the farms surveyed, except for two farms with purebred Tsigai. The nutrient content was following the minimum ingredient content in the milk according to Regulation (EC) No 853/2004 European legislation. However, a more detailed study is needed to see the relationship between high SCC and the presence of microorganisms to better understanding the reasons for the physiological and pathological SCC in the udder. Individual milk SCC represents a useful tool for the detection of subclinical mastitis in dairy ewes. It is recommended to evaluate a series of SCC, take into account the stage of lactation, and use two thresholds allowing to distinguish three classes of ewes: healthy, doubtful (or briefly infected), and infected (or persistently infected).

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# THE STUDY OF RAPESEEDS ASH COMPOSITION IN THE CONDITIONS OF THE AGROECOLOGICAL EXPERIMENT

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## ABSTRACT

A comparative analysis of the seeds ash composition of the breed Rif (*Brassica napus* L.) rapeseeds grown in the Lipetskaya region was held. The plants were grown in the conditions of the agroecological experiment using mineral (NPK and zeolite) and organic (hen droppings) fertilizers. 6 variants of the experiment were studied – the plants are grown without fertilizers application (the control); the mineral fertilizer ($N_{60}$:$P_{60}$:$K_{60}$) separately and together with zeolite (5 $t.ha^{-1}$); the zeolite in pure form (5 $t.ha^{-1}$); hen droppings (5 $t.ha^{-1}$) separately and together with zeolite (5 $t.ha^{-1}$). We studied the accumulation of 9 basic elements (in mass %) contained in Brassica napus. seeds ash using the method of energy-dispersive X-ray spectroscopy. The accumulation order of the elements was determined: P ≈ K > Mg ≥ Ca > Mo > S > Zn > Mn > Fe. The proportion of P fluctuated from 10.852 to 11.855 mass %; the proportion of K – from 9.933 till 12.343 mass %. The rapeseeds contained Mg, Ca, and Mo in similar concentrations within the range of 4.0 -5.8 mass %. The combined application of zeolite with organic fertilizer ensured the accumulation of the minerals in the seeds. Correlations between the elements were established. High correlation between elements K and Mo was found (r = 0.96); P and Mg (0.86); P and Fe (r = 0.94); C and Mo (r = 0.86). The positive effect of the combined organic-mineral fertilizers with poultry farms wastes usage on the mineral elements accumulation in rapeseeds was stated. It is noted that the accumulation of P, Ca, Mo, and S in rape seeds leads to a decrease in Zn.

**Keywords:** *Brassica napus*; seeds; analytical scanning electron microscopy; Energy Dispersive X-ray Analysis (EDS); ash elements

## INTRODUCTION

For the last twenty years, the world's rapeseed production has had a stable growth. This crop is an important source of renewable energy, and new conditions are created for the use of oilseed rape **(Ralphe et al., 2006; Günnur and Nilgün, 2013)**. The main manufacturers are China, India, Canada, and the EU **(Carre and Pouzet, 2014)**. This culture has been adapted to the western conditions of the USA. Edaphoclimatic conditions of Russia allow growing the rapeseed plants in all the regions **(Karpachev, 2009)**. The seeds fund development demands the constant improvement of this culture cultivation technology in the concrete edaphoclimatic conditions taking into account the breed peculiarities, the reaction on the different technological elements including the reaction on both mineral and organic fertilizers **(Pin Koh and Ghazoul, 2008; Rondanini et al., 2012)**. The researches results prove that the greatest impact on rapeseeds productivity is caused by the norm of a nitrogen fertilizer application, and the smallest – by the previous culture and the fertilizer type, as well as the interaction between these processing factors **(Rathke et al., 2005)**. Organic animal waste is recognized as a valuable source of plant nutritional chemicals in farming systems and plays a certain role in the improvement of the soil using organic substances **(Schoenau and Davis, 2006)**. Therefore, the further development of rapeseed cultivation technologies with organic and mineral fertilizers usage is currently important. Also, there is growing interest in the use of rapeseed for food development. The focus is on the composition of the protein and lipids contained in rapeseed. **(Gunhild et al., 2000; Arif et al., 2012)**. Moreover, there is very little research on the effect of zeolite and poultry manure when growing rapeseed on seed mineral composition.

Therefore, our research aimed to study the composition of ash elements in rapeseed under agroecological experiments.

### Scientific hypothesis

Information on the mineral composition of the seeds of *Brassica napus* of the breed Rif grown in the Lipetskaya region is not available. We checked whether there are differences in the content of macro - and microelements in rapeseed depending on the use of mineral and organic fertilizers.

## MATERIAL AND METHODOLOGY

### Biological material

The experiments were conducted in the experimental field of Bunin Yelets State University in 2018 – 2019. The spring-planted rapeseed precursor plant is winter-planted wheat. The experimental area soil is black earth with the following agrochemical characteristic of the tilth-top layer: pH 4.88, humus content – 5.76%, total content of N – 0.288%, P – 197.2 mg.kg$^{-1}$, K – 124.7 mg.kg$^{-1}$, Ca – 25.7 mg.kg$^{-1}$ and Mg – 2.4 mg.kg$^{-1}$. The research object was the spring-planted breed Rif rapeseed, combining high potential for productivity and adaptability with high quality of oil and seeds and resistance to major diseases. The rapeseed was seeded on the following experiment scheme: 1 – control; 2 – $N_{60}$:$P_{60}$:$K_{60}$; 3 - zeolite, 5 t.ha$^{-1}$; 4 – hen droppings, 5 t.ha$^{-1}$; 5 – $N_{60}$:$P_{60}$:$K_{60}$ + zeolite 5 t.ha$^{-1}$; 6 – hen droppings 5 t.ha$^{-1}$ + zeolite 5 t.ha$^{-1}$.

### Samples

The seeds used for analysis were cleaned of all foreign substances, such as dust, dirt, stones, immature and damaged seeds.

### Preparing seeds for analysis

Preliminary dried at T = 40 – 50 ºC seeds weighing with the mass of 10 g was mineralized in the muffle furnace Naberterm (Germany) at T = 400 ºC. The received ash was dispersed by ultrasound at 18 kHz frequency for 15 minutes. The dispergate even layer was applied on the object table covered with carbonic scotch.

### EDS - analysis

The chemical composition of the basic ash components (P, S, K, Mn, Fe, Mg, Ca, Zn, Mo) was determined by the method of energy dispersive spectrometry (ESD) on the analytical scanning electron microscope (SEM) EVO LA 15 (Zaiss, Germany). While performing the elemental analysis the working distance (WD) is 10 mm. 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 spectra, and histograms (Figure 1). The fractional accuracy of the chemical analysis is spread in the following way: at the element concentration from to 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%. Analyzed 6 samples of rape seeds in 3 replicates. 3 ash areas of each sample were studied.

### Statistical Analysis

The data obtained during the experiments were processed using mathematical methods of variation statistics using the Microsoft Excel software and Statistika 6; we used the Student-Fisher method. To assess the effect of growing conditions on the mineral composition of seeds the threshold of reliability of the obtained data is designated as ($p$ <0.05).

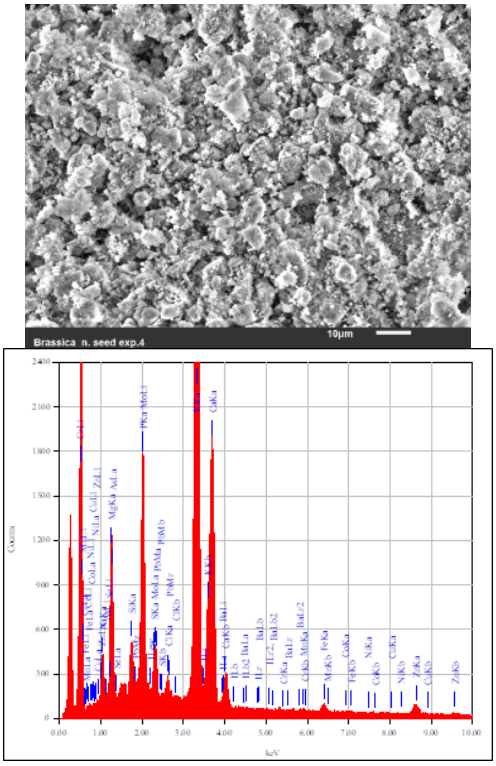


**Figure 1** he microstructurepicture of the sample under study (at the top) and the general view of the X-ray spectrum lines that show the elements presence inthe analyzing area (at the bottom).

## RESULTS AND DISCUSSION

We analyzed the content of 9 elements in the rapeseed ash - K, P, Mg, Ca, Mo, Mn, Zn, Fe, and S (shown in Table 1). The predominant elements were P and K.

The decreasing series of elements in the rapeseed ash is as follows: P ≈ K > Mg ≥ Ca > Mo > S > Zn > Mn > Fe. Our results are comparable to the data obtained by the authors **(Calısır et al., 2005)**. Studies **(Gins et al., 2018)** showed that the main elements in the seeds of amaranth are also K, Ca, but the order of their accumulation is different.

The proportion of P is from 10.852 mass % (control) till 11.855 mass % (zeolite 5 t.ha$^{-1}$). High content of P in the rapeseed ash was also noted in the experimental variants using hen droppings 5 t.ha$^{-1}$; $N_{60}$:$P_{60}$:$K_{60}$ + zeozeolite 5 t.ha$^{-1}$ and hen droppings 5 t.ha$^{-1}$ + zeolite 5 t.ha$^{-1}$. (Figure 2). The content of P in seeds is an important and only source available to maintain the initial growth of seedlings of future plants **(Manske et al., 2001; Masoni et al., 2007; Fageria, Baligar and Jones, 2010)**. The accumulation of P in the seeds is determined by bioavailability, the ability of the roots to absorb P from the soil, and plant growth rates **(Lynch 2007; Hammond and White 2008; White, 2013)**.

According to **White and Veneklaas (2012)**, increasing P content in seeds can improve plant formation and increase yield rape. K in the seed ash contains from 9.933 mass % (zeolite 5 t.ha$^{-1}$) till 12.34 mass % ($N_{60}$:$P_{60}$:$K_{60}$) (Figure 2).

It was determined that the accumulation of K in the control was 25% lower than in the variant with the mineral fertilizer ($N_{60}$:$P_{60}$:$K_{60}$) and 20% lower than in the variant with the combined mineral fertilizer ($N_{60}$:$P_{60}$:$K_{60}$ + zeolite 5 t.ha$^{-1}$) application. K is a macroelement that is responsible for the regulation of the majority of metabolic reactions that

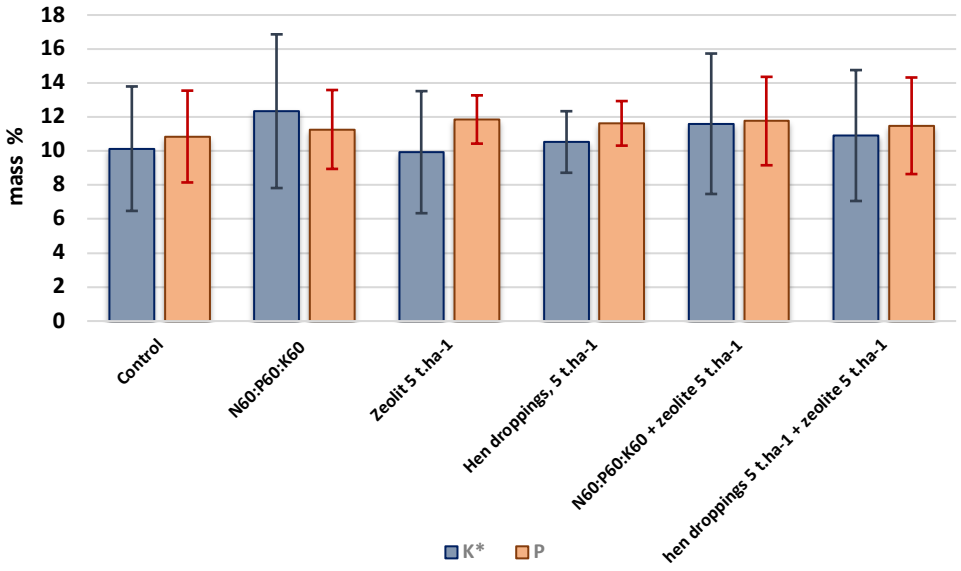


**Figure 2** The comparative concentration of K and P in the ash of the rape.

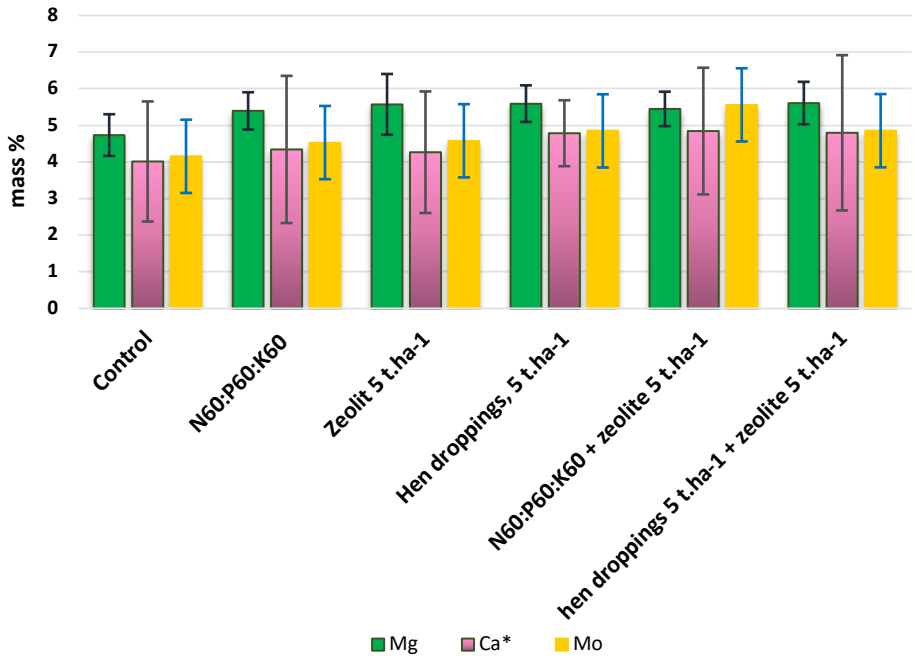


**Figure 3** The comparative concentration of Mg, Ca and Mo in the ash of the rape.

low in living organisms. K controls osmotic pressure transmembrane potential, charges equilibrium, cathode-anion balance, pH – everything that the homeostasis of cells and tissues consists of.

In the ionic form, K can be found in all the organs, tissues, and cell structures in concentrations that exceed the concentration of other ions **(Meathnis et al., 1997)**.

In Figure 3 the comparative data of Mg, Ca, and Mo concentrations are presented. Mg activates a large number of enzymes that take part in the processes of $CO_2$ and N assimilation. Mg is necessary for the keeping up of the cathode-anion balance and pH regulation. In the cell wall and the seeds membrane, $Mg^{2+}$ is coordinately connected with carboxylic groups of pectin substances and takes part in the creation of the inner physiological environment of plants. Mg, Ca and N are localized in the seed membrane. ATP, phosphoinositol (phytin) in combination with Mg are accumulated in the seeds in the form which is comfortable for storage **(Nechaev, Trauberg and Kochetkova, 2007)**. The concentration of Mg in the rape speed fluctuates from 4.733 mass % (control) to 5.575 (zeolite 5 $t.ha^{-1}$). In the variants of the experiment with the mineral and organic N application the concentration of Mg in the rapeseeds increases by 20 – 25% in comparison with the control.

Ca is indispensable within the plant for the stabilization of cell walls and maintenance of membrane integrity. A high proportion of calcium is located in the cell walls. It is involved in the regulation of the cation/anion-balance (IPI Bulletin). The concentration of Ca in the rapeseed fluctuates from 4.012 mass % (control) to 4.843 mass % ($N_{60}$:$P_{60}$:$K_{60}$+ zeolite 5 $t.ha^{-1}$).

Mo fulfills many useful functions for the organism: it is a cofactor and an activator of oxidases (xanthine oxidase and serine oxidase), which takes part in the synthesis of the

**Table 1** The elemental composition of *Brassica napus* seeds, breed Rif, mass % in the ash.

| Elements | Experience options | | | | | |
|---|---|---|---|---|---|---|
| | **Control** | **$N_{60}$:$P_{60}$:$K_{60}$** | **zeolite, 5 t.ha$^{-1}$** | **hen droppings, 5 t.ha$^{-1}$** | **$N_{60}$:$P_{60}$:$K_{60}$ + zeolite 5 t.ha$^{-1}$** | **$N_{60}$:$P_{60}$:$K_{60}$ + hen droppings, 5 t.ha$^{-1}$** |
| K | 10.14 | 12.34 | 9.96 | 10.52 | 11.61 | 10.91 |
| min – max | 6.75 – 13.17 | 7.52 – 7.92 | 6.64 – 10.27 | 7.33 – 4.21 | 7.47 – 16.09 | 4.77 – 13.91 |
| V % | 36.1 | 34.5 | 35.2 | 34.5 | 35.5 | 42.1 |
| P | 10.85 | 11.27 | 11.85 | 11.69 | 11.73 | 11.85 |
| min – max | 8.58 – 4.21 | 8.32 – 3.42 | 9.03 – 4.74 | 9.18 –14.61 | 9.16 – 14.22 | 5.98 – 12.89 |
| V % | 23.9 | 21.6 | 23.8 | 22.3 | 22.1 | 30.52 |
| Mg | 4.73 | 5.39 | 5.58 | 5.19 | 5.15 | 5.21 |
| min – max | 4.77 – 6.06 | 4.18 – 5.41 | 4.98 – 6.13 | 4.67 – 5.84 | 4.55 – 5.68 | 3.89 – 5.24 |
| V % | 10.9 | 10.8 | 10.1 | 9.6 | 9.2 | 12.4 |
| Ca | 5.82 | 4.34 | 4.07 | 4.78 | 4.89 | 4.77 |
| min – max | 3.15 – 6.11 | 3.87 – 7.81 | 2.55 – 5.72 | 2.94 – 6.84 | 3.24 – 6.39 | 2.55 – 7.54 |
| V % | 37.9 | 34.6 | 38.2 | 37.5 | 35.8 | 43.8 |
| Mo | 5.65 | 4.53 | 4.18 | 4.85 | 5.56 | 4.75 |
| min – max | 1.74 – 8.09 | 2.02 – 9.67 | 1.4 – 7.16 | 1.56 – 8.69 | 1.52 – 7.85 | 1.68 – 8.75 |
| V % | 75.8 | 74.8 | 75.2 | 74.2 | 74.1 | 75.3 |
| S | 1.28 | 1.18 | 1.11 | 1.25 | 1.15 | 1.16 |
| min – max | 0.97 – 1.51 | 0.98 – 1.63 | 0.48 – 1.51 | 0.67 – 1.97 | 0.9 – 1.43 | 0.97 – 1.48 |
| V % | 25.8 | 23.6 | 26.7 | 23.9 | 23.9 | 17.4 |
| Zn | 0.31 | 0.16 | 0.27 | 0.21 | 0.16 | 0.22 |
| min – max | 0.08 – 0.26 | 0.09 – 0.65 | 0.13 – 0.42 | 0.08 – 0.36 | 0.07 – 0.26 | 0.11 – 0.61 |
| V % | 62.4 | 73.3 | 61.2 | 63.3 | 61.1 | 72.2 |
| Mn | 0.06 | 0.06 | 0.12 | 0.31 | 0.19 | 0.15 |
| min – max | 0.04 – 0.11 | 0.03 – 0.09 | 0.06 – 0.24 | 0.04 – 0.24 | 0.07 – 0.37 | 0.08 – 0.24 |
| V % | 52.9 | 50.2 | 69.7 | 67.4 | 67.5 | 52.4 |
| Fe | 0.03 | 0.03 | 0.19 | 0.11 | 0.22 | 0.16 |
| min – max | 0.01 – 0.07 | 0.01 – 0.06 | 0.09 – 0.32 | 03 – 0.12 | 0.12 – 0.31 | 0.09 – 0.27 |
| V % | 40.6 | 52.1 | 55.5 | 57.2 | 58.4 | 60.1 |

Note: Means within a column with at least one identical superscript are not significantly different by Student`s *t*-test ($p$ <0.05).

**Table 2** Correlation matrix for the 9 elements in the ash of the seed *Brassica napus*

| Elements | P | Mg | Ca | Mo | S | Zn | Mn | Fe |
|---|---|---|---|---|---|---|---|---|
| K | -0.03 | 0.19 | 0.32 | 0.96 | 0.57 | 0.76 | -0.11 | 0.13 |
| P | | 0.85 | 0.61 | 0.69 | -0.49 | -0.28 | 0.58 | 0.94 |
| Mg | | | 0.72 | 0.57 | -0.17 | 0.001 | 0.56 | 0.76 |
| Ca | | | | 0.86 | 0.17 | -0.23 | 0.78 | 0.72 |
| Mo | | | | | 0.06 | -0.23 | 0.60 | 0,78 |
| S | | | | | | 0.52 | 0.16 | -0.50 |
| Zn | | | | | | | 0.16 | -0.52 |
| Mn | | | | | | | | 0.67 |

amino acid **(Avtsyn et al., 1991)**. The concentration of Mo in the rapeseed fluctuates from 4.178 mass % (control) to 5.558 mass % ($N_{60}$:$P_{60}$:$K_{60}$ +zeolite 5 t.ha$^{-1}$).

Sulfur plays an indispensable role in rape plant metabolism as a component of proteins and glucosinolates. S is essential for protein formation, important for high protein content in rapeseeds. Not only the amount of protein but also the quality of protein is influenced by the S-status of plants. Because of the central role of sulfur and nitrogen in the production of proteins, there is a close relationship between the supplies of S and N in plants **(Blake-Kalff et al., 2000)**. The concentration of S in the rapeseeds is rather high and varies from 0.993 to 1.275 mass %. Depending on the experimental variant the content of S in the seeds fluctuates insignificantly (Table 1). The proportion of Zn does not exceed 0.305% mass and also fluctuates insignificantly depending on the variant of the experiment. Mn is a co-factor and activator of many enzymes and possesses antioxidant activity. In the rapeseeds the concentration of Mn is 0.058 mass % (control) – 0.303 mass % 9 (hen droppings 5 t.ha$^{-1}$). In the experimental variants with the application of the fertilizer, the concentration of Mn is 2 – 6 times higher than the control (Table 1). Organic Fe is an essential compound for the human organism this element s a part of catalytic centres of many oxidation-reduction enzymes **(Shmalko and Roslyakov, 2011)**.

In the rapeseeds, the concentration of Fe varies from 0.034 mass % (the control) to 0.159 – 0.215 (the variant with the

complex composition of fertilizers), Table 1. Thus, we have found that the content of manganese and iron in rape seeds increases significantly when grown in the variants of the experiment with the introduction of zeolite and hen droppings. **Fordoński et al. (2016)** research also showed that the micronutrient content (Mn, Zn, Cu, and Fe) of winter oilseed rape increased significantly in response to an increase in nitrogen fertilization doses.

The low variation coefficient 10 – 25% is determined for P, Mg, and S; K and Ca are characterized with the middle variation coefficient 35 – 36%. The low and average variation coefficient is typical for biologically significant elements of seed rape and indicates the accumulation stability of the elements. Zn, Mn, and Fe are marked with the high variation coefficient

Calculated correlation coefficients between elements (Table 2). It is established that there is a high correlation between elements, for example, K and Mo (r = 0.96); P and Mg (0.86) and P and Fe (r = 0.94); C and Mo (r = 0.86). The mean correlation is between Ca and Mn, Mo, and Fe (r = 0.78); between K and Zn, Mg and Fe (r = 0.76); between P and Mo (r = 0.69); Mn and Fe (r = 0.67); P and Ca (r = 0.61); Mo and Mn (r = 0.60); S and Zn (r = 0.56 – 0.57). Weak correlation found between between S and Zn (r = 0.52); between K and Ca (r = 0.32); K and Mg (r = 0.19): Ca and S (r = 0.17) and S and Mn (r = 0.16).

It is noted that the accumulation of P, Ca, Mo, and S in seeds leads to a decrease in Zn. These results are consistent with data from **Szczepaniak et al. (2017)**.

## CONCLUSION

Research conducted in agro-ecological experience the influence of zeolite and chicken droppings on the formation of the mineral composition of rapeseed has been established. Using the method of the energy dispersive X-ray spectrometry the new data about the variety of the rapeseeds mineral composition were received, the proportion of the elements in the ash was determined, the variation coefficients were calculated. In variants of the experiment with hen droppings, 5 $t.ha^{-1}$, and $N_{60}:P_{60}:K_{60}$ +hen droppings, 5 $t.ha^{-1}$ the content of P, Ca, Mo, S, Mn, and Fe are 2 – 4 times higher than in the control. The influence of the experimental variants on the accumulation of potassium in rape seeds has not been established. A high correlation has been established between the elements K and Mo, P and Mg, P and Fe, C and Mo. It was noted that the accumulation of P, Ca, Mo, and S in rape seeds leads to a decrease in the Zn content.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE STUDY ON THE PROCESS OF DEHYDRATING LEGUMES DURING HIGH-TEMPERATURE MICRONIZATION WITH INFRARED RAYS

*Otari Sesikashvili, Nodari Mardaleishvili, Elene Gamkrelidze, Shalva Tsagareishvili*

## ABSTRACT

Heat treatment is a common operation in grain processing technology. Thermal action on grain is characterized by temperature level and duration. Also, the grain changes all its complex properties. Frequently, the temperature in grain activates the process of change in the moisture content and is accompanied by moisture loss. The processes of change in the heat and moisture are interrelated. Based on the experiments conducted on legumes (different varieties of bean, white lupin), a mathematical model of these processes has been developed and the appropriate non-linear differential equations have been used. The obtained equations cannot be solved either analytically or numerically, because some of the coefficients are unknown. The equations are solved based on certain assumptions. Based on these assumptions, the paper provides the calculation of the change in temperature and moisture content over time in legumes, such as bean and white lupin. The obtained results have been compared with experimental data.

**Keywords:** IR radiation; heat treatment; bean; legume grain; dehydration; moisture content

## INTRODUCTION

The heat treatment, in particular, with the use of the radiation (infrared) energy input, is a fairly common operation in the grain processing technological processes. This is the method that is most frequently used by large enterprises producing instant cereals and cereal flakes and is known as high-temperature micronization **(Zverev, 2009)**. In the production of grain products, many methods of heat treatment (HT) of grain have been proposed, such as roasting, steaming, extrusion, micronization, etc. **(Zverev, Sesikashvili and Bulakh, 2017)**. In any case, the process is associated with the rising temperature of the product within a certain time interval. The use of electromagnetic radiation for heating and heat treatment of products is based on the phenomenon of the absorption of radiation incident on the product and conversion of its energy into heat **(Rogov and Nekrutman, 1986)**. When producing products from legumes, it is economically feasible to use heat-treated full-fat soybeans in livestock feeding. It has proven to be difficult to cut the cost of these products in other ways **(Shulaev and Betin, 2010)**. Since the unwanted components in soy are thermolabile components, it is acceptable to process them in a relatively inexpensive way-such as the thermal method, to increase the nutritional value of the soybean and to inactivate the unwanted components **(Perez-Maldonado, 2002)**. During the heating process, the product is dehydrated. The temperature and final moisture content of legumes during heating by infrared (IR) radiation are determined by several factors: heating time, initial moisture content of beans, and heat treatment conditions (irradiance, the ambient tempera¬ture in the working area) **(Zverev and Sesikashvili, 2018)**. Usually, these conditions in industrial facilities are dependent on their designs and rarely change during operation, even if this possibility exists. Moisture source raw material depends on its condition when delivered, storage conditions and can vary, sometimes widely. The outlet temperature of the product is regulated by its residence time in the working area **(Zverev and Sesikashvili, 2018)**. The heating process, as a rule, is carried out under a high thermal head and is limited to the time when the grain surface begins to darken. The product temperature changes continuously throughout the entire period of treatment that is the process is essentially non-isothermal **(Lykov and Mikhailov, 1972)**.

This work aimed to determine the experimental relationships and develop a model of heating legumes while varying moisture, irradiance and the ambient temperature in the heat treatment zone of infrared heating installations for legumes, as well as to establish the optimal heat treatment conditions to improve the nutritional value of legumes.

### Scientific hypothesis

There is a correlation dependence of moisture content on the temperature of the product, as well as the influence of the above-listed factors on it. The temperature and final moisture content of legumes during heating by infrared (IR) radiation are determined by several factors: heating time, initial moisture content of beans, and heat treatment conditions (irradiance, the ambient temperature in the working area).

## MATERIAL AND METHODOLOGY

During experiments for heat treatment (HT) of the grain, a QP1 model (Elcer, Odesa, Ukraine) panel for halogen quartz emitters was used as a source of infrared (IR) radiation (see Figure 1). The dimensions of a panel for quartz emitter were 62 x 247 mm, rated power W = 1 kW, emitter temperature 750 °C.

By the nature of the structural design of the heat treatment zone, along with radiation heat exchange, convective heat transfer occurs between pulse product and air, while the convective heat transfer is more intense, the higher the temperature in the working area. The upper limit of this temperature is determined by the technical capabilities of the structural elements.

The ambient temperature in the heat treatment zone is included in all mathematical models of the heating process. Moreover, in several works, it was assumed that this temperature is proportional to the irradiance, $\Delta T_c = K_c E$, which required experimental verification.

The possibility to change the emitter height $h_l$ above the product allowed to vary the irradiance.

The linear dimensions of the bean were determined using an electronic digital caliper VINCA DCLA-0605 Electronic Digital Caliper, 150 mm. (AMAZON.CO. UK, Tbilisi, Deliver to Georgia).

The surface temperature of the sample-weight of beans was measured under the middle lamp of the emitter was determined as follows: the bean was arranged on the pallet in a monolayer, which, for a fixed amount of time (30, 60, 90, and 120 s) was placed in a heated IR treatment zone. Then the pallet was quickly removed and the temperature of beans was determined using an AR360A+ infrared laser thermometer (IntellSafe, Shenzhen cheerman technology CO., LTD, Guangdong, China), the temperature measurement interval was -50 – +360 °C, and the accuracy was 0.5 °C Error in the temperature due to heat loss when the bean moves from the treatment zone to the measurement area is insignificant.

The temperature inside the beans was determined using a thermocouple with a wire diameter of 0.1 – 0.2 mm, which was connected to a DT 9208A (ANENG, Nanchang Huatuo Industrial Co., Ltd. China) digital multimeter (the temperature measurement range was -40 – +1370 °C) (see Figure 2). To that end, the grain was drilled through until its midpoint with a drill 1 mm in diameter, and then a thermocouple was inserted. The temperature was recorded every 30 s. To minimize the effect of direct radiation on the readings, the thermocouple was placed in a tube made of a material with a reflection coefficient of R = 0.9 in a wavelength range of λ = 1μm.

The initial moisture content of grain was determined using an electronic digital meter of grain and seed moisture (moisture meter) VSP-100 (Proautotools, Kharkiv, Ukraine).

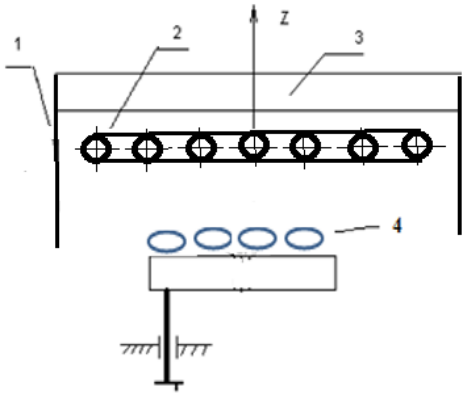


**Figure 1** Scheme for (The IR panel QP1-type) measuring the temperature of a monolayer of beans: 1 – Lateral reflective screens; 2 – block of emitters; 3 – Upper reflective screen; 4 – Monolayer of beans on a stage.

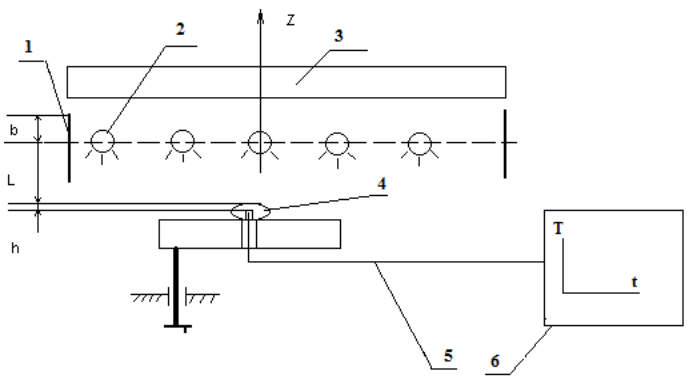


**Figure 2** Temperature measurement design of a single bean: 1 – The lateral reflecting barriers; 2 – A QP1 model IR linear generator; 3 – An upper reflecting barrier; 4 – A bean on the object stage; 5 – Thermocouple (Ni-Cr, Ni-Al); 6 – The registering device.

The moisture loss during IR heating was estimated as the difference between the initial sample weight and its weight after heat treatment.

The sample weight of the bean before and after heat treatment was determined using an SF – 400C (Banggood, China, Kyiv, Deliver to Ukraine), model electronic digital analytical balance, with a weighing accuracy of 0.01 g. The moisture content (%), after heat treatment, was calculated based on the initial moisture content and mass loss according to the formula derived by prof. Zverev based on **GOST 13586.5 (2015)** and **ISO 3166 (2015)**.

$$W = 100\,[W_0/100 - \Delta m/m_0]/[1 - \Delta m/m_0] \quad (1)$$

Where:

$W_0$ – initial moisture, %; $m_0$ – initial sample weight, g; $\Delta m$ – sample weight loss, g.

## Statistical Analysis

Non-linear modeling was carried out by using an application software package "STADIA-6", developed at the M. V. Lomonosov Moscow State University **Kulaychev (1999)**. Scoping the adequacy of the models is a complex procedure, requiring high computational costs, which are rapidly growing with dimensions of space of external parameters. By the volume, this task may greatly exceed the task of parametric optimization of a model itself (especially in the case of a nonlinear model), that's why for the newly-designed objects, it may not be resolved. Some indication of the adequacy of the models is provided by the squared multiple correlations, $R^2$. To describe the ordered sample, we used statistical functions of the average arithmetic value and the average standard error. We selected the value of reliability $p < 0.05$.

## RESULTS AND DISCUSSION

The author recommends the method of high-temperature micronization (HTM) - heating in infrared fluxs for the heat treatment of food products and legumes to obtain products with a higher nutritional value **(Zverev, 2009; Pan and Atungalu, 2010)**. The author provides data obtained during heat treatment of soybeans in a laboratory facility with infrared (IR) emitters KGT-220-1000 **(Zverev and Kozin, 2008)**. The authors consider the method of high-temperature micronization (HTM) - heating in an infrared stream to obtain functional food products and increased shelf life **(Zverev and Zvereva, 2006)**. Infrared (IR) heating provides significant advantages over conventional heating, including reduced heating time, uniform heating, reduced quality losses, absence of solute migration in food material, versatile, simple, and compact equipment, and significant energy saving. Infrared heating can be applied to various food processing operations, namely, drying, baking, roasting, blanching, pasteurization, and sterilization **Lee (2020)**. The authors **(Pan, Venkitasamy and Li, 2016)** note that Infrared heating is used on an industrial and pilot scale for baking (roasting), drying, defrosting, pasteurizing, blanching, and peeling. For each of these operations, infrared heating equipment is either being used commercially or is currently in pilot development. We use infrared radiation to improve the nutritional value of legumes. The authors **Žilić et al., (2009)** argue that micronization of corn (at a temperature of 140 °C within 40 s) did not affect the standard chemical composition flour from white, yellow, and red grains to a large extent. However, a slight decrease in ash, protein, and cellulose content was found in micronized red corn flour. Crude protein changes the content was insignificant, reduced solubility of some fractions indicated changes in the structure of the protein. Micronization negatively affects biologically active compounds (tocopherols, b-carotene) is natura¬lly found in raw grains. This thermal process increased antioxidant activity and changed the pastel properties of the selected corn flour samples. Due to the reduced POD levels in micronized flour samples are expected to be increased storage stability. Despite the modified diet and technological characteristics, micronized flour is a good raw material for gluten production-free products. The authors note **Arntfield et al. (1997)** the use of micronization (infrared treatment) on grain legumes tempered with water has the potential for reducing cooking times. Using Laird lentils, the effects of tempering conditions and moisture content following micronization were evaluated in terms of color, chemical properties, and texture after 15 min cooking. The texture of cooked lentils softened as tempering moisture increased. This was related to increasing starch gelatinization and decreasing protein solubility during micronization. IR heating for food and agricultural processing holds great potential for improved processing efficiency and product quality. The significant attractive merits of IR heating over conventional heating methods for various food-processing applications include high heat delivery rate, no need for heating medium, reduced processing time, improved energy efficiency, and enhanced product quality and safety without the significant negative environmental footprint. Selective heating can result in higher-quality food products that are safe for consumption **(Pan and Li, 2014)**. The authors confirm that a simple infrared system consists of an emitter, which is a heat source (infrared lamp) and a reflector (used for temperature control). The efficiency of infrared equipment mainly depends on the type of heat source used. The emitter determines the color of the light, the wavelength of the process radiation, the process temperature, and the power density. Electric and gas heaters are commonly used for process heating **(Vaidyanathan and Krishnamurthy 2020)**. The described IR equipment converges to our installation. The authors note **Niu, Classen and Scott (2003)** that five experiments were conducted to study the effects of micronizing, tempering, and flaking of wheat (cv. Genesis) on the chemical characteristics and its feeding value for broiler chicks. Samples of wheat were micronized (an infrared invisible band with wavelengths of 1.8 to 3.4 μ) at 90, 105, and 120 °C for 50 s, with or without tempering to 20% moisture, and with or without flaking. Micronization at 120 °C produced negative effects on weight gain and feed efficiency (0 – 18 days). Tempering reduced protein solubility in 0.2% KOH, increased the solubility of dietary fibre, and increased in vivo digesta viscosity and in vitro extract viscosity as well as diet AME. Flaking wheat did not affect broiler performance in experiments 3 and 4, but decreased growth rate and feed efficiency in the experiment. The authors note that IR heating has demonstrated the advantage of efficient heat transfer to food products by reducing processing time and energy costs. The major obstacles to the adoption of most IR technologies in the food

industry are the lack of in-depth understanding of IR heating fundamentals and the capital cost required in the initial setup by replacing the existing conventional processing equipment **Erdoğdu et al. (2011)**. The authors note **Kayitesi et al. (2013)** that background cowpea is mostly utilized as cooked whole seeds. This is often achieved only after boiling for up to 2 h, resulting in high energy consumption and a long time for food preparation. Micronisation of pre-conditioned cowpeas reduces their cooking time. Micronisation changes the physicochemical properties of cowpea seeds, which may affect the sensory properties of cooked cowpeas. Consumer acceptance and utilization of micronized cowpeas depend on their sensory properties. The authors **(Ogundele and Kayitesi, 2019)** emphasize the importance of using infrared heating as a method of shortening the cooking-time of African legume seeds and flour, by increasing their water absorption rates and pasting viscosity is emphasized in the literature. Structural changes caused by infrared heating of moisture-conditioned African legumes include microstructural, molecular, and interaction of the biomolecules in the seeds.

The authors **Sakare et al. (2020)** provide a critical discussion of the principle, applications, and comparative performance of IR energy for drying a range of food materials including grains, fruits, vegetables, and seafood in the recent past. The effects of process variables on energy consumption, drying time, rate of drying, and quality of the dried product are explained in detail.

Using animal food with lupine, the authors **Tuśnio et al. (2020)** note that the animals fed diets with high lupin levels had significantly smaller bodyweight gain ($p = 0.001$) than those fed diets with low lupin level aside from the micronizing process.

The authors provide also comparative data on the effectiveness of one or another method of heat treatment of legumes. It has been established that the efficiencies of thermo--wet, infrared, and extrusion treatment in the final result are comparable – it's all a question of the cost price **(Shurchkova, Ganzenko and Radchenko, 2007)**.

The authors claim that the treatment method significantly affects the level of exchange energy (including due to the inactivation of anti-nutritional substances). For example, if raw soybeans have an exchange energy level of 13.5 MJ.kg$^{-1}$, then after wet extrusion the level of exchange energy for chickens is 17.9 MJ.kg$^{-1}$, dry extrusion – 17.4 MJ.kg$^{-1}$, micron-zation – 15.4 MJ.kg$^{-1}$ and for roasting – 15.6 MJ.kg$^{-1}$ **(Shershunov, Cherviakov and Kurzenkov, 2004)**.

The paper describes the method of heat treatment with a radiation energy supply (high-temperature micronization or IR heat treatment), the main directions of heat treatment of legumes and cereals have been determined. The high-temperature micronization with IR rays has been adopted as a major method **Kozin (2011)**.

The measurement of the temperature and moisture content of grain was done at three levels of the height of the emitters set above the monolayer of grain (100 mm, 75 mm, and 50 mm) and at three values of the initial moisture content. The experimental results are presented in Tables 1-3.

## Changes in the temperature of beans during the process of IR heat treatment

The influence of the length of exposure of IR heat treatment on the temperature increment of the sample weight of the „Tsanava" bean variety at the emitters' panel height of 100 mm is shown in Figure 3.

Influence of the length of exposure of IR heat treatment on the temperature increment of the sample weight of the „Tsanava" bean variety at the emitters' panel height of 75 mm is shown in Figure 4.

The influence of the length of exposure of IR heat treatment on the temperature increment of the sample weight of the „Tsanava" bean variety at the emitters' panel height of 50 mm is shown in Figure 5.

The results obtained are in good agreement with the results obtained for soybeans and cereals in the work **Kozin (2011)**.

The authors **(Semwal and Meera, 2020)** investigated the change in the properties of grain starch when exposed to infrared radiation.

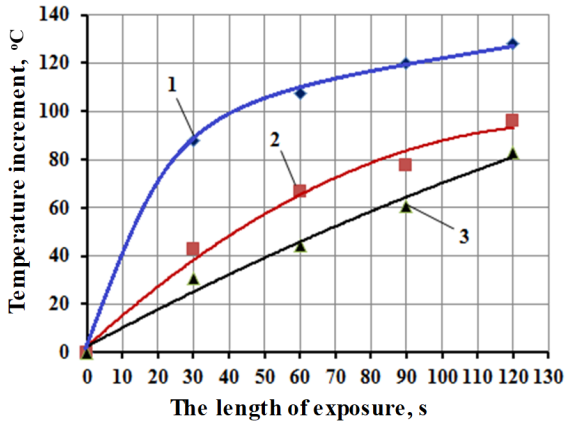


**Figure 3** The influence of the length of exposure of IR heat treatment on the temperature increment of the sample weight of „Tsanava" bean variety at the height of 100 mm from the QP1 model emitters set to the pallet, with the initial average moisture contents, %: 1 – 12.7; 2 – 17.7; 3 – 29.2.

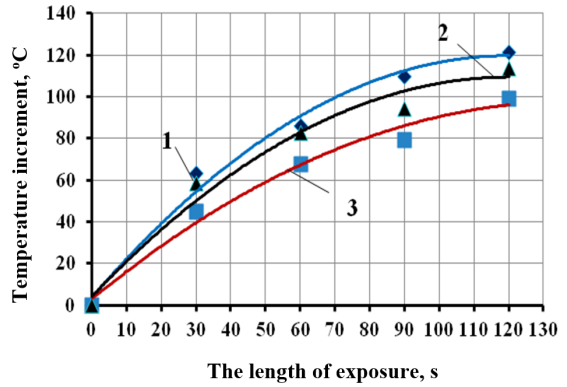


**Figure 4** The influence of the length of exposure of IR heat treatment on the temperature increment of the sample weight of „Tsanava" bean variety at the height of 75 mm from the QP1 model emitters set to the pallet, with the initial average moisture contents, %: 1 – 12.7; 2 – 17.7; 3 – 29.2.

**Table 1** „Tsanava“ bean variety.

| Time, sec | Initial temperature, ºC | Initial moisture content W% | Final temperature, ºC | Final moisture content, W% |
|---|---|---|---|---|
| 0 | 30.4 | 18.5 | 30.4 | 18.4 |
| 30 | 30.5 | 18.6 | 73.4 | 14.4 |
| 60 | 29.8 | 17.0 | 96.3 | 16.9 |
| 90 | 30.4 | 17.1 | 108.1 | 16.9 |
| 120 | 31.4 | 17 | 127.0 | 16.6 |

**Table 2** „Field read“ bean variety.

| Time, sec | Initial temperature, ºC | Initial moisture content W% | Final temperature, ºC | Final moisture content, W% |
|---|---|---|---|---|
| 0 | 27.2 | 31.25 | 27.2 | 31.1 |
| 30 | 28.1 | 29.8 | 65.1 | 25.8 |
| 60 | 27.5 | 30.5 | 84.6 | 24.9 |
| 90 | 26.8 | 30.2 | 98.1 | 24.2 |
| 120 | 26.6 | 29.8 | 114.5 | 23.6 |

**Table 3** White lupin beans.

| Time, sec | Initial temperature, ºC | Initial moisture content W,% | Final temperature, ºC | Final moisture content, W% |
|---|---|---|---|---|
| 0 | 27.8 | 17.5 | 28.0 | 17.3 |
| 30 | 27.9 | 19.6 | 78.2 | 16.4 |
| 60 | 28.4 | 21.5 | 108.0 | 13.0 |
| 90 | 27.8 | 24,5 | 109.7 | 13.3 |
| 120 | 27.9 | 19.1 | 125.5 | 11.7 |

The effects of changes in the structural characteristics of starch on mechanical and functional properties, as well as on the characteristics of the digestibility of starch were discussed. They attempted to present the theoretical foundations of the interaction of infrared radiation with grain and its components.

Micronization and pre germination of peas before milling resulted in changes to flour particle size, co¬lor, and compositional and functional properties of the flours. Peas tempered to 18% – 20% and micronized to 105 – 110°C produced flour that was similar in baking properties to the flour milled from untre¬ated peas except for crumb firmness and aroma and flavor of the bread **(Frohlich et al., 2019)**.

The authors **(Deepa and Hebbar, 2017)** evaluated the effectiveness of micronization of corn (Arjun, EH-434042) in inactivating enzymes respon¬sible for lipid breakdown and extending the shelf life of whole corn flour. Micronization at 200 °C for 4 min led to complete inactivation of the peroxidase enzyme and a decrease in lipase activity by 84%. The results showed no significant change in the free fatty acid content of the micronized flour, whereas a significant increase was observed in the raw flour under both storage conditions. The color and carotenoid content of cornflour did not differ greatly during storage. Whereas micronized cornmeal was stored for 60 and 120 days under accelerated and ambient conditions, respectively, without significant impact on quality, the shelf life for raw cornmeal under similar conditions was only 15 and 30 days.

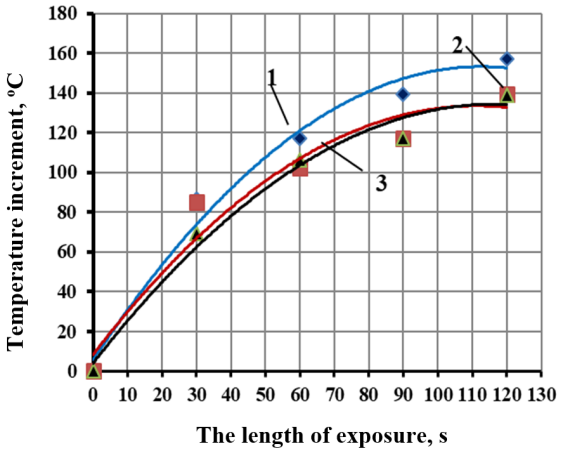


**Figure 5** The influence of the length of exposure of IR heat treatment on the temperature increment of the sample weight of „Tsanava“ bean variety at the height of 50 mm from the QP1 model emitters set to the pallet, with the initial average moisture contents, % :1 – 12.7; 2 – 17.7; 3 – 29.2.

## Changes in the moisture content of bean during the IR heat treatment process

The influence of the length of exposure of IR heat treatment on the moisture content of the sample weight of the „Tsanava“ bean variety at the different heights shown in Figure 6 – 8.

The results obtained are in good agreement with the results obtained for soybeans and cereals in the work **Kozin (2011)**.

The authors **Campos-Vega et al. (2018)** pay special attention to the post-harvest processing of dry beans necessary to ensure high-quality cooking, storage, and food safety. It also provides details on low-energy technologies as well as new processes for developing functional food ingredients from dry beans concerning composition and nutritional changes and their impact on human health and well-being.

## Process modeling during heat treatment

### *A lumped model of heating*

As the simplest model, a first-order differential equation is used, reflecting the heat balance as a whole for the caryopsis, in the form as follows:

$$CMd(\Delta T)/dt = P_E + P_k - P_d \quad (2)$$

$$\Delta T = T - T_0$$

Where:

$P_E$ – absorbed radiation power, W; $P_k$ – convective heat transfer power, W; $P_d$ – power spent on desorption of moisture (evaporation), W; T – the average current temperature, K; $T_0$ – the initial temperature of the product and the ambient temperature, K; C – specific heat capacity, $J.kg^{-1}$; M – the mass of caryopsis, kg.

Radiation power absorbed by the product:

$$P_E(t) = K_s S_1 AE(t) \quad (3)$$

Where:

$S_1$ – the surface area of caryopsis, $m^2$; A – absorption factor; $K_S$ – coefficient of proportionality; E(t) – irradiance (a function of time in general), $W.m^{-2}$; t – treatment time, s.

Convective heat transfer power with the environment:

$$Pk = K_\alpha S_1[\Delta T_c(t) - K_g \Delta T] \quad (4)$$

$$\Delta Tc(t) = T_c(t) - T0$$

Where:

$K_\alpha$ – coefficient that takes account of heat exchange medium, W/K; Kg – coefficient that takes account of the difference between the average temperature and the temperature on the surface; Tc(t) – ambient temperature,°C (a function of time in general).

Power spent on desorption of moisture (evaporation):

$$P_d = K_d(\lambda + \varepsilon/\mu)dm_в/dt \quad (5)$$

Where:

$m_в$ – the mass of moisture, kg; $\lambda = (2.503 - 2.255).10^6$ – the latent heat of free water evaporation, $J.kg^{-1}$; ε – adsorption potential, $J.mol^{-1}$; $\mu = 0{,}018$ – molar mass of vapor, $kg.mol^{-1}$; $K_d$ – coefficient of proportionality.

In the general case, during the heating process, neither the mass of caryopsis M (as a result of moisture evaporation), nor the heat capacity C, nor the heat transfer coefficient $K_\alpha$, nor the coefficient Kg remains constant due to their dependence on the current temperature and moisture content.

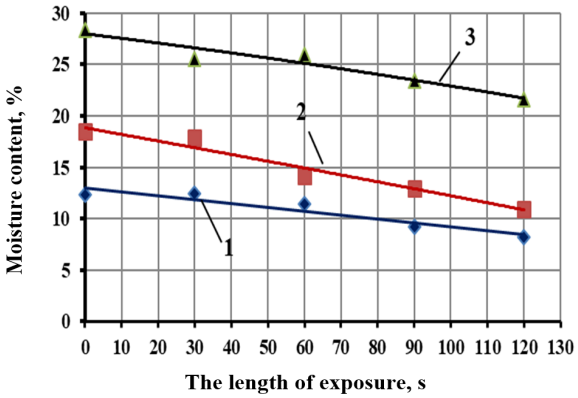


**Figure 6** The influence of the length of exposure of IR heat treatment on the moisture content of the sample weight of „Tsanava" bean variety at the height of 100 mm from the QP1 model emitters set to the pallet, with the initial average moisture contents1 – 12.7; 2 – 17.7; 3 – 29.2.

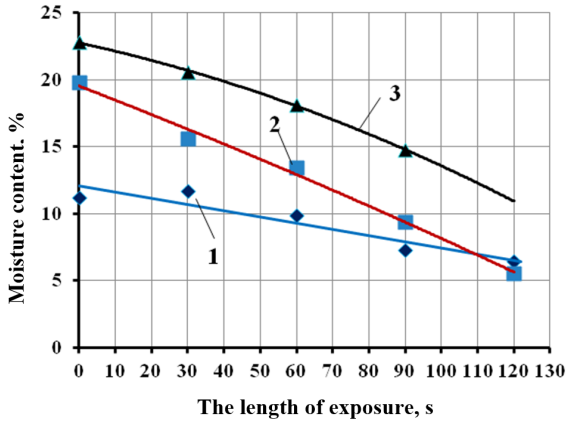


**Figure 7** The influence of the length of exposure of IR heat treatment on the moisture content of the sample weight of „Tsanava" bean variety at the height of 75 mm from the emitters' panel to the pallet, with the initial average moisture contents, %1 – 12.7; 2 – 17.7; 3 – 29.2.

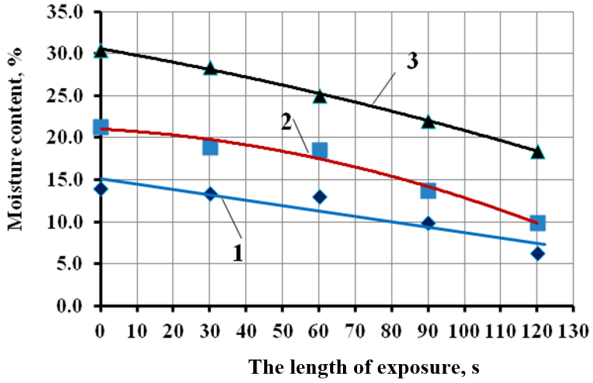


**Figure 8** The influence of the length of exposure of IR heat treatment on the moisture content of the sample weight of „Tsanava" bean variety at the height of 50 mm from the emitters' panel to the pallet, with the initial average moisture contents: 1 – 12.7; 2 – 17.7; 3 – 29.2.

The moisture content of the product undergoing treatment also affects the absorption factor A, with the adsorption potential ε.

The processes of heat and moisture exchange are interrelated and, in the general case, are described by a system of corresponding non-linear differential equations. Essentially, in the case of taking into account heat losses due to evaporation, and in the general case, other exothermic reactions as well, occurring when the product is heated, to determine the temperature in a function of time, it is necessary to solve the system of non-linear differential equations:

$$CMd(\Delta T)/dt = S_1\{K_sAE(t) + K_\alpha[\Delta T_c - K_g\Delta T] - Kd(\lambda+\varepsilon/\mu)dm_в/dt\},$$
$$dm_в/dt = -f_W[T(t), m_в] \quad (6)$$

$$C(T)[M+m_в(t)]d(\Delta T)/dt = S_1\{K_sAE(t) + K_\alpha [\Delta T_c - K_g\Delta T] - Kd(\lambda+\varepsilon/\mu)\, dm_в/dt\},$$
$$dm_в/dt = - W[T(t), m_в] \quad (7)$$

$$d(\Delta T)/dt + S_1\ K_\alpha Kg\Delta T/C(T)[M+m_в(t)] = \{S_1[K_sAE(t) + K_\alpha\Delta T_cT - Kd(\lambda+\varepsilon/\mu)dm_в/dt]\}/C(T)[M+m_в(t)] \quad (8)$$

The function $f_W[T(t),m_в]$ is non-linear and reflects the process of moisture evaporation. Usually, it is not possible to solve the system of equations (6) in an analytical form. The use of numerical methods is also practically impossible since the number of coefficients included in the equations is not determined. Therefore, we have to resort to various kinds of assumptions and simplifications, often rather rough ones.

Suppose that:

$$S_1K_\alpha Kg/C(T)[M+m_в(t)] = Kt = const,\ \{S_1[K_sAE(t) + K_\alpha\Delta T_cT - Kd(\lambda+\varepsilon/\mu)\ dm_в/dt]\}/C(T)[M+m_в(t)] = a_1 = const \quad (9)$$

Where:

$\Delta T = T- T_0$, T – temperature, °C; $T_0$ – initial temperature, °C; $T_\infty$ – temperature, °C when $t \to \infty$; t – time, s;

$$\Delta T_\infty = T_\infty - T_0$$

In the case of the constancy of these quantities, we have for the initial conditions $\Delta T(0) = 0$, the model of heating can be represented as:

$$\Delta T(t) = K_0[1 - \exp(-K_t t)] \quad (10)$$

Or

$$t(\Delta T) = -\ln[1-\Delta T/K_0]/K_t \quad (11)$$

Where:

$K_0 = \Delta T_\infty$, °C and Kt, $c^{-1}$ – the empirical coefficients.

The coefficients are taken as constant (independent of time), but dependent on the heating conditions (initial moisture content, irradiance, and the ambient temperature in the treatment zone). The condition of constancy (weak change) of the coefficients is not satisfied in the full temperature range. It is known from experiments that close to a product temperature of 100 ºC, intensive dehydration begins, and the rate of moisture loss sharply increases. Therefore, the proposed dependence can be considered to be an initial, rather rough approximation.

A similar solution can be obtained using the solution obtained by A.V. Lykov in the form of the exponential series, taking the first two terms **(Lykov and Mikhailov, 1972)**

$$\Delta T/(T_\infty - T_0) = 1 + a_1 \exp(-k_1 t) + a_i \exp(-k_i t) \quad (12)$$

Where:

a and k are coefficients.

Note that when identifying the parameters of models (10) for the temperature increment and (11) for time, according to the same experimental data, the numerical values of $K_0$ and $K_t$ in the expressions will differ.

## A model that takes account of the influence of moisture content

Typically, in industrial HTM installations, the irradiance and the ambient temperature that characterize the energy activity of the medium, are derived from the design of the treatment zone and are constant. Treatment time and product moisture content are varying.

Taking into account the prevailing influence of moisture content on coefficient $K_0$ the model (10) can be represented as:

$$\Delta T(t) = K_0W^C\ [1 - \exp(-K_t t)] \quad (13)$$
$$\Delta T(t) = K_0(1-W)^C\ [1 - \exp(-K_t t)]$$
$$\Delta T(t) = K_0WC^W\ [1 - \exp(-K_t t)]$$

Where:

C – empirical coefficient; W – moisture content, %.

## A model of dehydration

The authors **(Heydari, Kauldhar and Meda, 2020)** present the characteristics of drying lentils using thin layer microwave drying with and without hot air pre-drying. The characteristics were evaluated in a laboratory microwave dryer. Drying experiments were carried out at 300 and 750 W, and the pre-drying experiment was carried out at room temperature (23 °C). Of several thin-layer mathematical models evaluated using experimental data, Page's model was found to be the most appropriate model for predicting the drying process of lentils.

The authors **(Deepa and Hebbar, 2016)** note that when cereals/legumes with sufficient moisture are micronized, some beneficial changes are observed, such as partial gelatinization of starch, inactivation of enzymes that are responsible for degradation, and denaturation of anti-nutritional factors. Partial gelatinization due to micronization improves starch digestibility and palatability and shortens cooking time without significantly affecting other nutrients present in the beans.

**Table 4** The results of identification of coefficients of a model (13) for the „Tsanava“ bean variety.

| The distance between the emitter and product, H mm | Initial moisture content W,% | K0 | Kt | C | The squared pair correlation coefficient, R2 |
|---|---|---|---|---|---|
| 100 | 13-24 | 2602 | 0.0239 | -1.14 | 0.96 |
| 75 | 11-23 | 203 | 0.0184 | -0.182 | 0.96 |
| 50 | 14 -30 | 282 | 0.0242 | -0.218 | 0.98 |

**Table 5** The results of identification of coefficients of a model (13) for the „field red“ bean variety.

| The distance between the emitter and product, H mm | Initial moisture content W,% | $K_0$ | $K_t$ | C | The squared pair correlation coefficient, $R^2$ |
|---|---|---|---|---|---|
| 100 | 17.5-35.5 | 364 | 0.0126 | -0.31 | 0.93 |
| 75 | 14.1-28.4 | 192 | 0.0156 | -0.129 | 0.98 |
| 50 | 10.5-29.6 | 232 | 0.0237 | -0.159 | 0.98 |

**Table 6** The results of identification of coefficients of a model for (13) for lupin.

| The distance between the emitter and product, H mm | Initial moisture content W,% | $K_0$ | $K_t$ | The squared pair correlation coefficient, $R^2$ |
|---|---|---|---|---|
| 100 | 11 | 143 | 0.0103 | 0.99 |
| | 20 | 101 | 0.0233 | 0.99 |
| | 24 | 84 | 0.0248 | 0.98 |
| 75 | 11 | 157 | 0.0194 | 0.98 |
| | 17 | 130 | 0.0207 | 0.99 |
| | 34 | 128 | 0.0155 | 0.98 |
| 50 | 13 | 160 | 0.0251 | 0.99 |
| | 20 | 144 | 0.0193 | 0.98 |
| | 28 | 120 | 0.0197 | 0.99 |

**Table 7** The results of identification of coefficients of a model for (10) ($\Delta T(t) = K_0 W^C [1 – \exp(-K_t t)]$) for lupin.

| The distance between the emitter and product, H mm | Initial moisture content W,% | $K_0$ | $K_t$ | C | The squared pair correlation coefficient, $R^2$ |
|---|---|---|---|---|---|
| 100 | 11-24 | 142 | 0.0181 | -0.109 | 0.96 |
| 75 | 11-34 | 304 | 0.189 | -0.270 | 0.98 |
| 50 | 13-28 | 567 | 0.0208 | -0.465 | 0.98 |

We shall use the relationship (12). We shall confine ourselves to the first approximation, which, after substituting the model for the heating time (10) and the transformations, leads to a model for the relative current moisture content, as a function of a temperature increment:

$$U/U_0 = (1 – \Delta T/k_0)^C, \quad (14)$$

Where:
$\Delta T$ – temperature increment; $k_0$ and C – empirical coefficients.

The value of the parameter k0 can be taken from the results of the identification of the heating model parameters or, just as the parameters B and C, may be identified by the results of dehydration experiments. Identification of the parameters B and C by the results of the experiments with a wide variation in the initial moisture content, irradiance, and temperature in the treatment zone, has shown a dependence on the initial moisture content and the absence of correlation with heating modes.

As a result, a model is proposed

$$U/U_0 = (1 – \Delta T U_0/K_0)^C \quad (15)$$

Where:
U0 – initial moisture content.

However, it should be borne in mind that this model describes adequately the process of dehydration to an extent limited by the upper-temperature value of grain not much more than 100 ºC.

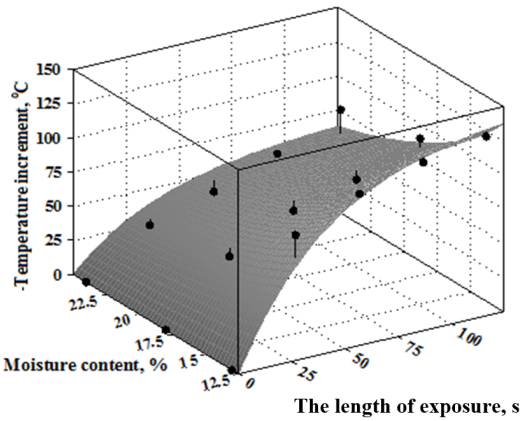


**Figure 9** The „Tsanava“ bean: The height of the emitters panel H=100 mm; Model $\Delta T(t) = K_0 W^C [1 – \exp(-K_t t)]$.

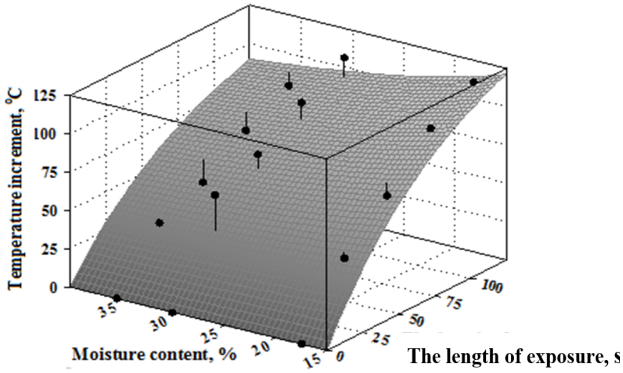


**Figure 10** The „field red“ bean: The height of the emitters panel H=100 mm; Model-$\Delta T(t) = K_0 W^C [1 – \exp(-K_t t)]$.

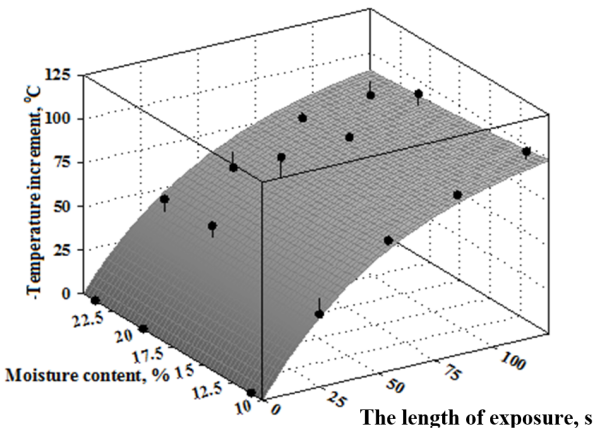


**Figure 11** The „White lupin“ beans: The height of the emitters panel H=100 мм; $\Delta T(t) = K_0 W^C [1 – \exp(-K_t t)]$.

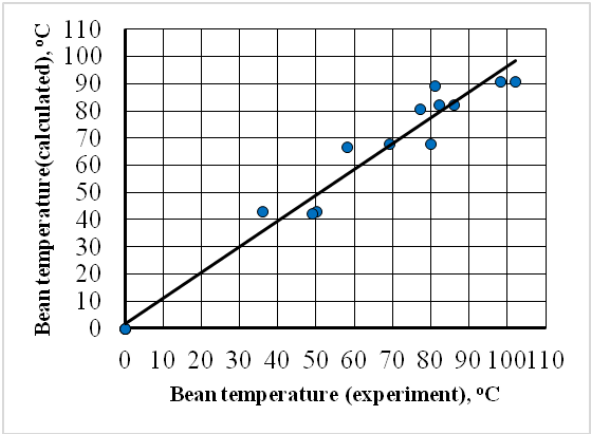


**Figure 12** The height from the emitters' panel to the pallet, H=100 mm.

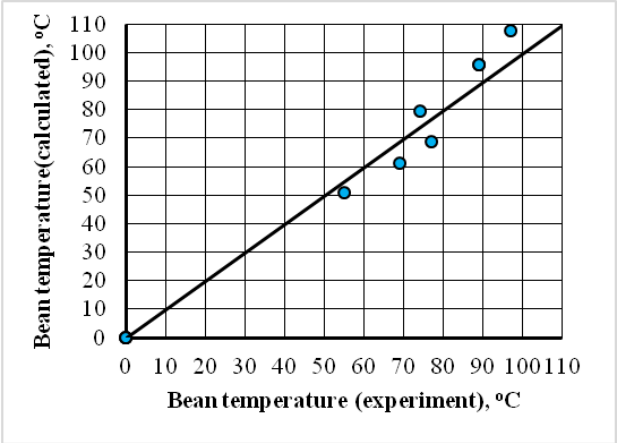


**Figure 13** The height from the emitters' panel to the pallet, H=75 mm,

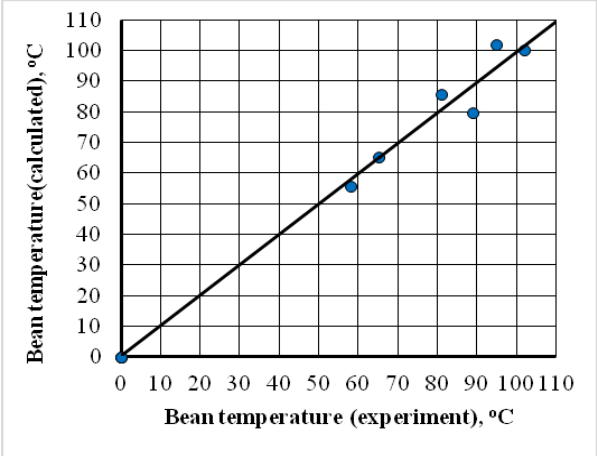


**Figure 14** The height from the emitters' panel to the pallet, H=50 mm.

From the presented graphs (Figure 6, Figure 7, and Figure 8) and earlier obtained results for barley and millet, it can be assumed that the model describes adequately experimental data on the small-sized (3 – 5 mm) objects.

As can be seen in Table 6 and Table 7, none of the models considered has any significant advantages. Hence, hereinafter, we will focus on the first of them.

Table 6 clearly shows the correlation $K_0(W)$. A similar effect was noted in the work **(Zverev and Sesikashvili, 2016)**.

The results of experiments and calculations are shown in Figure 9, Figure 10, and Figure 11.

Based on the accepted theory, a program for calculating the basic quantities was designed using a mathematical

package MathCad (Mathsoft, Cambridge, Massachusetts, USA) **(Makarov, 2011)**.

The accuracy of temperature estimates according to the proposed model ($DT(t) = K_0WC\ [1 - \exp(-K_t t)]$ can be judged following Figure 12, Figure 13, and Figure 14.

As is seen in Figure 12, Figure 13, and Figure 14 the calculated temperature values are in satisfactory agreement with the experimental values.

## CONCLUSION

Regardless of accepted assumptions, the models considered describe quite well the growth of the bean temperature during IR heating in the range of 30 – 100 ºC, at a higher moisture content.

An increase in the proportion of radiative heat input, due to a decrease in the distance between the radiator and the product's monolayer, leads to an increase in the irradiance and, consequently, to a reduction in the heating time to the setpoint temperature.

An increase in the initial moisture content of beans with unchanged other modes of HTM leads to an increase in temperature and, accordingly, to a reduction in the heating time to the fixed temperature. For a particular HTM installation, the proposed heating models allow to identify its coefficients through a series of simple experiments and then to use it for adjusting the heat treatment conditions.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE EFFECT OF TEMPEH FLOUR FROM TREE BEAN (*PARKIA TIMORIANA* (DC) MERR.) SEEDS ON LIPID PROFILE IN PROTEIN-ENERGY MALNUTRITION RATS

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## ABSTRACT
Children with protein-energy malnutrition (PEM) need a food product that can be easily absorbed by the body because they have impaired nutrient utilization and lipid profile disorder. The provision of fermented products such as tempeh is an alternative approach. Seeds of tree bean (*Parkia timoriana* (DC) Merr.) are a good source of various nutrients needed by children with PEM. This study aimed to prove the effectiveness of the administration of tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds on the lipid profile in PEM rats. PEM in rats was induced by a zero-protein diet containing granulated sugar (20 g), vegetable oil (1 g), and cornstarch (24 g). The animals were categorized into a normal control group (K+), a PEM group without treatment (K−); a group fed tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds 1.5 g.kg$^{-1}$ BW (P1); and a group fed tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds 3 g.kg$^{-1}$ BW (P2). The intervention was administered for 30 days. There was a significant increase in HDL levels ($p$ = 0.001) and significant decreases in cholesterol ($p$ = 0.001), triglyceride ($p$ = 0.001), LDL levels ($p$ = 0.001), and atherogenic index of plasma (AIP) ($p$ = 0.000) in treatment groups. In conclusion, the administration of tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds increases HDL levels and decreases cholesterol, triglycerides, and LDL level, and atherogenic index of plasma (AIP) in rats with PEM.

**Keywords:** atherogenic index of plasma; lipid profile; protein-energy malnutrition; tempeh flour; tree bean

## INTRODUCTION
Protein-energy malnutrition (PEM) is defined as malnutrition in which energy and protein intake are under the nutritional adequacy rate **(Adriani and Wirjatmadi, 2016; Data and Information Center Ministry of Health Indonesia, 2015)**. It occurs because of a lack of consumption of foods containing calories and protein and the presence of infectious diseases that affect nutrient utilization **(Adriani and Wirjatmadi, 2016)**.

The highest prevalence of PEM in children under 5 years of age **(Adriani and Wirjatmadi, 2016)**. The prevalence of stunted and wasted children in 2019 in the world was 21.3% and 6.9%, respectively **(UNICEF, 2020)**. Based on Riskesdas 2018, in Indonesia, the prevalence of malnutrition and undernutrition was about 3.9% and 13.8%, respectively **(Center Ministry of Health Indonesia, 2018)**.

Undernutrition and the presence of infectious diseases can aggravate the diseases and worsen nutritional status. Poor nutrition in children leads to slow growth and affects cognitive abilities **(UNICEF, 2020)**. PEM causes hypoalbumin and decreases the immune system **(Hoffer, 2001)**. Also, this condition affects the lipid profile in which children who are malnourished will have high levels of triglycerides, LDL, and cholesterol and low HDL levels **(Alves et al., 2014; Velásquez and Cano, 2012)**.

Children with PEM need food products that are easily absorbed by the body because of impaired utilization of nutrients. Fermented foods contain nutrients that are easily absorbed by the intestines; one of the fermented foods is tempeh **(Masdarini, 2011)**. Tempeh is consumed by Indonesians, as a functional food. It is typically made from soybeans using Rhizopus sp. as its starter **(Cempaka et al., 2018)**.

Tree bean (*Parkia timoriana* (DC) Merr.) is a plant that is still classified as a legume or Leguminosae. Tree bean seeds are high in protein, minerals, essential amino acids, and fatty acids **(Mohan and Janardhanan, 1993)**. Several studies have demonstrated that tree bean seeds have anti-proliferative, antibacterial, and antioxidant functions. **(Angami et al., 2018)**. This study aimed to prove the effectiveness of the administration of tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds on the lipid profile in rat models of zero-protein-diet-induced PEM.



**Figure 1** Tree bean (*Parkia timoriana* (DC) Merr) seeds.



**Figure 2** Peeling the black part of tree bean seeds.



**Figure 3** Inoculation.



**Figure 4** Tempeh from tree bean seeds after fermentation 48 h.



**Figure 5** Drying tempeh.

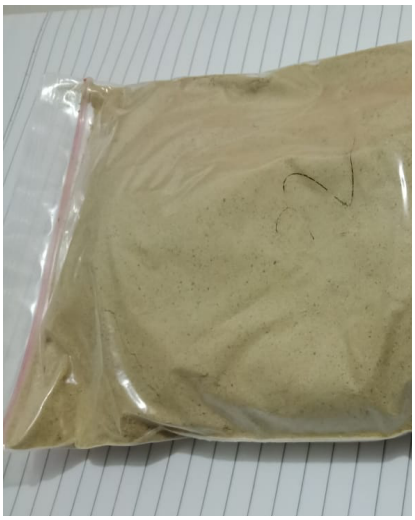

**Figure 6** Tempeh flour from tree bean seeds.

### Scientific hypothesis

Tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds increases HDL levels and decreases cholesterol, triglycerides, and LDL level and atherogenic index of plasma (AIP) in rats with PEM.

## MATERIAL AND METHODOLOGY

This study is included in the research project Study of the Administration of Tempeh Flour from Tree Bean (*Parkia timoriana* (DC) Merr.) Seeds in Nutritional Status, Hematological Profile, Immune Status, and Metabolic Response on PEM Rats.

### Samples

This study used tempeh flour from tree bean seeds as samples. Tree bean seeds (Figure 1) were obtained from suppliers in Klaten, Central Java, Indonesia.

### Animals

Male Wistar rats (8 – 12 weeks old, 150 – 200 g, n = 24), were acquired from the Centre for Food and Nutrition of Universitas Gadjah Mada, Yogyakarta, Indonesia.

### Laboratory Methods

Albumin and hemoglobin level measurements as criteria of PEM were performed after 14 days of zero-protein diet administration. Measurements of cholesterol, triglyceride, LDL, and HDL levels were performed before the intervention and at the end of the intervention. Blood sampling was done to analyze albumin, hemoglobin, cholesterol, triglyceride, LDL, and HDL through the plexus retroorbital. Examination of triglyceride used Glycerol 3 Phospate Oxidase-Peroxidase Amino Phenazone (GPO-AP) method, meanwhile cholesterol, LDL and HDL used Cholesterol Oxidase-Peroxidase Aminoantipyrine Phenol (CHOD-PAP) with enzymatic spectrophotometric basis. The atherogenic index of plasma (AIP) was calculated from the log (triglyceride/HDL). Blood serum was analyzed in the Centre for Food and Nutrition of Universitas Gadjah Mada, Yogyakarta, Indonesia.

### Description of the Experiment

#### *The Processing of Tempeh from Tree Bean Seeds*

The seeds were washed and soaked in water for 2 h. Then, the soaking water was removed, and the seeds were washed thoroughly. Tree bean seeds were boiled in water for 2 h. Then, the water was removed and tree bean seeds were drained. Tree bean seeds were peeled (the black part) as seen in Figure 2 and soaked in boiled water for 18 h (the water was changed regularly every 6 h). Tree bean seeds that had been soaked, drained, and boiled for 30 min were then removed and drained again. Tree bean seeds were aerated to dry and then split into two pieces. The tempeh starter was given to tree bean seeds and mixed well. The mixture was placed in plastic bags with a small hole on two opposite sides (Figure 3). It was then left for two nights until mushrooms appeared on the tempeh and began to solidify (Figure 4).

#### *Processing of Tempeh Flour from Tree Bean Seeds*

Tempeh from tree bean seeds was sliced using a slicer and then blanched with hot steam for 2 min. Next, tempeh was dried by oven at 60 °C for 6 h (Figure 5), ground with a grinder, and then sieved using a 60 mesh sieve (Figure 6). Tempeh flour was stored in airtight containers. The process was carried out at the Centre for Food and Nutrition of Universitas Gadjah Mada, Yogyakarta, Indonesia.

#### *Animal Treatments*

The animals were individually housed and provided a standard feed of Comfeed II at 15 g per rat per day and water ad libitum. Rats were randomly divided into four groups (n = 6/group): the normal control group (K+), the PEM group without treatment (K−), the group fed tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds at 1.5 $g.kg^{-1}$ BW (P1), and the group fed tempeh flour from tree bean (*Parkia timoriana* (DC) Merr.) seeds at 3 $g.kg^{-1}$ BW (P2). All animals, except the K+ group animals, were fed a zero-protein diet for 14 days. This diet contained granulated sugar (20 g), vegetable oil (1 g), and cornstarch (24 g) and was administered orally at 10 g per rat per day. PEM was defined when the rats had albumin <3 $g.dL^{-1}$ and hemoglobin <10 $mg.dL^{-1}$.

### Statistical Analysis

Statistical analyses were performed using the IBM SPSS Statistic 22 software. Data are presented as mean ±SD or median. A paired *t*-test and a 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 U test was used, as appropriate, for non-parametric results.

## RESULTS AND DISCUSSION

The average body weight of the animals significantly increased in all the groups ($p$ <0.05), as seen in Table 1. The P1 group (29(29-31) g) experienced the highest weight gain after the administration of tempeh flour from tree bean seeds compared to the P2 group (27.5(25-28) g). K+ (33(31-35) g) and K− (10(7-12) g) groups that were not given any treatment experienced a significant increase in body weight ($p$ <0.05). Bodyweight gain is due to the nutrient content in the feed. Protein and fat content in tempeh flour from tree bean seeds may increase body weight in rats with PEM.

The results of collaboration research showed that the rats acquired PEM after the administration of the zero-protein diet for 14 days. Rats achieved PEM with albumin (1.21 – 1.33 $g.dL^{-1}$) and hemoglobin (11.86 – 12.13 $g.dL^{-1}$) **(Ulfa, 2020)**. In the previous study, the provision of a zero-protein diet for 2 weeks in rats indicates decreases in albumin and hemoglobin levels **(Anggraeny et al., 2016)**. PEM causes hypoalbuminemia where albumin levels are under a normal value <3.5 $g.dL^{-1}$. Hypoalbuminemia leads to impairment of albumin synthesis and other proteins by the liver **(Murray et al., 2003)**.

A significant difference in triglyceride was found after the administration of tempeh flour from tree bean seeds in the K+ ($p$ = 0.001), K− ($p$ = 0.036), P1 ($p$ = 0.001), and P2 ($p$ = 0.056) groups, as seen in Table 2. Significantly decreased triglyceride levels were found in the P1 and P2 groups, implying that two doses of tempeh flour from tree bean seeds could significantly decrease triglyceride levels in rats with PEM. Based on Table 3, there was a significant decrease in total cholesterol in the K+ ($p$ = 0.028), K− ($p$ = 0.028), P1 ($p$ = 0.028), and P2 ($p$ = 0.028) groups after the administration of tempeh flour from tree bean seeds.

**Table 1** The average body weight before and after treatments.

| Groups | Bodyweight (g) | | | |
|---|---|---|---|---|
| | Pre | Post | Δ | *p* |
| K+ | 124(120-128) | 156(154-161) | 33(31-35) | 0.026* |
| K- | 99(97-104) | 109(107-113) | 10(7-12) | 0.027* |
| P1 | 100(99-103) | 130(128-132) | 29(29-31) | 0.020* |
| P2 | 102.5(98-105) | 129(126-132) | 27.5(25-28) | 0.026* |
| $P^1$ | 0.002* | 0.000* | 0.000* | |

Note: normal control group (K+), PEM group without treatment (K-), tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 1.5 g.kg$^{-1}$ BW group (P1), and tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 3 g.kg$^{-1}$ BW group (P2); *p* = Wilcoxon test, $p^1$ = Kruskal Wallis test; * = significant ($p$ <0.05)

**Table 2** Triglycerides level before and after the administration of tempeh flour from tree bean seeds.

| Groups | Triglycerides (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Pre | Post | Δ | *p* |
| **K+** | 67.8 ±2.089$^{b,c,d}$ | 69.29 ±2.58$^{b,c,d}$ | 1.44 ±0.517$^{c}$ | 0.001* |
| **K-** | 105 ±3,7276$^{a}$ | 109 ±3.88$^{a,c,d}$ | 3.87 ±3.32$^{c}$ | 0.036* |
| **P1** | 105 ±2,888$^{a}$ | 80.82 ±2.46$^{a,b,d}$ | -24.45 ±3.68$^{a,b,d}$ | 0.001* |
| **P2** | 103 ±5,83$^{a}$ | 95.61 ±3.059$^{a,b,c}$ | -7.94 ±784$^{c}$ | 0.056* |
| ***p*$^1$** | 0.001* | 0.001* | 0.001* | |

Note: normal control group (K+), PEM group without treatment (K-), tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 1.5 g.kg$^{-1}$ BW group (P1), and tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 3 g.kg$^{-1}$ BW group (P2); *p* = Wilcoxon test, $p^1$ = Kruskal Wallis test; * = significant ($p$ <0.05).

**Table 3** Total cholesterol levels before and after the administration of tempeh flour from tree bean seeds.

| Groups | Total cholesterol levels (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Pre | Post | Δ | *p* |
| **K+** | 74.82(71.53-76.64) | 76.57(72.8-78.66)$^{b,c,d}$ | 1.75(0.97-2.54)$^{c,d}$ | 0.028* |
| **K-** | 1.56(151.82-167.88) | 1.59(155.65-168.2)$^{a,c,d}$ | 2.2(0.32-8.01)$^{c,d}$ | 0.028* |
| **P1** | 1.50(146.72-155.47) | 93.31(91.21-97.91)$^{a,b,d}$ | -55.44(-64.26-(-51.73))$^{a,b}$ | 0.028* |
| **P2** | 1.54(146.28-157.66) | 1.03(99.58-107.11)$^{a,b,c}$ | -49.04(-57.24-(-42.53))$^{a,b}$ | 0.028* |
| ***p*$^1$** | 0.001* | 0.001* | 0.001* | |

Note: normal control group (K+), PEM group without treatment (K-), tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 1.5 g.kg$^{-1}$ BW group (P1), and tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 3 g.kg$^{-1}$ BW group (P2); *p* = Wilcoxon test, $p^1$ = Kruskal Wallis test; * = significant ($p$ <0.05).

**Table 4** LDL levels before and after treatments tempeh flour from tree bean seeds.

| Groups | LDL levels (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Pre | Post | Δ | P |
| **K+** | 21.11(18.69-23.53) | 22.38(20.98-25.17)$^{b,c,d}$ | 0.94(0.22-3.69)$^{c,d}$ | 0.028* |
| **K-** | 56.05(54.67-58.13) | 57.34(53.85-59.44)$^{a,c,d}$ | 0.59(-0.82 -1.99)$^{c,d}$ | 0.172 |
| **P1** | 56.06(53.29-57.44) | 27.62(23.78-30.77)$^{a,b,d}$ | -27.74(-33.66-(-25.98))$^{a,b,d}$ | 0.028* |
| **P2** | 55.02(53.98-60.9) | 36.01(33.57-37.76)$^{a,b,c}$ | -20.05(-25.24-(-16.22))$^{a,b,c}$ | 0.028* |
| **p$^1$** | 0.004* | 0.001* | 0.001* | |

Note: normal control group (K+), PEM group without treatment (K-), tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 1.5 g.kg$^{-1}$ BW group (P1), and tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 3 g.kg$^{-1}$ BW group (P2); *p* = Wilcoxon test, $p^1$ = Kruskal Wallis test; * = significant ($p$ <0.05).

**Table 5** HDL levels before and after treatments tempeh flour from tree bean seeds.

| Groups | HDL levels (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Pre | Post | Δ | *p* |
| **K+** | 79.25(76.87-81.63) | 77.775(72.03-80.46)$^{b.c.d}$ | -1.34(-6.2-(-0.24))$^{c.d}$ | 0.028* |
| **K-** | 46.26(44.22-51.02) | 46.36(43.68-48.28)$^{a.c.d}$ | -0.5(-2.74-1.16)$^{c.d}$ | 0.249 |
| **P1** | 45.24(42.86-47.62) | 66.285(64.37-70.5)$^{a.b.d}$ | 21.55(20.32-22.88)$^{a.b.c}$ | 0.028* |
| **P2** | 44.9(42.86-47.62) | 58.62(55.17-61.3)$^{a.b.c}$ | 12.57(9.85-18.44)$^{a.b.d}$ | 0.028* |
| ***p*$^1$** | 0.003* | 0.001* | 0.001* | |

Note: normal control group (K+), PEM group without treatment (K-), tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 1.5 g.kg$^{-1}$ BW group (P1), and tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 3 g.kg$^{-1}$ BW group (P2); *p* = Wilcoxon test, $p^1$ = Kruskal Wallis test; * = significant ($p$ <0.05).

**Table 6** AIP before and after treatments tempeh flour from tree bean seeds.

| Groups | AIP (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Pre | Post | Δ | *p* |
| **K+** | -0.07±0.01 | -0.048±0.03 | 0.02(0.01-0.05) | 0.048* |
| **K-** | 0.35±0.01 | 0.38±0.01 | 0.03±0.01 | 0.001* |
| **P1** | 0.36(0.35-0.41) | 0.09±0.02 | -0.28±0.01 | 0.027* |
| **P2** | 0.36(0.34-0.42) | 0.21±0.03 | -0.13(-0.25-(-0.11)) | 0.027* |
| ***p*$^{1}$** | 0.004* | 0.000* | 0.000* | |

Note: normal control group (K+), PEM group without treatment (K-), tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 1.5 g.kg$^{-1}$ BW group (P1), and tempeh flour from seeds of *Parkia timoriana* (DC) Merr., 3 g.kg$^{-1}$ BW group (P2); *p* = Wilcoxon test, $p^1$ = Kruskal Wallis test; * = significant ($p$ <0.05).

The highest decrease in total cholesterol occurred in the P1 group, followed by that in the P2 group. LDL levels in the K+ ($p$ = 0.028), P1 ($p$ = 0.028), and P2 ($p$ = 0.028) groups showed significant differences after the administration of tempeh flour from tree bean seeds. Meanwhile, there was no significant difference in LDL levels in the K− group ($p$ = 0.172) before and after treatment (Table 4). Significantly decreased LDL levels were found in the P1 and P2 groups after treatment. A significant difference was observed in HDL levels after treatment in the K+ ($p$ = 0.028), P1 ($p$ = 0.028), and P2 ($p$ = 0.028) groups. There was no significant difference in the K− ($p$ = 0.249) group. HDL levels in the P1 group were higher than in the P2 group, as seen in Table 5.

Atherogenic index of plasma (AIP) in K+ ($p$ = 0.048), K− ($p$ = 0.001), P1 ($p$ = 0.027) and P2 ($p$ = 0.027) groups showed significant differences after treatments (Table 6). AIP in P1 and P2 groups decreased significantly after the administration of tempeh flour from tree bean seeds. Significantly decreased triglyceride levels and increased HDL levels in the P1 and P2 groups caused AIP in both groups to decrease. AIP is the strongest marker to predict cardiovascular disease (CVD) risk (Khazaál, 2013). Malnutrition worsens the prognosis of heart failure **(Amare, Hamza, and Asefa, 2015)**.

Total cholesterol, triglyceride, and LDL levels increased in rats with PEM; meanwhile, HDL levels decreased compared to the normal control group. Chronic malnutrition causes endocrine changes leading to metabolic profile disorders. Previous studies demonstrated that 98.9% of children have dyslipidemia with low HDL and hypertriglyceridemia **(Veiga et al., 2010)**. A previous study that was conducted on preschool children indicated that malnourished children had lower HDL levels than normal children, and higher triglyceride levels were found in malnourished children **(Velásquez and Cano, 2012)**.

Tree bean seeds in this study were processed into fermented food as tempeh **(Masdarini, 2011)**. Fermentation can increase protein digestibility through the breakdown of complex proteins into a more soluble form **(Nkhata et al., 2018)**. Besides that, tempeh is one of the functional foods that have long been consumed by Indonesians **(Cempaka et al., 2018)**. Tree bean seeds contain rich protein (albumin and globulin), minerals (magnesium, potassium, phosphorus, zinc, iron, and manganese), essential amino acids (phenylalanine, leucine, isoleucine, and tyrosine), and fatty acids such as linoleic acid and oleic acid **(Mohan and Janardhanan, 1993)**. Tree bean seeds contain a thioproline compound that gives them a distinctive odor **(Thangjam and Sahoo, 2012)**. Thioproline is an antioxidant compound and a free radical scavenger. These compounds inhibit the production of nitroso compounds by reducing bacteria and the reaction of nitrogen species **(Kumagai, 2003)**.

In this study, the administration of tempeh flour from tree bean seeds reduced triglycerides, cholesterol, and LDL levels and increased HDL levels in PEM rats. These results are consistent with previous studies, in which, the administration of tempeh flour decreased total cholesterol in high-saturated fat-diet induced rats **(Ridwan, 2015)**. The provision of black soybean tempeh and yellow soybean tempeh decreased LDL cholesterol **(Priastiti and Puruhita, 2013)**. The administration of ethanol extract of tree bean seeds in hyperlipidemia rats could reduce triglycerides, cholesterol, and LDL levels and increase HDL levels **(Dewi, 2018; Fitriani, 2018)**. In a previous study, the administration of a tree bean seed extract decreased high levels of triglycerides and total cholesterol **(Sheikh et al., 2016)**. Flavonoids in tree bean seeds have a hypolipidemic effect that can reduce cholesterol, triglyceride, and LDL levels and increase HDL levels **(Bao et al., 2016; Dubey et al., 2020)**. Flavonoids can inhibit the expression of fatty acid synthase in the liver, which is stimulated by AMPK activity on hepatocyte cells through the kinase B1 pathway **(Pil Hwang et al., 2013)**. Besides, flavonoids reduce fatty acid synthesis in the liver and fat accumulation **(Do et al., 2013)**.

The content of essential amino acids found in tree bean seeds also plays a role in reducing triglyceride, cholesterol, and LDL levels in PEM rats. A previous study explained that the provision of essential amino acid supplements and phytosterols are taken 3 times a day for 4 weeks can reduce triglyceride, cholesterol, and LDL levels in the subject **(Coker et al., 2015)**. The administration of a protein-rich mucuna product in hyperlipidemic rats reduced LDL cholesterol, total cholesterol, and triglyceride levels. Proteins have hypolipidemic activity due to the presence of secondary metabolites, such as phytates and polyphenols. Polyphenols induce metabolic hypolipidemia with their ability to reduce cholesterol acyltransferase and HMG-CoA reductase activity **(Ngatchic et al., 2016)**. The administration of leucine and valine in high-fat-diet rats for 8 weeks can increase HDL cholesterol levels **(Cojocaru et al., 2012)**.

Tree bean seeds contain a thioproline compound that produces a distinctive odor in tree bean seeds. Thioproline contains amino acids and is a natural metabolite that acts as an intracellular sulfhydryl antioxidant and a free radical

scavenger and protects cell membranes from damage caused by oxygen derivatives **(Cavallini et al., 1956)**. Thioproline that binds to proteins by hydrogen bonds and van der Waals forces exhibits a strong inhibitory effect. Thioproline is also effective in protecting against oxidative damage in rat brain tissue **(Lyu et al., 2020)**. Antioxidants play a role in decreasing LDL levels and increasing HDL levels through several mechanisms, such as activating the transcription factor NrF2 and increasing antioxidant enzymes, as well as neutralizing oxidative species and inhibiting the activation of the nuclear transcription factor-κB (NF-κB) signaling pathway **(Ademuyiwa et al., 2005; Brunzell et al., 2008; Hegazy et al., 2019; Oršolić and Car, 2014; Oršolić et al., 2019)**. Thioproline in dietary supplements can protect the body from diseases associated with oxidative stress, such as atherosclerosis **(Ham, Jason Chan and Chan, 2020)**.

## CONCLUSION

The administration of tempeh flour from tree bean (*Parkia timorina* (DC) Merr.) seeds increases HDL levels and decreases cholesterol, triglycerides, LDL level, and atherogenic index of plasma (AIP) in rats with PEM with the most effective dose is 1.5 g.kg$^{-1}$ BW.

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The authors declare no conflict of interest.

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The experiments were approved by The Ethical Committee of Medical Research of the Faculty of Medicine, Universitas Diponegoro (no. 22/EC/H/FKUNDIP/IV/2020), Indonesia.

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# THE STUDY OF THE VARIABILITY OF MORPHOBIOLOGICAL INDICATORS OF DIFFERENT SIZE AND WEIGHT GROUPS OF HYBRID SILVER CARP (*HYPOPHTHALMICHTHYS SPP.*) AS A PROMISING DIRECTION OF DEVELOPMENT OF THE FISH PROCESSING INDUSTRY

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## ABSTRACT

In water reservoirs, the size and characteristics of fish, in the first place, affect – the composition and clarity of food objects, the number of food competitors and predators, their numbers, industry, and others. Besides, the rate of linear and weight growth are the arteries that determine the value of the species as an object of commercial fishing. The hybrid of silver and bighead carp easily adapts to different growing conditions (from the point of view of the formation of fish-biological indicators), protein in different years depending on feeding conditions, in one reservoir indicators of length and body weight of ribs are inadequate. Evaluating the influence of feeding conditions on the linear growth of the rib, the method of generalized evaluation was used. An indicator equal to the sum of potential fish productivity for phyto-zooplankton was used to characterize the foraging status of a particular condition in a certain period. In terms of the quality of growth indicators, the indicator of the absolute weight of nature with differentiation of farms, age groups, and years was used. The results of the research revealed significant variability in the size and weight characteristics of different groups of silver and bighead carp, which were isolated from ponds and reservoirs. According to the results of experimental studies established for the cultivation of hybrid silver and bighead carp in reservoirs, relatively high rates of linear and weight growth are expected, higher than for similar species, which is provided in the conditions of fish ponds.

**Keywords:** fish; length; mass; pond; reservoir

## INTRODUCTION

Due to the deterioration of the conditions of natural reproduction of populations of industrial fish species, aquaculture is becoming increasingly important in Ukraine as a crucial factor in meeting the needs of mankind in protein food **(Archibisova and Suslov, 2018)**. As fish and fish products occupy a significant place in human nutrition, fish receive about 40% of protein foods of animal origin mainly from aquatic organisms **(Jennings, 2016)**.

Ukraine has all the opportunities for efficient fisheries, increasing domestic production through the development of fish farming, which over the past 5 years, in contrast to the dynamics of industrial catches is characterized by certain stability and provides annual production of 20 – 25 thousand tons of fish products. At the same time, there is a steady trend to increase the share of freshwater grazing aquaculture products in a total catch in inland waters, which has increased to 30 – 35% over the past 10 years **(Kozakevych, 2017; Mushtruk et al., 2020)**.

In modern conditions of fish farming mainly polyculture of carp and herbivorous fish is used, which provides a balanced consumption of the whole complex of forage organisms and the formation of maximum fish productivity. The use of herbivorous fish cannot be limited to growing them only in ponds, these species are no less promising for reservoirs and other complex reservoirs. The technology of commercial fish production in small reservoirs due to grazing aquaculture involves obtaining the bulk of fish products by optimizing the use of natural feed resources, optimally selected species composition of introducers, which allows us to consider this area as a resource-saving and environmentally friendly technology **(Orel, 2020)**.

One of the promising objects of cultivation in reservoirs of different types is a hybrid of silver and bighead carp because it has a rapid rate of weight gain until the end of the growing season. High plasticity in the choice of food objects (phytoplankton, zooplankton, detritus, and in the ponds, also – the remnants of the dusty fraction of carp feed) allows

hybrids to have much less competition than other species **(Marenkov, 2018)** (Figure 1).

Hypophthalmichthys is a valuable commercial fish with a scrumptious taste. The meat is tender and delicious, belongs to the group of medium-fat **(Buchtová and Ježek, 2011; Jia et al., 2018)**. In terms of calories, it is inferior to grass carp and common carp, but its value increases with age, with increasing linear size of fish, the meat becomes fatter. The fat content of meat varies from 4.5 to 29.0%, a significant amount of it accumulates on the internal organs of fish. A large amount of fat in the body of the silver carp allows it to adapt to starvation in winter and early spring.

Due to the peculiarities of the diet of silver carp meat contains amino acids, polyunsaturated fatty acids of omega-3 and omega-6, with regular consumption, they help prevent the development of malignant tumors, nervous disorders, improve heart function, strengthen blood vessel walls, lower cholesterol and blood pressure in patients with hypertension **(Ferguson et al., 2020)**

### Scientific hypothesis

Morphometric parameters can serve as an integral characteristic of the living conditions of fish in a particular water body. At the same time, among the relevant studies, this area remains the least studied. Even though ichthyological work in ponds and reservoirs was previously widely carried out.

## MATERIAL AND METHODOLOGY

The research was conducted in the spring, summer, and autumn periods from 2017 to 2019 in ponds based on the training-research-production laboratory of fish farming of the National University of Life and Environmental Sciences (TRPLF NULES of Ukraine) of Ukraine, village Nemishayevo, Kyiv region (zone Polissya); State Enterprise "Experimental Farm" Nyvka " of the Institute of Fisheries of the National Academy of Agrarian Sciences (SEEF "Nyvka" IF NAAS) of Ukraine, Kyiv is located on the border of the zones (it is on the river Nivka that the Forest-Steppe is divided - to the south and Polissya - to the north); Bila Tserkva Experimental Hydrobiological Station of the Institute of Hydrobiology of the National Academy of Sciences (BEHS IHB NAS) of Ukraine, Bila Tserkva (Forest-steppe zone), Kosiv, Kyiv region (Forest-steppe zone) and Velykoburlutsky, Kharkiv region (Forest-steppe zone) reservoirs.



**Figure 1** Two-year hybrid of white with motley thick-thyme, which was obtained in the autumn period during the oblah nahulna become No10 BEGS IGB NAS of Ukraine.



**Figure 2** Collection of ichthyological materials (the second anniversary of the hybrid of white with motley thick-haired) in the spring period during unloading of the wintering rate No1 of the NLDLR NUBIP of Ukraine.



**Figure 3** Conducting a morphometric analysis of two-years of the hybrid of white with motley thick-throes, which were obtained in the autumn period during the regional concubination system No1 NNVLR NUBIP of Ukraine.

**Samples**

The collection of ichthyological material was carried out during stocking and catching fish in ponds and reservoirs. The material for the study was young-of-the-year, annuals, biennials, and triennials of a hybrid of silver and bighead carp (Figure 2). The number of stocks in ponds was 6, and catches – 18; Velykoburlutsky Reservoir 1 stocking, catches – 2, Kosiv Reservoir – 2 each.

Phytoplankton sampling was performed by scooping water from 0.3 m surface water bodies into 0.5 $dm^3$ plastic containers. For preservation of samples 40% formaldehyde was added at the rate of 1 : 100.

Zooplankton samples were taken with an Apstein mesh (sieve No 72) in a container with a volume of 0.5 $dm^3$, while filtering 100 l of water, fixed with 4% formalin. During the research period, 30 samples of phytoplankton and zooplankton were taken from ponds (wintering and feeding).

**Chemicals**

Formaldehyde ($CH_2O$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Formalin (water solution formaldehyde, producer «Inter-Synthesis» Limited Liability Company, Ukraine).

**Animals and Biological Material:**

During the morphological analysis, 330 specimens of silver and bighead carp of different size and weight groups were caught from ponds, and 115 specimens from reservoirs were processed during the morphological analysis.

**Instruments**

Set of grids with a mesh step from 30 to 100 mm (producer "CrayFish" Limited Liability Company, Finland).

Electronic laboratory scales (TBE-0.15-0.001-a-2, producer «Inter-Synthesis» Limited Liability Company, Ukraine)

Technical electronic scales (BTHE-6-H1K-1, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Counting chamber of Najotta (producer "Laboratory equipment" Limited Liability Company, Ukraine).

Binocular microscope (XSP-139B LED Ulab, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Apstein's grid (producer "ADS-Lab" Limited Liability Company, Ukraine).

Bogorov counting chamber (producer "ADS-Lab" Limited Liability Company, Ukraine);

Stereoscopic microscope (MBS-9, producer "Laboratory equipment" Limited Liability Company, Ukraine).

**Laboratory Methods**

To achieve the task of the experiment used standard methods of morphometric analysis **GOST (1985)**.

Processing of ichthyological materials was performed according to standard methods generally accepted in ichthyology **(Sabaj, 2020)**.

Thickening of phytoplankton samples was performed by sedimentation.

Determination of the taxonomic composition of algae was carried out according to the determinants **(Mao et al., 2018)**.

Phytoplankton biomass was determined by the calculation-volume method **(Mao et al., 2018)**.

In-house processing of samples was carried out by the conventional hydrobiology counting-weight method in the Bogorov counting chamber under a stereoscopic microscope MBS-9.

Phytoplankton samples were examined in a special Najott counting chamber 0.01 $cm^3$ under a light microscope, all detected algae species were determined and counted at 1.0 $dm^3$.

Zooplankton organisms were identified to the species using determinants **(Mao et al., 2018)**.

To assess the impact of feeding conditions on linear growth, using the method of generalized estimation.

To characterize the forage productivity of a particular pond in a certain period used an indicator equal to the sum of potential fish productivity for phyto- and zooplankton. To characterize the growth of cultivated objects used the indicator of absolute weight gain with differentiation by age groups, years, and by farms.

**Description of the Experiment**

Over two years, 445 specimens of the white hybrid with variegated silver carp of different size and weight groups were studied. In ponds and Kosiv Reservoir, the number of

repetitions of each experiment was twofold, in Velykoburlutsky Reservoir - one-time.

### Statistic 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. We calculated the arithmetic mean (unweighted) value (M), the arithmetic mean error (± m), which made it possible to estimate with a certain probability the deviation of the arithmetic mean deviation, Fulton fatness rate. The statistical reliability of the results of the researches was provided by analyzing samples with the number of fish from 10 to 25 specimens.

## RESULTS AND DISCUSSION

After caught in the winter pond No 101 SEEF "Nyvka" IF NAAS of Ukraine, the value of the length and body weight of annual fish in 2017 were (min-max values): 116.0 – 204.0 mm, 12.50 – 90.80 g, and in 2018 – 160.0 –188.0 mm and 37.0 – 60.0 g (Table 1) **(Makarenko, Shevchenko and Sytnyk, 2018b)**.

The length of annual fish in 2017 after catching in the winter pond № 2 TRPLF NULES of Ukraine fluctuated within (min-max values) – 101.0 – 150.0 mm, with a variation in bodyweight – 8.10 – 30.70 g, and in 2018 – 90.0 – 111.0 mm, 5.90 – 11.40 g (Table 1).

The length and body weight of annual fish after catching in the winter pond No 14 BEHS IHB NAS of Ukraine in 2017 were (min-max values): 101.0 – 137.0 mm and 6.50 – 20.70 g, and in 2018 – 102.0 – 136.0 mm, 9.0 – 21.0 g (Table 1).

**Table 1** The average values of length and body weight of annuals of the hybrid of silver and bighead carp.

| Indexes | Pond No 101 | | Pond No 2 | | Pond No 14 | |
|---|---|---|---|---|---|---|
| | 2017 y. | 2018 y. | 2017 y. | 2018 y. | 2017 y. | 2018 y. |
| | M ±m | | M ±m | | M ±m | |
| Average body length, mm | 157.72 ±20.14 | 176.88 ±6.54 | 117.48 ±11.90 | 99.72 ±5.99 | 120.68 ±9.20 | 117.16 ±8.61 |
| Average body weight, g | 39.67 ±17.59 | 50.76 ±5.97 | 13.82 ±5.53 | 8.57 ±4.32 | 13.38 ±3.46 | 13.19 ±3.30 |
| Fulton fatness (K-factor) | 1.72 | 1.64 | 1.55 | 1.62 | 1.45 | 1.62 |

**Table 2** The average values of length and body weight of biennials of a hybrid of silver and bighead carp.

| Indexes | Pond No 2 | | Pond No 1 | | Pond No 10 | |
|---|---|---|---|---|---|---|
| | 2017 y. | 2018 y. | 2017 y. | 2018 y. | 2017 y. | 2018 y. |
| | M ±m | | M ±m | | M ±m | |
| Average body length, mm | 339.33 ±11.69 | 339.53 ±24.47 | 312.00 ±12.68 | 304.00 ±33.66 | 281.47 ±21.70 | 303.53 ±31.98 |
| Average body weight, g | 412.47 ±60.87 | 387.73 ±81.64 | 306.93 ±42.83 | 270.60 ±72.22 | 213.60 ±47.61 | 269.00 ±83.65 |
| Fulton fatness (K-factor) | 1.82 | 1.98 | 1.79 | 1.77 | 1.78 | 1.78 |

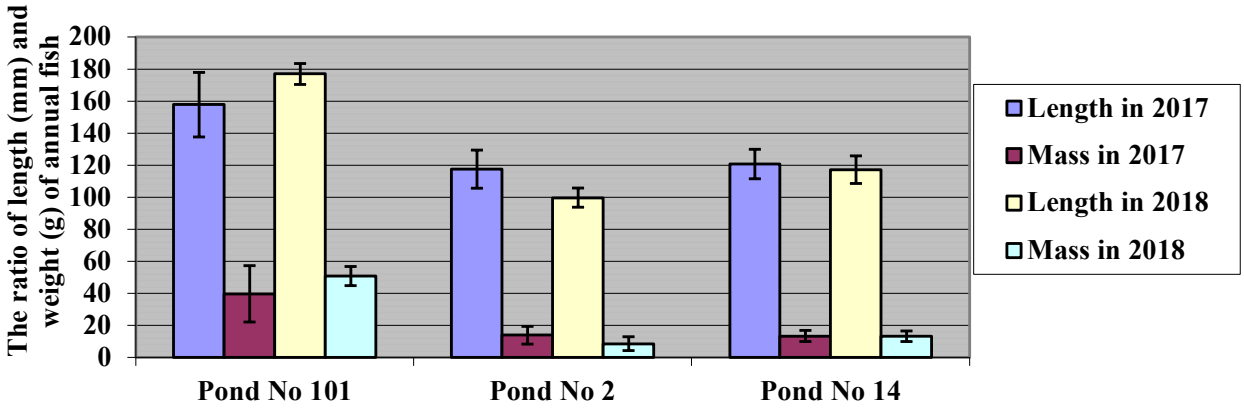


**Figure 4** The ratio of the average values of linear growth in length and weight of annuals of the hybrid of silver and bighead carp from the studied winter ponds in 2017 – 2018.

**Table 3** The average values of length and body weight of biennials of a hybrid of silver and bighead carp.

| Indexes | Pond No 119 | | Pond No 1 | | Pond No 13 | |
|---|---|---|---|---|---|---|
| | 2018 y. | 2019 y. | 2018 y. | 2019 y. | 2018 y. | 2019 y. |
| | M±m | | M±m | | M±m | |
| Average body length, mm | 388.80 ±27.37 | 340.40 ±54.49 | 332.73 ±26.95 | 316.47 ±20.96 | 301.67 ±39.54 | 252.07 ±28.38 |
| Average body weight, g | 637.40 ±125.65 | 407.25 ±187.08 | 368.47 ±113.07 | 327.07 ±89.33 | 256.33 ±105.05 | 142.18 ±48.89 |
| Fulton fatness (K-factor) | 1.84 | 1.83 | 1.76 | 1.68 | 1.76 | 1.71 |

**Table 4** The rate of linear-weight growth of a hybrid of silver and bighead carp in experimental ponds.

| Age group | Indexes | | SEEF "Nyvka" IF NAAS of Ukraine | | TRPLF NULES of Ukraine | | BEHS IHB NAS of Ukraine | |
|---|---|---|---|---|---|---|---|---|
| | | | 2017 – 2018 | 2018 – 2019 | 2017–2018 | 2018–2019 | 2017–2018 | 2018–2019 |
| 1-1+ | Linear growth rate | mm | 181.61 | 162.65 | 194.52 | 204.28 | 160.79 | 186.37 |
| | | % | 115.15 | 91.95 | 165.58 | 204.85 | 133.24 | 159.07 |
| 1+-2 | | mm | 49.47 | 0.87 | 20.73 | 12.47 | 20.20 | 0.51 |
| | | % | 14.58 | 0.26 | 6.64 | 4.10 | 7.18 | 0.83 |
| 1-1+ | Weight growth rate | g | 372.80 | 336.97 | 293.11 | 262.03 | 200.22 | 255.81 |
| | | % | 939.0 | 663.9 | 2120.0 | 3158.0 | 1596.0 | 2039.0 |
| 1+-2 | | g | 224.93 | 19.52 | 61.54 | 56.47 | 42.73 | – 126.82 |
| | | % | 54.53 | 5.03 | 20.05 | 20.87 | 20.00 | – 52.86 |

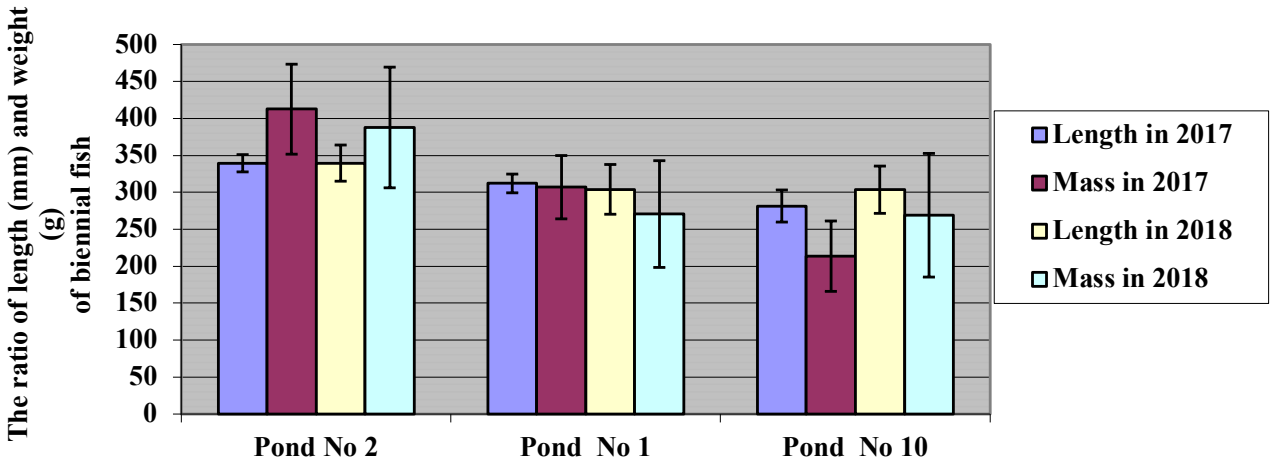


**Figure 5** The ratio of the average values of linear growth in length and weight of biennials of a hybrid of silver and bighead carp from the studied feeding ponds in 2017 – 2018.

The difference in the annual growth of a hybrid of silver and bighead carp (Figure 4) usually persists in their later lives, especially in the presence of competition between them for food, which is scarce **(Zebral, et al., 2018; Mion et al., 2018)**.

A similar trend of differences in biological parameters was observed in older age groups (biennials) of a hybrid of silver and bighead carp. In the autumn during the catch in the pond No 2 SEEF "Nyvka" IF NAAS of Ukraine indicators of length and weight of biennial fish in 2017 (min-max values) – 315.0 – 363.0 mm, 320.0 – 552.0 g, in 2018 – 283.0 – 362.0 mm, 212.0 – 463.0 g; in the pond № 1 TRPLF NULES of Ukraine in 2017 – 293.0 – 327.0 mm, 235.0 – 363.0 g, in 2018 – 264.0 – 398.0 mm, 175.0 – 414.0 g; in the pond No 10 BEHS IHB NAS of Ukraine in 2017 the indicators were in the range – 217.0 – 310.0 mm, 90.0 – 287.0 g, in 2018 – 266.0 – 378.0 mm, 171.0 – 458.0 g (Table 2).

In comparison with the hybrid of silver carp, the average standard weight of biennials grown on farms producing aquaculture products is white silver carp: in the Polissya zone – 300 g, forest-steppe zone – 350 g, variegated silver carp, respectively 350; 400 g **(Vdovenko, 2012; Vdovenko, 2013)**.

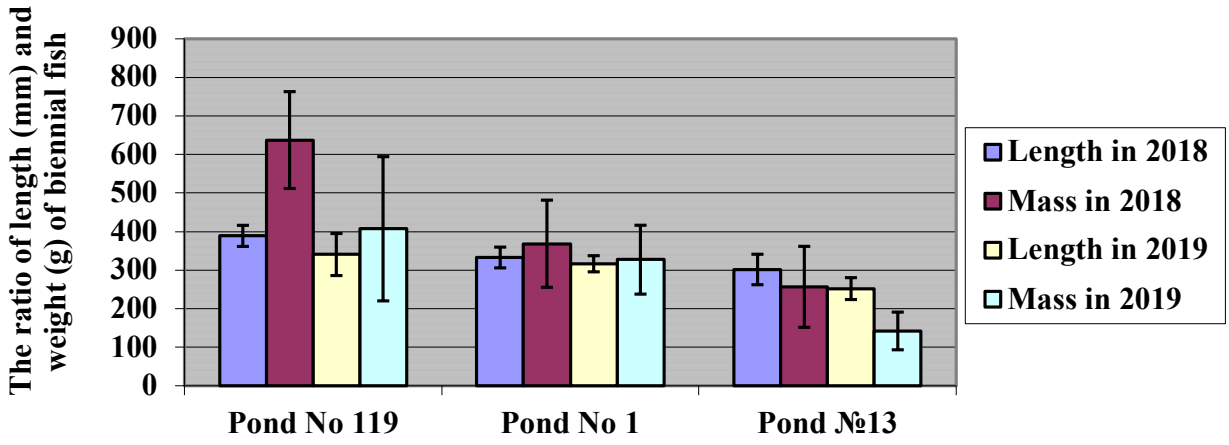


**Figure 6** The ratio of the average values of the linear growth of the length and weight of biennials of the hybrid of silver and bighead carp from the studied winter ponds in 2018 – 2019.

**Table 5** Performance by Phytoplankton and zoplankton,kg.ha$^{-1}$.

| | | DG "Nivka" IRG NAAN of Ukraine | | NNVLR NUBIP of Ukraine | | BEGS IGB NAS of Ukraine | |
|---|---|---|---|---|---|---|---|
| **Age groups** | | **1+** | **2** | **1+** | **2** | **1+** | **2** |
| 2017 y. | phytoplankton | 5.73 | 1.40 | 4.57 | 1.17 | 0.82 | 0.47 |
| | zooplankton | 1.75 | 0.47 | 1.00 | 0.30 | 1.30 | 0.70 |
| 2018 y. | phytoplankton | 0.94 | 0.67 | 0.48 | 0.71 | 1.40 | 8.10 |
| | zooplankton | 0.95 | 0.10 | 7.60 | 1.40 | 0.26 | 0.48 |
| 2017 y. | performance on the feed base | 164.60 | 41.43 | 119.97 | 31.97 | 53.54 | 29.40 |
| 2018 y. | performance on the feed base | 4.59 | 1.63 | 22.67 | 5.42 | 3.54 | 17.57 |

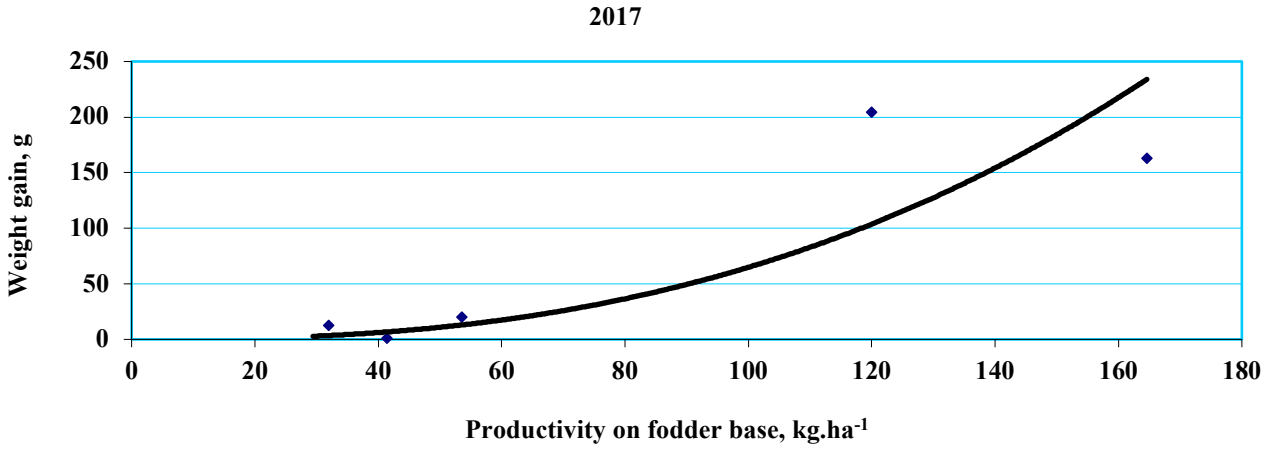


**Figure 7** The increase in the mass of younger age groups of the hybrid of silver and bighead carp from the potential productivity of the fodder base in 2017.

Figure 5 shows the ratio of the average values of linear growth in length and weight of biennials of a hybrid of silver and bighead carp.

In the spring during the catching in the winter pond No 119 SEEF "Nyvka" IF NAAS of Ukraine indicators of length and weight of biennials fish in 2018 fluctuated within the following limits (min-max values) – 285.0 –363.0 mm, 429.0 – 804.0 g, in 2019 – 225.0 – 360.0 mm, 178.30 – 754.60; wintering pond No 1 TRPLF NULES of Ukraine in

2018 – 251.0 – 333.0 mm, 238.0 – 633.0 g, in 2019 – 236.0 – 298.0 mm, 209.0 – 485.0 g; winter pond No 13.
BEHS IHB NAS of Ukraine in 2018 – 191.0 – 302.0 mm, 111.0 – 438.0 g, in 2019 – 155.0 – 236.0 mm, 63.30 –227.90 g (Table 3).

The ratio of the average values of linear growth in length and mass of biennials of a hybrid of silver and bighead carp is shown in Figure 6.

According to experimental studies in 2017 – 2018 in TRPLF NULES, it was found that from the stocking of annual fish to the catch of biennials, the rate of linear growth was the highest – 194.52 mm, 165.58%; 204.28 mm, 204.85%, respectively, and the rate of weight growth was found to be significantly higher in SEEF "Nyvka" IF NAAS of Ukraine – 372.80 g, 939.0%; 336.97 g, 663.9%. In 2018 – 2019, after wintering in biennials from the pond BEHS IHB NAS of Ukraine, compared with biennials, the weight of fish decreased (– 126.82 g, – 52.86%), due to wintering of fish. In other experimental ponds (SEEF "Nyvka" IF NAAS of Ukraine and TRPLF NULES of Ukraine) after fish wintering, an increase in linear and weight indicators was observed. This may be because the ponds were caught in September-October and the data was also obtained during this period. When transplanting fish into winter ponds, it could still feed on detritus for some time and thus increase in length and weight. A significant difference in the size and weight of fish indicates a difference in living conditions in the ponds (Table 4).

Fish production of ponds for grazing fish retention is determined exclusively by the state of the natural feed base of ponds, the availability of fish feed organisms, the effectiveness of their use by various objects of polyculture **(Liangzberg, 2016; Makarenko, 2018; Passos, Vidal and D'Anatro, 2019)**.

To characterize the foraging of a particular pond in a certain period, an indicator equal to the sum of potential fish productivity for phytoplankton and zooplankton was used (P/V – the coefficient for phytoplankton was taken as 100, zooplankton – 20, forage coefficient: 50 and 7, respectively). As a characteristic of growth, we used the indicator of absolute weight gain with differentiation by farms, age groups, and years (Table 5). The result of regression analysis is presented in Figure 7 and Figure 8.

The analysis of the obtained data showed that the dependence of the weight gain of the hybrid of silver and bighead carp (ΔM) of younger age groups on the potential productivity (P) on the feed base is satisfactorily (coefficient of determination is 0.64 – 0.66) described by the equation $\Delta M = a * Pb$, where a = 0.001 – 1.182; b = 1.85 – 2.57.

It was found that grown in pond fish farms hybrid of silver and bighead carp had a higher rate of linear and weight growth, compared with the parental forms kept in similar conditions of pond farms **(Sukhenko et al., 2019)**.

Today, much attention is paid to the study of aquatic bioresources of large reservoirs of the Dnieper cascade, small reservoirs of southern Ukraine, and other artificial reservoirs. **(Klymenko and Statnyk, 2012; Makarenko, Shevchenko and Sytnyk, 2018a; Liu et al., 2017; Tereshchenko et al., 2016; Tsaryk et al., 2020; Petruk, 2013)**.

The study of hydrobiocenoses in small reservoirs within the zones of Polissya and Forest Steppe is currently not comprehensive, its studies are isolated and do not contain a clear interconnection.

When comparing different groups of the hybrid of silver and bighead carp caught from experimental small reservoirs, there is also a significant difference in their size and weight characteristics. In the spring of 2018, during the stocking of the Kosiv Reservoir, the length and weight of annual fish was (min-max values) – 159.0 – 204.0 mm, 35.80 – 74.30 g, and in 2019 – 135.0 – 190.0 mm, 19.90 – 67.90 g. In the autumn of 2019, during the overfishing of the Kosiv Reservoir, the length and weight of triennial fish, fish stock (annuals) stocked in 2018, fluctuated within the following limits (min-max values) – 535.0 – 573.0 mm, 1493.70 – 1907.50 g; triennial fish, stocking of fish planting material (biennials) in 2019, varied – 577.0 – 640.0 mm, 2124.50 – 2722.60 g (Table 6).

The ratio of the average values of linear growth in length and weight of annuals and triennials of hybrid of silver and bighead carp is shown in Figure 9 and Figure 10.

In autumn, during the stocking of the Velykoburlutske Reservoir, the value of the length and body weight of young-of-the-year fish in 2017 was (min-max values): 118.0 – 157.0 mm, 13.30 – 28.80 g; in the autumn when catching biennials in 2018 – 385.0 – 434.0 mm and 592.0 – 884.0 g; in the summer when catching triennial fish in 2019 – 369.0 – 605.0 mm, 601.20 – 1754.20 g (Table 7). During the study pechenizka reservoir (Kharkiv region), it was found that the mass caught three years was 920 g. **(Gogol, 2014)**.

The rate of linear-weight growth of different size-weight groups of a hybrid of silver and bighead carp from the Kosiv Reservoir has not been determined, as not enough information has been collected.

In the study of the Velykoburlutske Reservoir, it was found that during 2017 – 2018 the average linear increase (from young-of-the-year fish to biennials of a hybrid of silver and bighead carp) was 227.18 mm, the absolute increase – 302.74%; for weight growth, these indicators were respectively: 757.74 g and 3.858%. In 2018 – 2019, in triennials, compared with biennials, the length increased by 66.30 mm, 116.02%, and weight – 459.64 g, 159.09%, which may be due to the high feed base of the reservoir (Table 8) **(Buzevych and Makarenko, 2020)**.

An important practical aspect of grazing aquaculture of herbivorous fish is to determine the optimal amount of planting material, in particular, it concerns stocking of reservoirs, which can be carried out in two strategic directions: mass stocking of young fish with low stock and less stocking with large planting material. One of the advantages of the first direction may be a higher growth rate of planting material in reservoirs (compared to ponds). This allows forming a more resistance to the conditions of a particular reservoir initial herd of aquaculture facilities **(Raoult, Gaston, and Taylor, 2018; Buzevych and Buzevych, 2017; Khrystenko et al., 2012; Sukhenko et al., 2017; Zakharchenko et al., 2019; Didenko, 2007)**. To verify this factor, we conducted a comparative analysis of the rates of linear and weight growth of annuals and biennials of hybrids of silver and bighead carp grown in pond conditions and young-of-the-year – triennials in the reservoir.

**Table 6** The average values of length and body weight of a hybrid of silver and bighead carp.

| Indexes | Kosiv Reservoir | | | |
|---|---|---|---|---|
| | Annuals | | Triennials | |
| | Spring 2018 | Spring 2019 | Autumn 2019 (Fish stocking in 2018) | Autumn 2019 (Fish stocking in 2019) |
| | M±m | M±m | M±m | M±m |
| Average body length, mm | 172.12 ±9.94 | 158.20 ±19.19 | 548.30 ±13.35 | 617.70 ±20.75 |
| Average body weight, g | 45.88 ±8.79 | 38.81 ±15.03 | 1714.05 ±148.46 | 2402.43 ±226.90 |
| Fulton fatness (K-factor) | 1.71 | 1.91 | 1.71 | 1.63 |

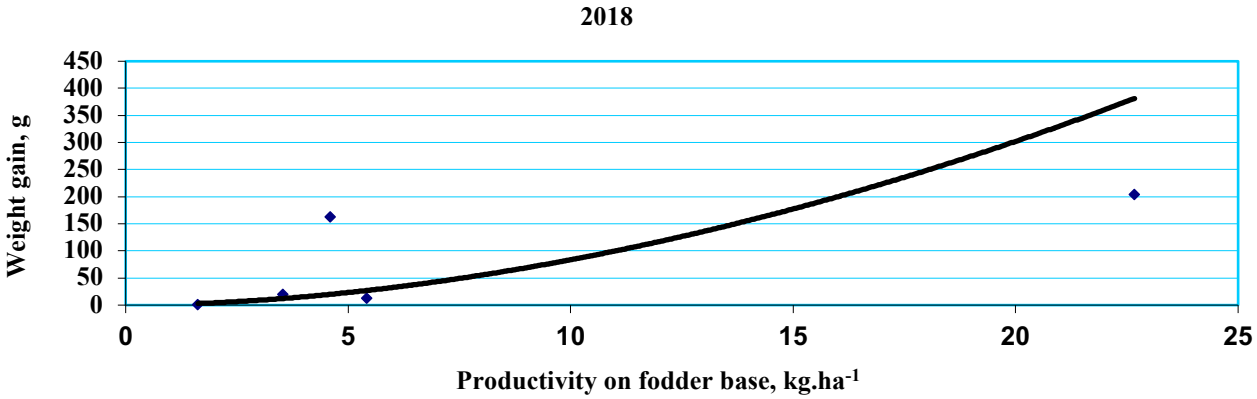


**Figure 8** The increase in the mass of younger age groups of the hybrid of silver and bighead carp from the potential productivity of the fodder base in 2018.

**Table 7** The average values of length and body weight of a hybrid of silver and bighead carp

| Indexes | Velykoburlutske Reservoir | | |
|---|---|---|---|
| | Young-of-the-year | Biennials | Triennial |
| | Autumn 2017 | Autumn 2018 | Summer 2019 |
| | M±m | M±m | M±m |
| Average body length, mm | 136.72 ±63.30 | 413.90 ±17.74 | 480.20 ±95.00 |
| Average body weight, g | 20.16 ±5.12 | 777.90 ±114.70 | 1237.54 ±509.56 |
| Fulton fatness (K-factor) | 1.75 | 1.83 | 2.12 |

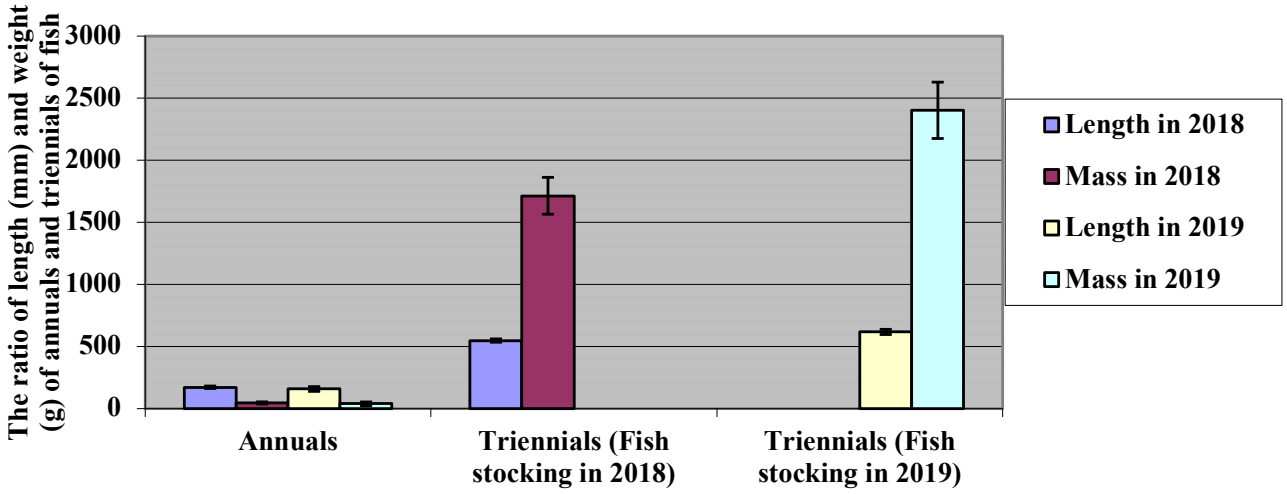


**Figure 9** The ratio of the average values of linear growth in length and mass of annuals and triennials of hybrid of silver and bighead carp from the Kosiv Reservoir in 2018 – 2019.

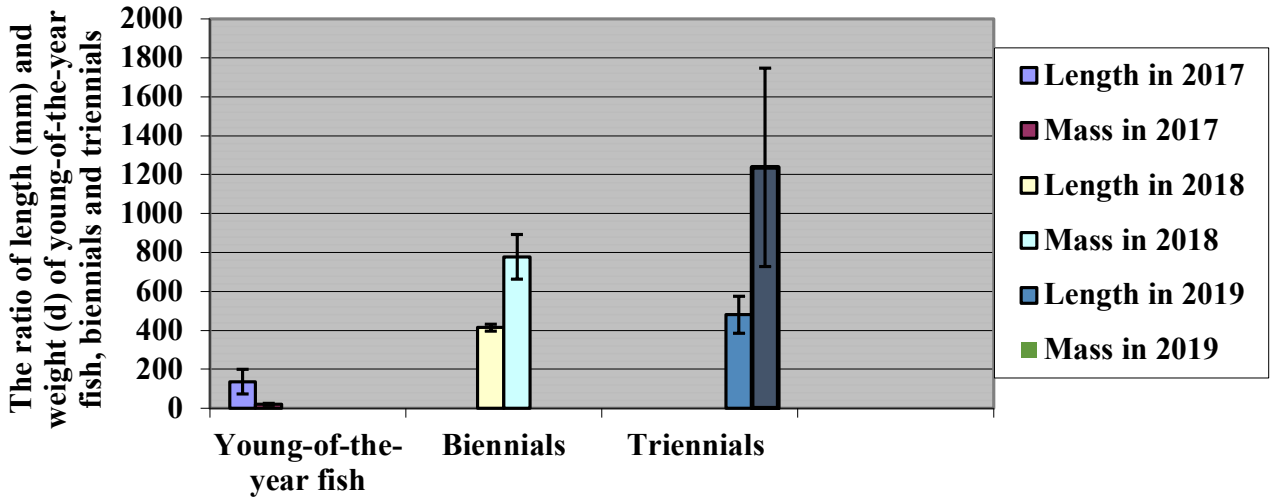


**Figure 10** The ratio of the average values of linear growth in length and weight of young-of-the-year fish, biennials, and triennials of a hybrid of silver and bighead carp from the Velykoburlutske reservoir 2017 – 2019.

**Table 8** The rate of linear-weight growth of a hybrid of silver and bighead carp from the Velykoburlutske reservoir.

| Age group | Indexes | | 2017 – 2019 |
|---|---|---|---|
| 0+ – 1+ | Linear growth rate | mm | 277.18 |
| | | % | 302.74 |
| 1+ – 2+ | | mm | 66.30 |
| | | % | 116.02 |
| 0+ – 1+ | Weight growth rate | g | 757.74 |
| | | % | 3.858 |
| 1+ – 2+ | | g | 459.64 |
| | | % | 159.09 |

## CONCLUSION

It was found that in 2017 – 2018 in TRPLF NULES from stocking annual fish to the catch of biennial fish the rate of linear growth was the highest – 194.52 mm, 165.58%; 204.28 mm, 204.85%, and the rate of weight growth was found to be significantly higher in DG "Nyvka" IRG NAAS of Ukraine – 372.80 g, 939.0%; 336.97 g, 663.9%. According to the research in 2018 –2019, after wintering in biennial fish from the pond BEGS IGB of Ukraine, compared with biennial fish, the weight of fish decreased (– 126.82 g, – 52.86%), which is due to fish wintering. In the ponds of DG "Nyvka," IRG NAAS of Ukraine, and TRPLF NULES of Ukraine after wintering a significant increase in linear and weight indicators of fish was observed.

The dependence of the mass gain of a hybrid of silver and bighead carp (ΔM) of younger age groups on the potential productivity (P) on the forage base, which is satisfactorily described by the equation was established.

For the Velykoburlutsky Reservoir, it is shown that during 2017 – 2018 the average linear increase (from young-of-the-year fish to biennials of a hybrid of silver and bighead carp) was 227.18 mm, the absolute increase was 302.74%; for weight growth, these figures were respectively: 757.74 g, 3.858%. In 2018 – 2019, in the studied triennial fish, compared to biennial fish, the length increased by 66.30 mm, 116.02%, and the weight – 459.64 g, 159.09%, which may be due to the high feed base of the reservoir.

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This article does not contain any studies that would require an ethical statement.

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# THE EFFECT OF TREATMENT CONDITIONS ON COLOR CHARACTERISTICS AND MEASURE OF CHOLESTEROL REMOVAL FROM MILK BY BETA-CYCLODEXTRIN APPLICATION

*Lukáš Kolarič, Peter Šimko*

## ABSTRACT
Long-term high cholesterol intake is one of the most critical risk factors of cardiovascular diseases (CVD). As milk and dairy products are rich in cholesterol and are consumed on a large scale, the production of low-cholesterol content products could decrease effectively high cholesterol intake what would be one of the crucial steps in CVD prevention. Thus, this study is aimed at optimization of treatment conditions (mixing speed, time, and temperature) and β-cyclodextrin addition affecting the measure of cholesterol removal in milk. As found, the optimal conditions were identified such as mixing speed 840 rpm, mixing time 10 min, and the temperature of mixing 25 °C while the most effectivity in cholesterol decrease content (98.1%) was observed after 2.0% β-cyclodextrin addition. The cholesterol removal process did not affect considerably the lightness values $L^*$ of treated milk, slight differences were noticed in terms of $a^*$ and $b^*$ color values but $\Delta E$ values were statistically insignificant, i.e., the process of cholesterol removal did not affect visual characteristics of treated milk. So, these conditions can be applied for the production of milk base functional foods with the decreased cholesterol content.

**Keywords:** milk; β-cyclodextrin; cholesterol; color; functional foods

## INTRODUCTION
Milk fat is probably the most complex of all common fats consisting of numerous fatty acids, especially saturated. Because of high content of saturated fatty acids, milk has been associated with a variety of human diseases **(Seçkin et al., 2005)** thus several international dietary recommendations advise lowering of saturated fat and cholesterol intake mainly for the reduction of cardiovascular diseases (CVD) **(Larsson, Virtamo and Wolk, 2012)**. The most frequently proposed mechanism through which saturated fats negatively influence the risk for CVD is the increase of blood lipids content, especially total cholesterol, and low-density lipoproteins **(Pereira, 2014)**. As milk and dairy products are consumed on a large scale, the importance of the reduction of dietary cholesterol content in these products has risen in the past years. Several methods were developed for the reduction of cholesterol content in dairy products such as the enzymatic conversion of cholesterol, steam distillation, adsorption on several sorbents, supercritical extraction, or the removal of lipids and cholesterol using liquid solvents **(Mohamed et al., 2000)**. However, these methods lack specificity and affect negatively the nutritional and textural properties of final products. Therefore, the most promising method is based on the selective adsorption of cholesterol molecules onto a cyclodextrin cavity. Cyclodextrins (CDs) are cyclic oligomers of α-D-glucopyranose formed by the transformation of starch, and because of their structure, they can be used as encapsulating agents for protecting flavors, vitamins or natural colors, food preservatives, or cholesterol sequestrant, respectively **(Astray et al., 2009)**.

According to **Kukula, Kolarič and Šimko (2020)**, the application of CDs in the dairy industry could decrease the total per capita daily cholesterol intake in the Slovakian population from 369.8 mg to 296.3 mg, which is below the value (300 mg per day for adults) recommended by the World Health Organization **(Bertolín et al., 2018)**. The CD ring is a conical cylinder of an amphiphilic nature, with a hydrophilic outer part and lipophilic cavity **(Matencio et al., 2020)**, and because of that, they can form inclusion complexes with molecules of low hydrophilicity and proper geometrical size **(Fenyvesi, Vikmon and Szente, 2016)**. The most common CDs are the natural α, β, and γ-forms with six, seven, and eight glucose units, respectively **(Matencio et al., 2020)**. In the β-CD molecule, a complete secondary belt is formed by the hydrogen bonds, therefore, the β-CD has the lowest water solubility of all CDs **(Szejtli, 2004; Astray et al., 2009)**. The H-bond belt is incomplete in the α-CD molecule because one glucopyranose unit is in a distorted position, and the γ-CD is a non-coplanar with a more flexible structure thus it is the most soluble of the CDs **(Astray et al., 2009)**. According to the solubility in water, the β-CD is more preferred in cholesterol removal in milk.

The traditional procedure of cholesterol removal in milk by CDs consists of steps such as mixing the milk with the proper concentration of CD, settling the CD-cholesterol complex, centrifugation, and the removal of the CD-cholesterol complex. The measure of cholesterol removal will be thus influenced by the conditions of these processes. According to **Ahn and Kwak (1999)**, the effectiveness of cholesterol adsorption is dependent mainly on β-CD concentration, mixing time, and mixing speed. **Kwak et al. (2003)** reached a 79.3% reduction of cholesterol in raw milk by the application of 1% (w/v) β-CD, mixing time 10 min at 4 °C, and centrifugation speed 166 g. On the other side, **Alonso, Calvo and Fontecha (2019)** reached higher cholesterol removal in raw milk (up to 91.6%) by the application of a lower concentration of β-CD (0.8%, w/v) and longer mixing time (30 min). **Lee, Ahn and Kwak (1999)** suggested that the optimum conditions consisted of 1.5% β-CD, mixing time 10 min at 10 °C, and centrifugation at 166 g for 10 min. Based on their results, the removal rate was influenced neither by mixing time nor by the time of centrifugation.

Therefore, the objective of this study was to find suitable treatment conditions and β-CD concentration on the measure of cholesterol removal from homogenized milk as well as the effect of the cholesterol removal on color characteristics of treated milk.

## Scientific hypothesis

The measure of cholesterol removal from homogenized milk is affected by the conditions of mixing and the concentration of β-CD. The application of β-CD does not affect the milk color characteristics.

## MATERIAL AND METHODOLOGY

### Samples

Commercial milk (3.5% declared fat content) bought in a local market.

### Chemicals

β-cyclodextrin (Wacker Chemie AG, Burghausen, Germany, ≥95.0%, HPLC).
Cholesterol (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, Sigma Grade, ≥99%).
Chloroform (Centralchem s.r.o., Bratislava, Slovakia, p.a.).
n-hexane (Centralchem s.r.o., Bratislava, Slovakia, p.a.).
Ethanol (Centralchem s.r.o., Bratislava, Slovakia, p.a.).
Sodium sulfate anhydrous (Centralchem s.r.o., Bratislava, Slovakia, p.a.).
Potassium hydroxide (Mikrochem, Pezinok, Slovakia, p.a.).
Methanol (Fisher Chemical, Loughborough, UK, HPLC grade).
Acetonitrile (Fisher Chemical, Loughborough, UK, HPLC grade).

### Instruments

HPLC system (Agilent Technologies 1260 Infinity, Santa Clara, CA, USA).
Vacuum degasser (Agilent Technologies, Santa Clara, CA, USA).
Quarterly pump (Agilent Technologies, Santa Clara, CA, USA).
Autosampler (Agilent Technologies, Santa Clara, CA, USA).
UV-DAD detector (Agilent Technologies, Santa Clara, CA, USA).
Chromatography column (Zorbax Eclipse Plus $C_{18}$, 2.1x50 mm, 5 µm particle size, Agilent, Santa Clara, CA, USA).
Guard column (Zorbax SB-$C_{18}$, 4.6x12.5 mm, 5 µm particle size, Agilent, Santa Clara, CA, USA).
Rotary vacuum evaporator (Heidolph, Germany).
Centrifuge (Hettich Zentrifugen, Germany)
Magnetic stirrer (Arex-6 Connect Pro, Velp Scientifica, Italy).
Analytical balance (Sartorius, Goettingen, Germany)
UV-VIS spectrophotometer (Cary 300, Agilent Technologies, Santa Clara, CA, USA).

### Laboratory Methods

***HPLC conditions for the determination of cholesterol content***

The determination of cholesterol content in milk samples was performed by HPLC analysis using an Agilent Technologies 1260 infinity system (USA) equipped with a vacuum degasser, a quarterly pump, an autosampler, and the UV-DAD detector, set at 205 nm. Isocratic elution was performed at a flow rate of 0.5 $mL.min^{-1}$ using the mobile phase consisted of acetonitrile/methanol 60:40 (v/v). The injection volume was 10 µL and the temperature was set at 30 °C. As a stationary phase, Zorbax Eclipse Plus $C_{18}$ column (2.1×50 mm, 5 µm particle size, Agilent, USA) was used with the guard column Zorbax SB-$C_{18}$ (4.6×12.5 mm, 5 µm particle size, Agilent, USA). The total run time of analysis was 5 min with the retention time of cholesterol in 2.2 min. The calibration curve was recorded from 10 working standard solutions with the concentrations of 2, 6, 10, 25, 40, 50, 75, 100, 300, and 350 $mg.L^{-1}$.

The results were recorded using the OpenLab CDS software, ChemStationEdition for LC, and LC/MS systems (product version A.01.08.108).

***Analysis of color***

The color of the treated milk was measured using a UV-VIS spectrophotometer Cary 300 (Agilent Technologies, USA) with DRA-CA-30I (internal) sphere accessory. The color itself was evaluated using the CIELAB coordinate system regarding illuminant D65 and a visual angle of 10°. The parameters measured were: L* (lightness factor), a* (–a* = green, +a* = red) and b* (–b* = blue, +b* = yellow). The spectrophotometer was standardized with a white plate, supplied with the equipment. The results were treated with Cary WinUV software (version 4.20(468)) **(Kolarič et al., 2020)**. All procedures were triplicated. ΔE was calculated to estimate the visual differences between the control milk and treated milk using Equation (1):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (1)$$

### Description of the Experiment

### Sample preparation:

***Cholesterol removal:***

Cholesterol removal in milk samples was performed according to the modified method by **Kukula, Kolarič and Šimko (2020)**. To study the effects of mixing conditions, 25 g of milk was placed in a beaker, and 1.0% of β-CD was added. The mixtures were stirred at 5 different mixing speeds (480, 600, 720, 840, and 960 rpm) using a magnetic stirrer (Arex-6 Connect Pro, Velp Scientifica, Italy) for 4 different times (5, 10, 15, and 20 min), and at 3 different

temperatures (25, 40, and 50 °C). The mixtures were then centrifuged (Hettich Zentrifugen, Germany) at 200 g for 20 min. After centrifugation, the milk supernatant was analyzed for cholesterol content and color. After setting optimal mixing conditions, the milk samples were stirred with 5 different β-CD concentrations (0.5, 1.0, 1.5, 2.0, and 2.5%). All procedures were triplicated.

***Analysis of cholesterol content:***

The analysis of cholesterol content in treated milk was performed according to **Kolarič and Šimko (2020)**. 5.0 g of samples were refluxed with 0.015 L of 1 mol.$L^{-1}$ KOH methanolic solution for 15 min. The extraction process was performed with the 0.015 L of the extraction solvent composed of n-hexane and chloroform (1:1, v/v) in duplicate. The extracts were filtrated through anhydrous $Na_2SO_4$ and evaporated using a rotary vacuum evaporator (Heidolph, Germany). The residue was then dissolved in 5 mL of methanol and filtered using a syringe PTFE filter with 0.2 μm membrane (Agilent Technologies, USA), and analyzed by HPLC. All procedures were triplicated.

**Number of samples analyzed:** 17
**Number of repeated analyses:** 3
**Number of experiment replication:** 1

## Statistical Analysis

Results are expressed as mean ±standard deviation or as a percentage. Statistical analysis was performed using the XLSTAT tool of Microsoft Excel 365 (version 2012, Microsoft, USA). The obtained data from the cholesterol content analysis and the analysis of color were subjected to one-way analysis of variance (ANOVA) and the Student´s test, and the values were considered significantly different when $p$ <0.05. From the ANOVA and Student´s test, it was able to determine if there are some significant differences between the results obtained by the analysis of the effect of mixing conditions (speed, time, and temperature), CD concentration, and color changes.

# RESULTS AND DISCUSSION

## Effect of mixing speed

The cholesterol removal was significantly affected by mixing speed. It was noticed significant ($p$ <0.05) differences between the results obtained at 480 to 600 rpm, and 720, 840, and 960 rpm. The results were not significantly different at 720, 840, and 960 rpm, suggested that the optimum mixing speed begins at 720 rpm. However, the highest removal efficiency (73.3%) was observed at 840 rpm thus this speed was set as the most suitable one, as shown in Figure 1A. The results are similar to other studies. **Kwak, Nam and Ahn (2001)** stirred homogenized milk with β-CD at 800 rpm and reached cholesterol removal up to 78.2% (1.0% β-CD). **Lee, Ahn and Kwak (1999)** reported that mixing speed at 800 rpm is efficient to remove 94.0% of the cholesterol in commercial milk by β-CD (1.0%), also supported by **Lee et al. (2007)**. According to **Maskooki et al. (2013)**, by the application of mixing speed at 700 rpm, it is possible to remove 67.7% of the cholesterol in homogenized milk by β-CD in the same concentration. Lower mixing speed (530 rpm) is reported by **Gianni et al. (2020)** with the cholesterol removal rate varied from 33 to 89%. According to **Kim, Ahn and Kwak (2004)**, the mixing speed is not an important factor in cholesterol removal in milk by crosslinked β-CD as it was not noticed a significant difference with various mixing speeds. The mixing speed is also influenced by the type of sample. E.g., higher speed is required in cream treatment as **Jung, Ko and Kwak (2013)** used 1400 rpm, **Ahn and Kwak (1999)** 800 – 1600 rpm, or **Shim, Ahn and Kwak (2003)** 400 – 1200 rpm. **Tahir, Bokhari and Adnan (2015)** extracted the cholesterol from ghee by mixing with β-CD immobilized on glass beads at 170 rpm.

## Effect of mixing time

A significant difference ($p$ <0.05) was noticed between the mixing time of 5 min and 10, 15, and 20 min. The highest measure of cholesterol removal was observed after 20 min of mixing (79.8%) but similar values were monitored in 10 and 15 min (79.3 and 79.2%, respectively). The lowest value of 73.0% was noticed at a mixing time of 5 min (Figure 1B). As found, a mixing time over 10 min is enough for sufficient removal. In comparison to this, slightly different results were reported by **Lee, Ahn and Kwak (1999)**. Based on their results, the cholesterol removal was not affected by a mixing time of 5 to 20 min, but it was noticed a reduction at 25 min of mixing. A similar trend was also mentioned by **Alonso et al. (2009)**. A slight reduction was observed when the mixing time increased to >20 min for 0.8 and 1.0% β-CD. The above results suggest that at longer mixing times, the inclusive complex between β-CD and cholesterol could be unstable. Therefore, from the other studies, it can be concluded that the mixing time between 10 and 20 min is the most suitable for the sufficient removal of cholesterol from milk. **Kwak et al. (2003)** reported that by the mixing time of 10 min it is possible to remove 79.3% of cholesterol content in milk by β-CD addition (1.0%), which is similar to our study. **Lee, Ganesan and Kwak (2012)** used 10 min mixing of milk with crosslinked β-CD and reached the removal of cholesterol up to 93%. Higher mixing times are reported in the case of cream. **Ahn and Kwak (1999)** achieved the highest removal of cholesterol in cream by the mixing time of 30 min. This time was also used in other studies **(Shim, Ahn and Kwak, 2003**; **Jeon et al., 2012**; **Galante et al., 2017)**.

## Effect of mixing temperature

Besides the importance of mixing speed and duration, temperature is reported as another factor affecting the measure of cholesterol removal. According to **Matencio et al. (2020)**, the most important factors to consider in the mechanism of CD/guest reaction are the structure of the guest molecule, solvent, and temperature. The various results are noticed among published reports. According to this study (Figure 1C), no significant differences ($p$ <0.05) between the mixing temperatures of 25, 40, and 50 °C, were observed, when the measure of cholesterol removal varied at 79.3, 78.2, and 77.9%, respectively. Similar results were mentioned by **Lee, Ahn and Kwak (1999)** as they also found no differences in cholesterol removal at different temperatures but suggested that mixing at 10 and 25 °C tends to increase cholesterol removal compared to lower values of temperature. A different mixing temperature was used in studies by **Alonso et al. (2009)**, **Alonso et al. (2018)**, and **Alonso, Calvo and Fontecha (2019)**. According to them, the mixing in at 4 °C is sufficient for

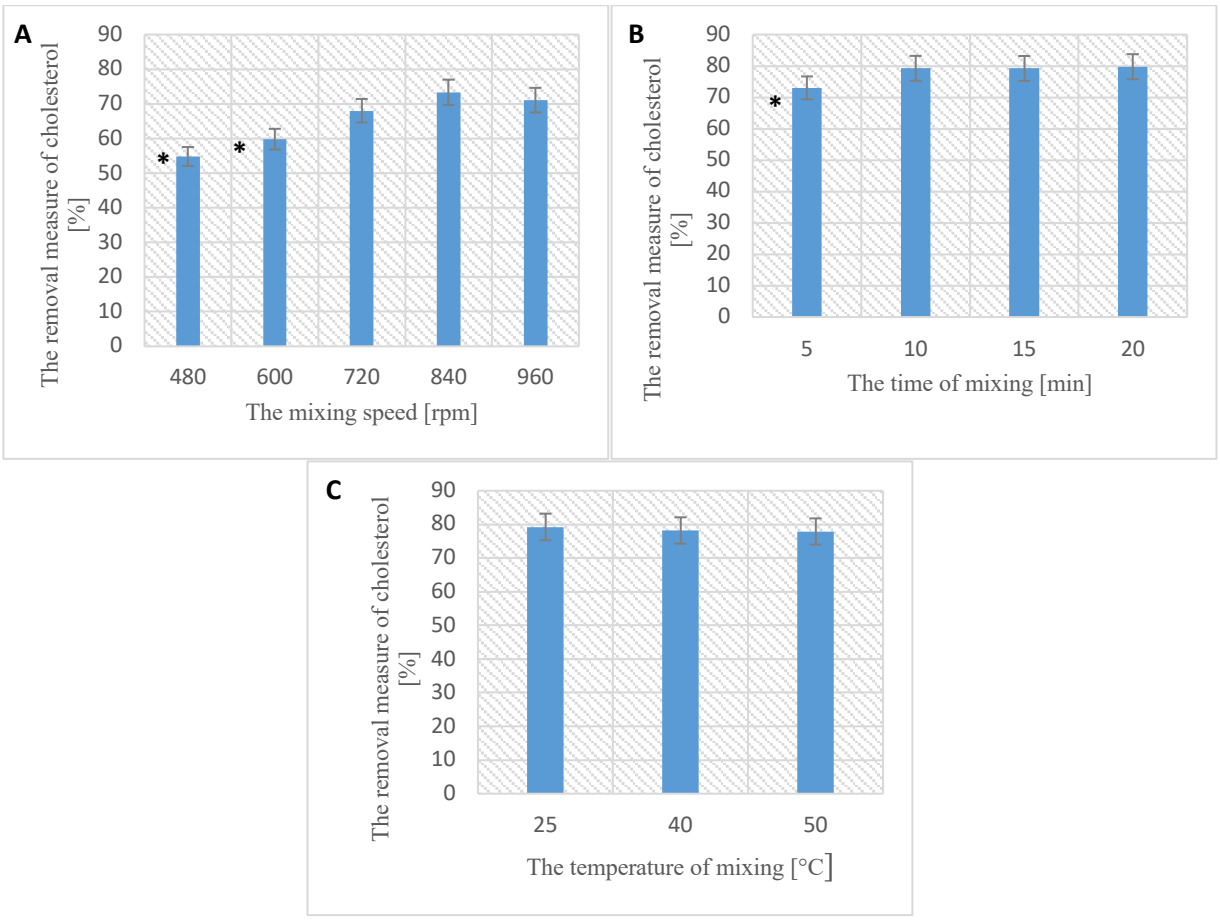


**Figure 1** The influence of the mixing conditions (speed (A), time (B), and temperature (C)) on the measure of cholesterol removal. Note: *indicates a statistically significant differences ($p < 0.05$). The other conditions were as follow: A - the concentration of β-CD 1%, mixing for 5 min at 25 °C, centrifuging at 200 g for 10 min. B – the concentration of β-CD 1%, mixing at 840 rpm at 25 °C, centrifuging at 200 g for 10 min, C – the concentration of β-CD 1%, mixing at 840 rpm for 10 min, centrifuging at 200 g for 10 min.

**Table 1** The effect of β-CD concentration on the cholesterol removal and color of milk.

| β-CD concentration [%] | Cholesterol content [mg.kg$^{-1}$] | $^{++}$Cholesterol removal [%] | $L^*$ coordinate | $a^*$ coordinate | $b^*$ coordinate | $\Delta E$ |
|---|---|---|---|---|---|---|
| 0.0 | 127.3 ±2.1 | - | 67.01 ±0.18 | -0.70 ±0.03 | 1.57 ±0.11 | - |
| 0.5 | 119.4 ±0.5 | $^{+}$6.2 ±0.4 | 67.53 ±0.37 | -0.82 ±0.05 | $^{+}$1.18 ±0.05 | 0.77 ±0.47 |
| 1.0 | 27.2 ±0.7 | $^{+}$78.6 ±4.3 | 67.26 ±0.48 | $^{+}$-1.00 ±0.06 | $^{+}$1.13 ±0.02 | 0.59 ±0.29 |
| 1.5 | 2.4 ±0.9 | 98.1 ±0.7 | 67.32 ±0.48 | -0.76 ±0.02 | $^{+}$2.20 ±0.04 | 0.52 ±0.01 |
| 2.0 | 2.4 ±0.4 | 98.1 ±0.3 | 66.86 ±0.27 | -0.75 ±0.02 | $^{+}$2.33 ±0.13 | 0.79 ±0.03 |
| 2.5 | 2.6 ±0.5 | 97.9 ±0.4 | 67.25 ±0.04 | -0.79 ±0.03 | $^{+}$2.32 ±0.09 | 0.69 ±0.04 |

Note: mean ±standard deviation (n = 3). Color coordinates: L (lightness factor), a (–a = green, +a = red), b (–b = blue, +b = yellow); $^{+}$indicates a statistically significant differences ($p < 0.05$); $^{++}$ The conditions of removal were as follow: mixing at 840 rpm for 10 min at 25 °C, centrifuging at 200 g for 10 min.

cholesterol removal up to 95%, which is effective in maintaining the quality of milk. **Maskooki et al. (2013)** reported that the mixing temperature of 20 °C is more suitable than 8 °C because the aggregations of milk lipoproteins at higher temperatures are lower, and consequently there is a better chance for the entrapment of cholesterol molecules in β-CD cavities.

The effect of milk lipoproteins aggregation may play a greater role by the processing of cream as the cream is mixing with β-CD usually at temperatures between 40 to 50 °C. E.g., **Shim, Ahn and Kwak, 2003**, **Ha et al. (2010)**, or **Lee, Ganesan and Kwak (2012)** used the temperature of mixing at 40 °C and achieved effective removal of cholesterol content in cream.

## Effect of β-CD concentration

After the optimization of mixing conditions, the influence of β-CD concentration was also studied, as shown in Table 1. The chromatographic records of the analysis of cholesterol in control milk without the addition of β-CD and with 2.0% β-CD addition are shown in Figure 2. As follows from Table 1, the lowest value was observed at 0.5% of β-

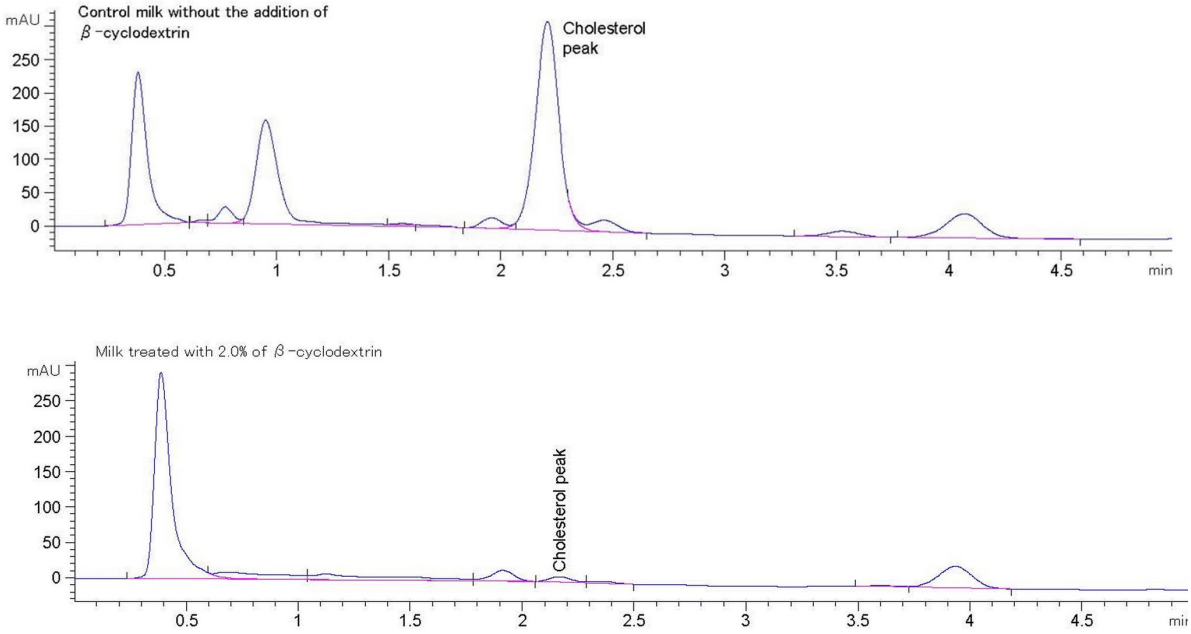


**Figure 2** The chromatographic records of the analysis of cholesterol in control milk without the addition of β-cyclodextrin and with 2.0% addition of β-cyclodextrin.

CD (6.2%) and the highest at 2.0% (98.1%). A slight decrease was noticed at 2.5% of β-CD (97.4%). The results were significantly ($p$ <0.05) different at 0.5 and 1.0% of β-CD addition and did not differ at the concentrations of 1.5, 2.0, and 2.5%. Therefore, the optimal β-CD concentration was thus determined at 1.5%. Similar results are reported by **Kwak, Nam and Ahn (2001)**. According to them, a high increase of cholesterol removal was also found between 0.5 and 1.0% β-CD addition. They suggested that 1.0% of β-CD addition may be effective enough for cholesterol removal. However, lower optimal concentrations are published by other authors. **Lee, Ahn and Kwak (1999)** noticed that the least efficiency of removal showed the addition of β-CD at 2.0%. According to **Alonso et al. (2009)**, no significant differences in cholesterol reduction were observed at concentrations of 0.6, 0.8, and 1.0%. According to **Maskooki et al. (2013)**, the measure of cholesterol adsorption was reduced at 1.5% β-CD due to excess of β-CD, which could compete with itself to bind to cholesterol molecules, and consequently reduced the cholesterol adsorption effectivity **(Lee, Ahn and Kwak, 1999; Maskooki et al., 2013)**. Besides that, from the published results, it can be stated that the variation of the optimal β-CD concentrations may be caused by different operational conditions, e.g., mixing temperature or mixing and centrifugal speeds. The β-CD concentration depends also on the fat/cholesterol content of food items. E.g., higher additions are required by the processing of cream. **Shim, Ahn and Kwak (2003)** used 10% of β-CD in the extraction of cholesterol in cream while **Dias et al. (2010)** reported that 20% of β-CD addition showed the best removal rate of cholesterol in commercial butter.

### Effect of treatment on color characteristics

Since the color characteristics of treated milk are scarce, these were studied by analyzing measured values in *CIEL*a*b** color space. As follows from Table 1, treatment with β-CD did not affect the lightness values ($L^*$), which is in accordance with results reported by **Gianni et al. (2020)**. Slight differences were noticed in $a^*$ coordinate values between the control sample and the sample treated with 1% of β-CD. The significant differences ($p$ <0.05) were shown between the control and 1.5, 2, and 2.5% of β-CD in $b^*$ coordinate values. In previous studies, there is no evidence about the influence of β-CD on the yellowness factor of milk. Despite higher values of this coordinate, it was not noticed any significant changes in the visual appearance of treated milk, which was also proven by the calculation of $\Delta E$ values. According to **Bhatia et al. (2019)**, the process of cholesterol removal by β-CD does not affect the typical color of cow ghee so it has no impact on the visual consumer perception of the product.

## CONCLUSION

This study proved that the removal of cholesterol from milk is affected by treatment parameters such as mixing duration, mixing speed, and the temperature of mixing as well as β-CD concentration. On the basis of experimental data, it was found that optimal mixing conditions were the speed at 840 rpm, the time 10 min, and the temperature at 25 °C when the mixing speed was noticed as the most significant ($p$ <0.05). After the optimization of mixing conditions, the effect of β-CD addition was monitored. The highest decrease of cholesterol content was found at 2.0% β-CD addition (98.1%), but the results between 1.5, 2.0, and 2.5% were insignificant ($p$ <0.05). The treatment did not affect the lightness values $L^*$ of treated milk, the slight differences were observed in both $a^*$ and $b^*$ color values but $\Delta E$ values were statistically insignificant ($p$ <0.05). Thus, this study confirmed the excellent possibility of the production of cholesterol-free milk by application of β-CD without any effect of this treatment on milk color characteristics.

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This article does not contain any studies that would require an ethical statement.

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# A PILOT PLANT SCALE OF YELLOW KONJAC (*AMORPHOPHALLUS MUELLERI* BLUME) FLOUR PRODUCTION BY A CENTRIFUGAL MILL USING RESPONSE SURFACE METHODOLOGY

*Jatmiko Eko Witoyo, Bambang Dwi Argo, Sudarminto Setyo Yuwono, Simon Bambang Widjanarko*

## ABSTRACT

This study aimed at investigating the effects of polishing conditions on the physicochemical properties of polished yellow konjac flour (PYKF) with a centrifugal mill using Central Composite Design-Response Surface Methodology (CCD-RSM). Micro-mill milled yellow konjac flour (MMYKF) mass and polishing cycles were the independent variables, with four observed responses (calcium oxalate, viscosity, degree of whiteness (DoW), and glucomannan). The lower limit (-1) and upper limit (+1) for MMYKF mass in this study are 10 and 15 kg, respectively, while the -1 and +1 for the polishing cycle are three times, and seven times, respectively. The optimum prediction occurred at 10 kg of MMYKF mass and six times the polishing cycle with the following characteristics: 0.52 ±0.00% w.b. calcium oxalate, 20362.00 ±16.00 cP viscosity, 62.22 ±0.01 DoW, and 69.43 ±0.02% d.b. glucomannan content, which agreed with the verification data with *p*-value >0.05 for all observed responses using the paired T-test. Polishing using a centrifugal mill is feasible and promises to be scaled up to industrial scale for yellow konjac flour polishing before the wet extraction process.

**Keywords:** Centrifugal mill; polishing; *Amorphophallus muelleri* Blume; Response Surface Methodology

## INTRODUCTION

Crude yellow konjac flour (CYKF) is the flour produced by the processing of yellow konjac chips using a mechanical mill **(Widjanarko, Faridah and Sutrisno, 2014)**, including using micro-mill assisted cyclone separator and known as micro-mill milled yellow konjac flour (MMYKF). It contains ≤67.02% dry base (d.b.) glucomannan **(Faridah and Widjanarko, 2013; Witoyo et al., 2020)** and is classified as a fermentable, water-soluble, and high-viscosity food fibre **(Alonso-Sande et al., 2009)**. Hence, it is widely applied in the food industry to improve texture, as a thickener, as a gelling agent **(Zhang, Chen and Yang, 2014)**, and as a fat replacement **(Jiménez-Colmenero et al., 2013)**. However, the glucomannan content in CYKF is lower than the international standard for top and first grades (≥70 ≥90% d.b) **(Liu et al., 2002)**. The lower glucomannan content in CYKF is related to the fact that the glucomannan granules in the CYKF are still covered by impurities, such as starch, fibre, protein, fat, and ash **(Kurt and Kahyaoglu, 2017)**. The toxic substances which may be harmful to the human body also appear in CYKF. The main reason is that calcium oxalate can cause mouth and oesophagus irritation **(Kumoro, Budiyati and Retnowati, 2014)**, interference with Ca and Fe withdrawal, and other health problems **(James et al., 2013)**. Calcium oxalate content in CYKF ranged from 0.398 to 5.17% **(Faridah, 2016; Witoyo, Widjanarko and Argo, 2019)**, which was not acceptable for extensive application in food products.

Polishing is a subsequent stage aiming at removing impurities on glucomannan granules surface of yellow konjac flour after the milling process. In general, polishing removes bran in paddy during the rice milling process **(Mohapatra and Bal, 2010; Paiva et al., 2016; Reddy et al., 2017)**. This treatment is also reported as being effective in reducing anti-nutritional (tannin contents) from 1.05% to 0.09 – 0.095% and improving color in the polished sorghum grains. This process is still limited to removing impurities covering glucomannan granules in yellow konjac flour. **Wachyuningsih (2011)** and **Sary (2018)** report a polishing yellow konjac flour that uses an abrasive polisher. However, this method does not control the temperature during the process and is detrimental to the glucomannan in yellow konjac flour.

The centrifugal mill (also known as a centrifugal grinder) is one of the modified friction types of polishing machines equipped with a water-cooling system in the milling chamber to minimize heat produced during processing. This machine has the same principle as its predecessor, while the application is limited to the laboratory scale process in producing wheat and durum flour from wheat grains **(Deng and Manthey, 2017; Khalid, Manthey and Simsek, 2018)**. Polishing yellow konjac flour with a tool like that is still rare. Using a centrifugal mill for yellow konjac flour

polishing was initially evaluated by **Witoyo et al. (2020)** using MMYKF as a raw material. The results showed that polishing conditions affected polished yellow konjac flour (PYKF), namely its physicochemical properties, such as calcium oxalate content, viscosity, degree of whiteness (DoW), and glucomannan content. However, the optimum conditions for the polishing of CYKF have not been determined. Thus, the Response Surface Methodology (RSM) approach is needed to obtain optimum conditions for all observed responses.

RSM is an empirical statistical modeling technique based on multiple regression analysis using quantitative data from experiments designed to solve multivariate equations **(Madamba, 2002)** simultaneously. This technique is commonly used to process and optimize biochemical and chemical processes, using fewer data **(Baş and Boyaci, 2007)**. This study aims to obtain the optimum condition of MMYKF mass and polishing cycle to PYKF using a centrifugal mill with Central Composite Design (CCD)-RSM Approach.

## Scientific Hypothesis

The RSM-CCD approach can predict the optimal polishing conditions to obtain PYKF with the best characteristics.

## MATERIAL AND METHODOLOGY

### Samples

This study applies the optimum of MMYKF through 40 mesh sieve (13.65% w.b. moisture content) derived from yellow konjac chips milled using micro-mill assisted cyclone separator as the raw materials. Yellow konjac chips were purchased from farmers growing yellow konjac in Klangon Village, Saradan District, Madiun Regency, East Java, Indonesia (Figure 1 and Figure 2).

### Chemicals

Potassium permanganate (Sigma-Aldric, USA), D-Glucose (Sigma-Aldric, USA), distilled water, $H_2SO_4$ (Merck KGaA, Darmstadt, Germany), NaOH (Merck KGaA, Darmstadt, Germany), HCl (Merck KGaA, Darmstadt, Germany), ammonium hydroxide (Merck KGaA, Darmstadt, Germany), methyl red (Sigma-Aldric, USA), $CaCl_2$ (Merck KgaA, Darmsadt, Germany), and other chemicals were purchased in analytical grade from Merck KGaA, Darmstadt, Germany, and used without further purification.

### Instruments

Color reader (Minolta CR-10, Minolta, Japan), analytical balances (Mettler Toledo Inc., USA), Shaker water-bath (Memmert WNB14, Germany), Centrifuge (Thermo Scientific SL40R, Thermo Fisher Scientific, Inc.), viscometer (NDJ-1 rotational viscometer, China), Shimadzu UV-Visible Spectrophotometer UVmini-1240 (Shimadzu cooperation, Kyoto, Japan), Oven (Memmert, Germany), Vortex (IKA Vortex 3, ProfilLab24 GmbH, Berlin, Germany), Electrical Stove (Maspion, Indonesia), Shaker (SI-1500 Orbital-Genie Orbital Shaker, Marshall Scientific LLC)., Centrifugal mill (Workshop local made, CM 1 model, Indonesia), Disk mill (FFC 45 model, China), Micro-mill assisted cyclone separator (Workshop local made, MM 1 model, Indonesia).

### Description of the Experiment

**Sample preparation:** The study involved the polishing process described by **Witoyo et al. (2020)** using a centrifugal mill (Figure 2) with minor modifications. The 8.96 – 16.04 kg of MMYKF from the micro-mill, assisted by cyclone separator, was weighed using the digital balance (CMOS digital scale modelDS-30k) and put into input hopper (Figure 2, (4)). The feed rate (20 Hz) and fan blower speed (10 Hz) panel were turned on for 30 seconds for conditioning, and the rotor speed was set to 30 Hz for the polishing process. Each cycle process was completed (30 seconds after CYKF run out in the input hopper). Then the PYKF (the heavy fractions) was collected (Figure 2, in front of (13)) (1st polishing cycle) and put into the same input hopper for 2 to 8 polishing cycles using the same procedure as in the 1st polishing cycle based on experimental design (Table 1). The final PYKF (about 250 g) from each process was collected, sampled, and stored in a dark bag for further analysis.

**A number of samples analyzed:** 13 samples (Table 3) and 3 samples from the verification experiment (Table 2). So there were 16 samples to be analyzed.

**A number of repeated analyses:** Each sample was in Duplo analyses.

**A number of experiment replication:** The experiment was conducted using the CCD method, whereby 13 runs for the whole experiment consisted of four replications for the correlation treatment, four replications for the axial treatment and five replications for the centre treatment. The verification experiment used three replications to validate the optimum prediction treatment suggested by Design-Expert program 10 version trials.

## Determination of Calcium Oxalate

Calcium oxalate content in yellow konjac flour was evaluated based on methods described by **Iwuoha and Kalu (1995)**. Briefly, 2 g of yellow konjac flour was mixed with 10 mL of HCl and 190 mL of distilled water, followed by heating at 100 °C for 1 h. The mixed solution was cooled and diluted with distilled water to 250 mL using a volumetric flask. The mixture was filtered and divided into two portions. Each filtrate portion was added with 4 drops of indicator methyl red and ammonium hydroxide ($NH_4OH$) until the color turned yellow. Then, it was reheated until it reached 90 °C, cooled, and filtered again. Each final filtrate was reheated until it reached 90 °C, and 10 mL of 5% $CaCl_2$ was added. The mixed solution was stored at 5 °C overnight. Each filtrate was centrifuged at 8000 rpm for 15 min to obtain pellets. The pellets were dissolved in 10 mL of 20% $H_2SO_4$ to obtain 10 mL of filtrate from each filtrate. The two filtrates were mixed and diluted with distilled water to 250 mL using a volumetric flask. 125 mL of mixed filtrate solution was then heated to almost boiling and titrated with 0.05 M standardized $KMnO_4$ to form a pink color and persisted for 30 seconds. The calcium oxalate content in yellow konjac flour was calculated by Eq. 1.

$$Calcium\ Oxalate\ Content\ (\%) = \frac{T \times (Vme)(Df) \times 10^5}{(ME) \times Mf \times 1000} \quad (1)$$

Where: T is the volume of $KMnO_4$ used for titration (mL), $V_{me}$ is the equivalent mass volume (1 $cm^3$ 0.05 M $KMnO_4$ equivalent to 0.0025 g anhydrous oxalic acid), Df is

a dilution factor, ME is the molar equivalent of $KMnO_4$ solution (0.05), and Mf is the mass of the yellow konjac flour (g).




**Figure 1** The yellow konjac plants (*Amorphophallus muelleri* Blume) (A), Yellow konjac tubers (B), and the optimum of polished yellow konjac flour (OPYKF) (C).

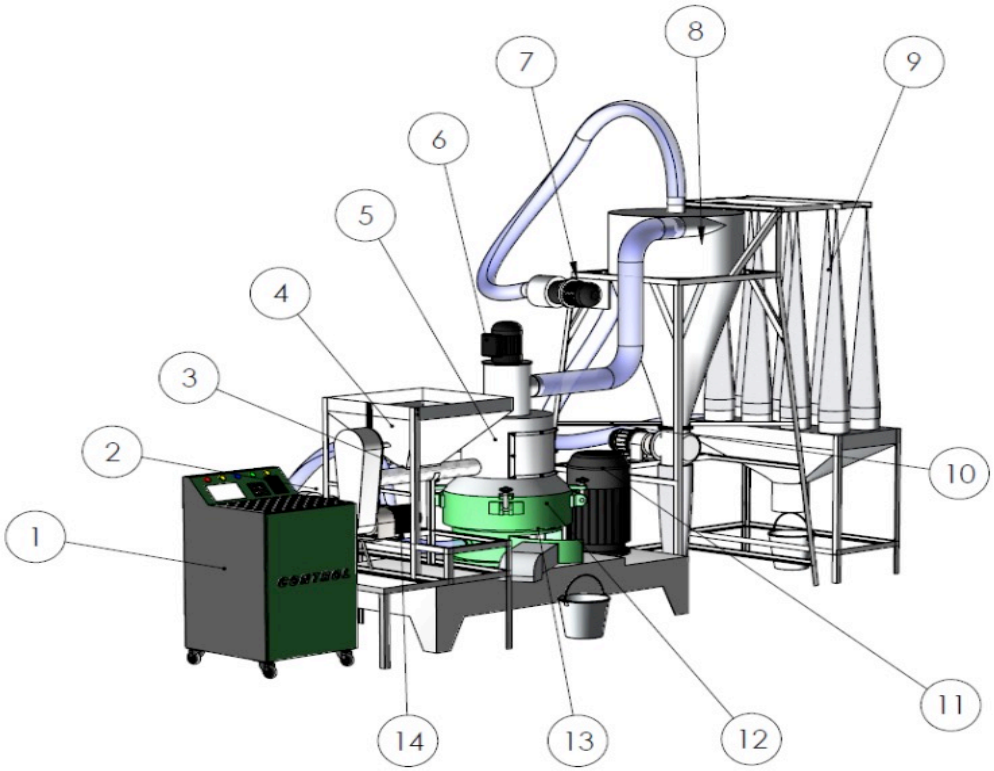


**Figure 2** The schematic drawing for centrifugal Mill (1) control panel, (2) suction blower, (3) screw feeder, (4) hopper input, (5) milling room, (6) fan blower, (7) blower cyclone, (8) cyclone separator, (9) dust collector, (10) motor for valve drive, (11) 3-phase motor, (12) milling room, (13) rotors, and (14) screw feeder motor.

### Determination of Viscosity

A solution (1% w/v) of yellow konjac flour was mixed using a constant temperature water-bath (75 °C) (Memmert, Germany) with a constant agitation speed (150 rpm) until perfectly hydrated **(Liu et al., 2002)**. The measurements were performed at room temperature using an NDJ-1 rotational viscometer with 12 rpm stirring speed and the spindle number 4 **(Witoyo et al., 2020)**.

### Determination of Degree of Whiteness (DoW)

Yellow konjac flour color was evaluated using Minolta CR-10 (Minolta, Japan). The yellow konjac flour's surface was monitored and expressed as Hunter value (L, a, and b). The DoW was calculated based on measured Hunter value using Eq. 2 **(Witoyo, Widjanarko and Argo, 2019)**.

$$\text{Degree of Whiteness} = 100 - \sqrt{(100-L)^2 + a^2 + b^2} \qquad (2)$$

### Determination of Glucomannan Content

With D-glucose as a standard curve, the spectrophotometric method was used to determine the glucomannan content in the yellow konjac flour **(Chua et al., 2012)**. In brief, 200 mg of yellow konjac flour sample was dissolved in 50 mL of NaOH-formic acid buffer, and mixed overnight at room temperature. The mixture was dissolved using a NaOH-formic acid buffer to 100 mL using a volumetric flask, and followed by centrifugation for 4000 rpm for 15 minutes to obtain glucomannan extract. The glucomannan hydrolyzate was obtained by hydrolyzing 5 mL of glucomannan extract with sulfuric acid (2.5 mL, 3 M), followed by boiling for 1.5 h in a water-bath. The mixture was cooled and added with NaOH solution (2.5 mL, 6M). The mixture was diluted again to 25 mL in a volumetric flask using distilled water. The absorbance of both the glucomannan extract and hydrolyzate was measured using a spectrophotometer at 540 nm. Compared with the D-glucose standard curve to obtained the glucose content (mg) in both samples. The calculation of glucomannan content (% d.b.) was following Eq. 3.

$$\text{GM content (\% d.b.)} = \frac{0.9 \times (5T - T_0) \times f}{m \times (100 - w)} \times 100 \qquad (3)$$

Where: m is the mass of yellow konjac flour (mg), w is water content in yellow konjac flour (%), $T_o$ is glucose content in yellow konjac flour extract (mg), T is glucose content in yellow konjac flour hydrolysate (mg), and f is dilution factor.

All physicochemical properties of 13 samples of polished yellow konjac flour (calcium oxalate content, viscosity, DoW, and glucomannan content) were analyzed in duplicate, as shown in Table 3.

### Experimental Design and Statistical Analysis

The CCD-RSM was applied in the polishing process of MMYKF using the centrifugal mill. At the same time, the MMYKF mass and polishing cycle were the independent variables. Following the report by **Witoyo et al. (2020)**, this study's central point was 12.5 kg with five times for MMYKF mass and polishing cycle, respectively. The range of independent variables used in this study is shown in Table 1. Physicochemical data of 13 randomized polishing processes were analyzed using Design-Expert 10 trial versions (State Ease, Inc.) based on multivariate analysis. The quadratic equation was used to calculate non-linear models of the observed system **(Faridah and Widjanarko, 2013)**, as shown in Eq 4. The appropriate model was determined based on $R^2$, adjusted $R^2$, lack of fit, and *p*-value of the model from the Design-Expert program's calculation **(Sugiono et al., 2019)**. The verification (Table 6) was carried out with three replications under the optimum polishing conditions prediction from the laboratory's Design-Expert program. The software predictions and verification data were analyzed and compared by paired T-test using Minitab 17 (Stat View, USA) to determine the optimum polishing conditions' accuracy.

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1,j=2}^{k-1,k} \beta_{i,j} X_i X_j + \varepsilon \qquad (4)$$

Where $\varepsilon$ is a random error, k is the number of independent parameters, $X_i$ and $X_J$ are the coded independent variables of MMYKF mass and polishing cycle, $\beta_0$, $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the regression coefficients of the intercept, linearity, quadratic, and interaction of the treatment, respectively, and Y is the observed response.

## RESULTS AND DISCUSSION

### Time of Polishing

The polishing time of each run of polishing is shown in Table 2. Table 2 shows that the MMYKF mass and number of polishing cycles increased the time of polishing. The longest polishing time was found at MMYKF mass of 15 kg and a polishing cycle of seven times with 59.55 minutes, whereas the shorter polishing time was found at MMYKF mass of 12.5 kg and the polishing cycle of two times with 16.25 minutes.

### Model Fitting and Selection

In this study, MMYKF mass ($X_1$) and polishing cycle ($X_2$) were applied to optimizing four responses of PYKF. The four PYKF responses were calcium oxalate, viscosity, DoW, and glucomannan content (Table 3). The selection model was evaluated for each response by the $R^2$, adjusted $R^2$, lack of fit, and *p*-value. Considering the criteria, the quadratic model was suitable and adequate to represents data from all responses of PYKF with a *p*-value <0.05. Table 4 shows the fitting model, coefficient regression, and significance of the second polynomial order for all responses. The $R^2$ of quadratic models were 0.9680, 0.9647, 0.9469, and 0.9808 for calcium oxalate, viscosity, degree of whiteness, and glucomannan content, respectively. Moreover, the adjusted $R^2$ value for calcium oxalate, viscosity, DoW, and glucomannan content in the sequence were 0.9452, 0.9394, 0.9090, and 0.9672. The higher $R^2$ and adjusted-$R^2$ indicated the quadratic model's adequacy to explain all response data **(Mohapatra and Bal, 2010)**. Moreover, for all responses the lack of fit ($p \geq 0.05$) values were calculated, indicating that the quadratic model was adequate to predict all observed responses **( Faridah, 2016; Mohapatra and Bal, 2010)**. The lack of fit for calcium oxalate, viscosity, DoW, and glucomannan responses were 0.7053, 0.3746, 0.2652, and 0.0521, respectively.

**Table 1** Range of polishing process.

| Independent Variable | Unit | -α | -1 | 0 | +1 | +α |
|---|---|---|---|---|---|---|
| Micro-mill Milled Yellow Konjac Flour (MMYKF) Mass | Kg | 8.96 | 10 | 12.5 | 15 | 16.04 |
| Polishing Cycle | Number of cycles | 2 | 3 | 5 | 7 | 8 |

**Table 2** The total time polishing each run.

| Std | Run | Actual Variable | | Coded Variable | | Total time of polishing (Minutes) |
|---|---|---|---|---|---|---|
| | | MMYKF Mass (Kg) | Polishing Cycle (Number of cycles) | $X_1$ | $X_2$ | |
| 2 | 1. | 15.00 | 3.00 | 1.00 | -1.00 | 27.62 |
| 13 | 2. | 12.50 | 5.00 | 0.00 | 0.00 | 37.77 |
| 8 | 3. | 12.50 | 8.00 | 0.00 | 1.41 | 57.78 |
| 12 | 4. | 12.50 | 5.00 | 0.00 | 0.00 | 37.00 |
| 1 | 5. | 10.00 | 3.00 | -1.00 | -1.00 | 17.88 |
| 5 | 6. | 8.96 | 5.00 | -1.41 | 0.00 | 24.57 |
| 10 | 7. | 12.50 | 5.00 | 0.00 | 0.00 | 37.35 |
| 6 | 8. | 16.04 | 5.00 | 1.41 | 0.00 | 46.92 |
| 4 | 9. | 15.00 | 7.00 | 1.00 | 1.00 | 59.55 |
| 3 | 10. | 10.00 | 7.00 | -1.00 | 1.00 | 38.53 |
| 7 | 11. | 12.50 | 2.00 | 0.00 | -1.41 | 16.25 |
| 11 | 12. | 12.50 | 5.00 | 0.000 | 0.000 | 36.97 |
| 9 | 13. | 12.50 | 5.00 | 0.000 | 0.000 | 37.77 |
| Ver.* | Ver.* | 10.00 | 6.00 | -1.00 | 0.586 | 35.56 ±0.35 |

Note: * Ver. means: Verification experiments.

The Effect of Independent Variables on Physicochemical Properties of PYKF

***Calcium Oxalate***

Calcium oxalate is one of the non-glucomannan components that is surrounding glucomannan cell granules and is toxic when consumed; thus, it needs to be removed. Calcium oxalate content in PYKF ranged from 0.45 to 1.12 % w.b. The present study results were higher than the data reported by **Faridah (2016)** and lower than the research data by **Witoyo, Widjanarko and Argo (2019)**. In this study, calcium oxalate was affected merely by the number of polishing cycles, as illustrated in Figure 3A. Calcium oxalate content decreased quadratically as a function of the polishing cycle increased. The decrease of calcium oxalate might be caused by erosion of non-glucomannan compounds covering the surface of yellow konjac flour granule during the polishing process, due to the frictional force between the rotor on the centrifugal mill and yellow konjac flour, and between yellow konjac flour, followed by the continuous fractionation process during the process **(Witoyo et al., 2020)**. According to **Nurhasanah, Antarlina and Syah (2017)** and **Nurhasanah et al. (2019)**, the polishing process can reduce the anti-nutritional compounds (tannins) in whole sorghum grains. **Wachyuningsih (2011)** stated that polishing yellow konjac flour using an abrasive polisher reduces the calcium oxalate in yellow konjac flour. Moreover, the calcium oxalate of PYKF was not affected by the MMYKF mass.

***Viscosity***

Viscosity is one of the important indicators in the utilization of yellow konjac flour in various applications, particularly in food products, as thickening agents, gelling agents, stabilizers, emulsifiers **(Impaprasert, Borompichaichartkul and Srzednicki, 2014)**, and fat replacers **(Jiménez-Colmenero et al., 2013)**, which require high viscosity. The viscosity of PYKF was in the range of 11.125 – 20.750 cP. This data was higher than the data reported by **Faridah (2016)** and lower than the data reported by **Xu et al. (2014)**. The data finding indicated the PYKF included in the second the first-grade classification for common konjac flour, which the Ministry of Agriculture issued, People's Republic of China **(Liu et al., 2002)**. The polishing cycle performed a quadratic effect on PYKF viscosity, as illustrated in Figure 3B. The PYKF viscosity increased five times in the polishing cycle and decreased afterward.

**Table 3** Physicochemical properties of Polished Yellow Konjac Flour (PYKF) using CCD-RSM.

| Std | Run | Actual Variable | | Coded Variable | | Responses | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MMYKF Mass (Kg) | Polishing Cycle (Number of cycles) | $X_1$ | $X_2$ | Calcium Oxalate (% w.b) | Viscosity (cP) | DoW | Glucomannan Content (% d.b.) |
| 2 | 1. | 15.00 | 3.00 | 1.00 | -1.00 | 0.95 | 15375.00 | 60.37 | 64.38 |
| 13 | 2. | 12.50 | 5.00 | 0.00 | 0.00 | 0.73 | 19125.00 | 61.41 | 69.18 |
| 8 | 3. | 12.50 | 8.00 | 0.00 | 1.41 | 0.45 | 17500.00 | 62.38 | 66.04 |
| 12 | 4. | 12.50 | 5.00 | 0.00 | 0.00 | 0.61 | 20750.00 | 61.83 | 69.92 |
| 1 | 5. | 10.00 | 3.00 | -1.00 | -1.00 | 0.89 | 15750.00 | 61.01 | 64.98 |
| 5 | 6. | 8.96 | 5.00 | -1.41 | 0.00 | 0.61 | 20125.00 | 61.78 | 69.19 |
| 10 | 7. | 12.50 | 5.00 | 0.00 | 0.00 | 0.61 | 20125.00 | 61.71 | 69.65 |
| 6 | 8. | 16.04 | 5.00 | 1.41 | 0.00 | 0.73 | 19750.00 | 61.65 | 68.84 |
| 4 | 9. | 15.00 | 7.00 | 1.00 | 1.00 | 0.56 | 17843.75 | 61.80 | 66.59 |
| 3 | 10. | 10.00 | 7.00 | -1.00 | 1.00 | 0.50 | 18875.00 | 62.34 | 67.34 |
| 7 | 11. | 12.50 | 2.00 | 0.00 | -1.41 | 1.12 | 11125.00 | 59.94 | 60.72 |
| 11 | 12. | 12.50 | 5.00 | 0.000 | 0.000 | 0.61 | 20500.00 | 61.78 | 69.63 |
| 9 | 13. | 12.50 | 5.00 | 0.000 | 0.000 | 0.67 | 19875.00 | 61.84 | 69.43 |

**Table 4** Fitting model, coefficient regression and significance of second polynomial order for PYKF's responses.

| Coefficient Regression | Calcium Oxalate (% w.b.) | Viscosity (cP) | DoW | Glucomannan (% d.b.) |
|---|---|---|---|---|
| **Intercept, $\beta_0$** | +0.65 | +20075.00 | +61.71 | +69.56 |
| **Linear** | | | | |
| $\beta_1$ (MMYKF Mass) | $+0.034^{ns}$ | $-242.07^{ns}$ | $-0.17^{ns}$ | $-0.23^{ns}$ |
| $\beta_2$ (Polishing Cycle) | -0.22* | +1826.17* | $+0.77^{*}$ | +1.51* |
| **Quadratic** | | | | |
| $\beta_1^2$ | $+0.011^{ns}$ | $-109.77^{ns}$ | $-0.014^{ns}$ | $-0.37^{ns}$ |
| $\beta_2^2$ | +0.067* | -2922.27* | -0.29* | -3.18* |
| **Interaction** | | | | |
| $\beta_{12}$ | $+7.752E\text{-}005^{ns}$ | $-164.06^{ns}$ | $+0.024^{ns}$ | $-0.039^{ns}$ |
| **Fitting Model** | | | | |
| $R^2$ | 0.9680 | 0.9647 | 0.9469 | 0.9808 |
| Adjusted $R^2$ | 0.9452 | 0.9394 | 0.9090 | 0.9672 |
| Lack of Fit | $0.7053^{ns}$ | $0.3746^{ns}$ | $0.2652^{ns}$ | $0.0521^{ns}$ |
| *P*-value | <0.0001 | <0.0001 | 0.0003 | <0.0001 |

Note: *significant, $^{ns}$not significant. Equation: $\beta_0 + \beta x_1 + \beta x_2 + \beta_{12} x_1 x_2 + \beta_1 x_1^2 + \beta_2 x_2^2$ .

In the study, glucomannan content affected the PYKF's viscosity. The higher the glucomannan content in PYKF the higher viscosity of its. Based on **Faridah (2016)** and **Wardhani et al. (2016)**, viscosity is positively correlated with glucomannan enclosed in yellow konjac flour. **Ryan, Yuan and Crosby (2004)** added that glucomannan had a higher viscosity than other polysaccharides at the same concentration. Besides, the MMYKF mass had not affected the viscosity of PYKF.

***Degree of Whiteness (DoW)***

The DoW of PYKF was in the range of 59.94 – 62.38. This result was lower than the results reported by **Impaprasert, Borompichaichartkul and Srzednicki (2014)** and higher than data reported by **Witoyo, Widjanarko and Argo (2019)** and **Witoyo et al. (2020)**. The DoW of PYKF was merely affected by the polishing cycle, as illustrated in Figure 3C. The polishing cycle provided a positive quadratic trend to PYKF's DoW. Decreasing impurity (non-glucomannan) components covering yellow konjac flour granules during the polishing process increase PYKF DoW. This result was consistent with the previous study by Nurhasanah and her team who claimed that the polishing process can increase the DoW of sorghum using abrasive polishing machines or modification of polishing machines with three-cylinder levels **(Nurhasanah, Antarlina and Syah, 2017; Nurhasanah et al., 2019)**. **Reddy et al. (2017)**, and **Paiva et al. (2016)** noted that improving color or DoW was closely related to the reduction of certain impurities during the milling process. **Witoyo et al. (2020)** reported that that the content of non-glucomannan compounds, such as proteins, fat, calcium oxalate, and starch had decreased during the polishing process of yellow konjac flour. However, in the present study, the MMYKF mass had a not significant effect on PYKF's DoW.

***Glucomannan Content***

Glucomannan is the most important parameter to determine the quality of yellow konjac or konjac flour based on international standards. According to the international standard for konjac flour, particularly concerning common konjac flour, glucomannan is divided into three classes, namely top-grade, first-grade, and second-grade with glucomannan content ≥75%, ≥65%, and ≥60% d.b, respectively **(Liu et al., 2002)**. Based on these standards, PYKF is ranking second in the category of first-grade common konjac flour with glucomannan content in the range of 60.72 – 69.92% d.b. This result is lower than KGM content by wet extraction from yellow konjac flour, as reported by **Impaprasert, Borompichaichartkul and Srzednicki (2013); Wardhani et al. (2016)** and similar to **Faridah, Widjanarko and Sutrisno (2011)** study.

In the present study, the polishing cycle affected glucomannan content. The number of polishing cycles exerted a quadratic effect on the glucomannan response, as shown in Figure 3D. The increase of the number of polishing cycles to five enhanced the glucomannan content, then decreased subsequently. The increased number of polishing cycles reduces distinct compounds, such as calcium oxalate, starch, protein, and other impurities surrounding the glucomannan cells in yellow konjac flour, due to friction between rotors of the centrifugal mill and flour as well as between the flour particles. According to **Witoyo et al. (2020)**, the polishing process destroys non-glucomannan compounds on the glucomannan cell surface in yellow konjac flour. Subsequently, the non-glucomannan components were detached and separated from glucomannan granules using a cyclone separator. Moreover, glucomannan content decreased after polishing treatment under the optimum conditions due to attrition of larger glucomannan particles that turned into fine particles and were eliminated in the cyclone separator. This finding was supported by **Faridah, Widjanarko and Sutrisno (2011)** and **Wachyuningsih (2011)** after milling yellow konjac chips using the stamp mill, and polishing yellow konjac flour using an abrasive polisher, respectively. Furthermore, the MMYKF mass had not affected the glucomannan of PYKF.

***Optimization and Verification of Prediction***

Polishing parameters were optimized to produce PYKF (Figure 1C) with high viscosity, DoW, glucomannan content, and low calcium oxalate content by using the centrifugal mill. The independent variables and response criteria to achieve the desired characteristics of PYKF are compiled in Table 5. The predicted optimum polishing condition was obtained at 10.00 kg and 6.17 polishing cycles (Table 6), with the following characteristics: 0.52 ±0.00% w.b. calcium oxalate, 20362.00 ±16.00 cP viscosity, 62.22 ±0.01 DoW, and 69.43 ±0.02% d.b. glucomannan content with desirability value of 0.927 (Figure 3E). The verification with modified conditions (Table 6) obtained the following characteristics: 0.56 ±0.05 % w.b. calcium oxalate, 20208.00 ±564.00 cP viscosity, 62.16 ±0.29 DoW, and 68.18 ±1.28% d.b. glucomannan content. Consequently, the verification data revealed no significant differences ($p$-value >0.05) for all responses compared to predicted values. This shows that the quadratic model was accurate and appropriate for optimizing yellow konjac flour polishing using the centrifugal mill **(Mohapatra and Bal, 2010)**. The desirability value of 0.927 expressed the prediction accuracy of the optimum polishing conditions equivalent to 92.70%.

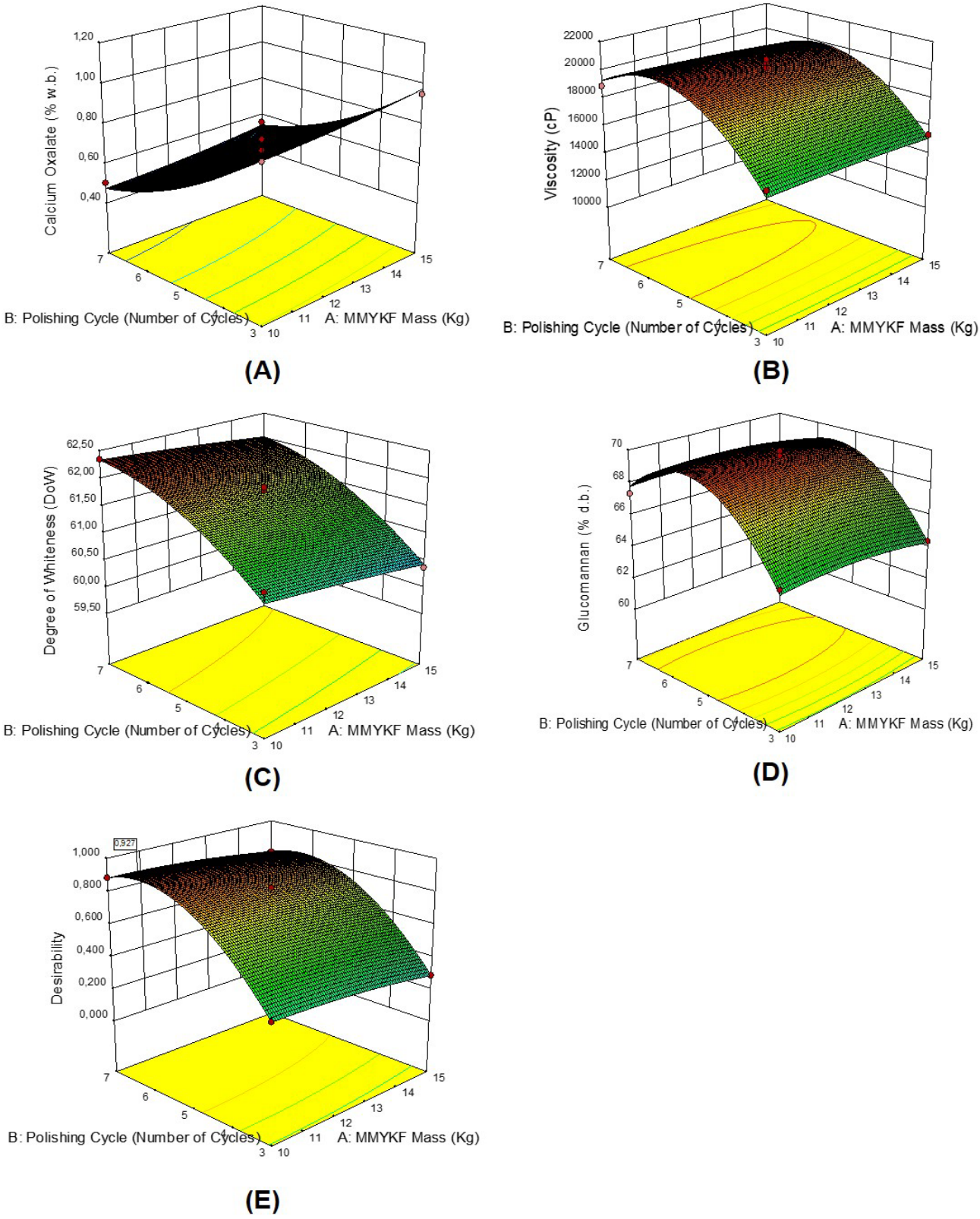


**Figure 3** The 3D-surface plot of calcium oxalate (A), viscosity (B), DoW (C), and glucomannan (D), and desirability of the optimum conditions (E) as affected by MMYKF mass and polishing cycle.

**Table 5** The independent variables, and responses criteria for polishing optimization.

| Constraints | Goal | Lower Limit | Upper Limit | Importance |
|---|---|---|---|---|
| **Independent Variable** | | | | |
| MMYKF Mass | Is within range | 10 | 15 | 3 |
| Polishing Cycle | Is within range | 3 | 7 | 3 |
| **Response** | | | | |
| Ca-Oxalate | Minimize | 0.45 | 1.12 | 3 |
| Viscosity | Maximize | 11125 | 20750 | 3 |
| DoW | Maximize | 59.94 | 62.38 | 3 |
| Glucomannan | Maximize | 60.72 | 69.91 | 3 |

**Table 6** The comparison of the software prediction and actual verification for all PYKF responses.

| | MMYKF Mass (Kg) | Polishing Cycle (Number of cycles) | Calcium Oxalate (% w.b.) | Viscosity (cP) | DoW | Glucomannan (% d.b) | Desirability |
|---|---|---|---|---|---|---|---|
| Software Prediction | 10.00 | 6.17 | 0.52 ±0.00 | 20362.00 ±16.00 | 62.22 ±0.01 | 69.43 ±0.02 | 0.927 |
| Verification | 10.00 | 6.00 | 0.56 ±0.05 | 20208.00 ±564.00 | 62.16 ±0.29 | 68.18 ±1.28 | - |
| | *P*-Value | | 0.305 | 0.678 | 0.791 | 0.219 | - |

## CONCLUSION

The optimization of yellow konjac flour polishing using a centrifugal mill was successfully investigated. In the present study, all responses of PYKF were only affected by the number of polishing cycles. The quadratic model represented all the responses of PYKF, with $R^2$ ≥0.94, and *Adjusted*-$R^2$ ≥0.90. The optimum polishing condition using a centrifugal mill was obtained at MMYKF mass of 10 kg and six polishing cycles, with characteristics as follow: 0.56 ±0.05% w.b. calcium oxalate, 20208 ±564 cP viscosity, 62.16 ±0.429 DoW, and 68.18 ±1.28% d.b. glucomannan content. As a result, polishing using a centrifugal mill appears feasible and promises to be scaled up to industrial scale for yellow konjac flour polishing before the wet extraction process.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# EVALUATION OF VAPOR-PHASE ANTIFUNGAL ACTIVITIES OF SELECTED PLANT ESSENTIAL OILS AGAINST FUNGAL STRAINS GROWING ON BREAD FOOD MODEL

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## ABSTRACT

The current study aimed to investigate antifungal activities of two commercially available essential oils (EOs), specifically Tea tree oil (*Melaleuca alternifolia*; TTEO) and St. John's wort oil (*Hypericum perforatum*; HPEO) against three *Penicillium* (*P.*) species: *P. citrinum*, *P. expansum*, and *P. crustosum* in *in situ* conditions. For this purpose, EOs were applied in the vapor phase to determine the growth inhibition of fungi artificially inoculated on sliced bread. Changes in colony growth rate were evaluated as markers for the mycelial growth inhibition (MGI) effect of the EOs. The antioxidant activities of the EOs were evaluated using the DPPH method. The moisture content (MC) and water activity ($a_w$) of bread as a substrate for fungal growth were also measured. From the DPPH assay, we have found that both EOs (TTEO, HPEO) exhibited strong antioxidant activity (64.94 ±7.34%; 70.36 ±1.57%, respectively). The values for bread MC and $a_w$ were 43.01 ±0.341% and 0.947 ±0.006, respectively. Our results suggest that HPEO is the only weak inhibitor of *P. citrinum* and *P. crustosum* colony growths. Also, the highest concentrations of TTEO display only the weak capability of mycelial growth inhibition of *P. citrinum* and *P. crustosum*. By contrast, the colony growth of *P. expansum* was enhanced by both EOs at all levels used. In conclusion, the application of both EOs in the vapor phase against selected *Penicillium* species seems not to be a promising alternative to chemical inhibitors used for bread preservation.

**Keywords:** essential oil; *Penicillium* sp.; antioxidant activity; antifungal activity; bakery product

## INTRODUCTION

Bread is a staple cereal-based product consumed worldwide. The shelf life of bread is reduced due to the action of many factors such as its moisture content (MC), water activity ($a_w$), and storage conditions **(Legan, 1993)**. Bread spoilage is often caused by microscopic fungi, among which the most common species are *Penicillium* (*P.*), *Aspergillus*, *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium*, and *Rhizopus* **(Passarinho et al., 2014)**. Although *P. expansum* is mainly associated with apple spoilage, many studies focused on the antimicrobial properties of bread have selected *P. expansum* as the target form because of its high resistance as compared to other microscopic fungi and mycotoxin production, as well **(Balaguer et. al., 2013; Belz et al., 2012; Luz et al., 2018)**.

To solve the problem with the shelf life of bakery products, diverse preservatives (such as propionic acid or sorbic acid) are added to the dough. Currently, however, most consumers avoid chemical preservatives present in daily foods in the context of a healthy lifestyle. Therefore, the use of EOs in packaging materials vaporized in the gas phase can be a solution for this concern **(Krisch, Tserennadmid and Vágvölgyi, 2011)**.

Essential oils (EOs) are defined as odorous and volatile products of plant secondary metabolism which are applicable in almost every field of life including folk medicine, food flavorings, and preservation **(Kalemba and Kunicka, 2003)**. These liquid, volatile, pure, and colored mixtures of several aromatic compounds are obtained from the different parts of plants, mainly from herbs and spices. However, various new sources of EOs such as food or secondary waste are currently being explored **(Ravindran and Jaiswal, 2016; Wu et al., 2017)**. EOs are extracted from different techniques and the most preferable method of extraction is hydrodistillation which is cheap and easy to use **(Irshad et al., 2020)**. About 3,000 different types of EOs are known to date, of which about 300 are in commercial use **(Burt and Reinders, 2003)**. Due to chemical composition, many EOs have strong antimicrobial activities, pharmacological and neuroprotective properties and some of them have been also shown to affect behavioral characteristics **(Lis-Balchin, Deans and Eaglesham, 1998)**.

Tea tree essential oil (TTEO) is obtained by distillation from the leaves and terminal branches of the narrow-leaf tea tree *Melaleuca alternifolia*. The EO is known mainly for its use in the treatment of many skin diseases. Also, it can be present in topical pharmaceuticals, cosmetics, and household products **(de Groot and Schmidt, 2016)**. The antifungal activity of TTEO is attributed to the presence of compounds such as terpinen-4-ol, α-terpineol, linalool, α-pinene, β-pinene, β-myrcene, and 1,8-cineole **(Puvača et al., 2018)**.

*Hypericum perforatum* (common St. John's wort) is one of the best-known and most commonly used herbs in recent years often associated with the treatment of anxiety and depression **(Lyles et al., 2017)**. In the plant, a wide range of biologically active substances such as naphthodianthrones **(Kitanov, 2001)**, acylphloroglucinol **(Verotta et al., 2000)**, xanthones **(Sparenberg, 1993)**, flavonoids **(Jürgenliemk and Nahrstedt, 2002)**, tannins **(Barnes, Anderson and Phillipson, 2001)**, and lipids **(Omarova and Artamonova, 1999)** has been identified. *Hypericum perforatu*m essential oil (HPEO) is composed mainly of 2-methyl octane, α-pinene, and caryophyllene **(Mathis and Ourisson, 1964; Schwob, Bessière and Viano, 2002)**. This EO is a popular home remedy for the recovery of cuts, burns as well as peptic ulcers **(Yeşilada et al., 1995)**.

In the current study, the antifungal activities of TTEO and HPEO against three *Penicillium* (*P.*) species (*P. expansum, P. crustosum*, and *P. citrinum*) inoculated on sliced bread were evaluated. To date, such research has not been done before our experiment. The efficacy of the selected EOs as antifungal preservatives in wheat bread will allow us to assess the possibility of their practical application as novel and safe preservatives.

## Scientific hypothesis

There is growing evidence that various types of essential oils (EOs) obtained from plants show effective antimicrobial and antifungal properties. Our study aimed to evaluate the antifungal properties of EOs (Tea tree oil and St. John's wort oil) against selected *Penicillium* species, providing the possibility of used these EOs for long self-life products in the food industry.

## MATERIAL AND METHODOLOGY

### Samples

Tea tree essential oil (TTEO) and St. John's wort (*Hypericum perforatum*) essential oil (HPEO) were purchased from Gratis Company (Ltd, Istanbul, Turkey).

### Chemicals

All chemicals were analytical grade and were purchased from Merck (Germany) and Sigma Aldrich (USA).

### Animals and Biological Material

The fungi *P. expansum*, *P. crustosum*, and *P. citrinum* were isolated from grape samples and identified with the MALDI-TOF MS Biotyper and 16S rRNA sequencing.

### Sample preparation:

Mass spectrophotometer **(**MALDI-TOF MS Biotyper, Bruker, United States).

Spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, United States).

Spiral mixer (Diosna SP 12 D, Diosna, Germany).

Fermentation cabinet (MIWE cube, Pekass s.r.o., Plzeň, Czech Republic).

Steamy oven (MIWE cube, Pekass s.r.o., Plzeň, Czech Republic).

Water activity analyzer (Lab Master $a_w$ Standard, Novasina, Switzerland).

Moisture analyzer (Kern DBS 60-3, Kern & Sohn GmbH, Germany).

### Laboratory Methods

#### *Radical Scavenging Activity—DPPH Method*

The radical scavenging activities of the EOs were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method according to **Sánchez-Moreno, Larrauri, and Saura-Calixto (1998)** with minor modifications. The 0.1 mL of each EO oil sample was mixed with 3.9 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the reaction mixture was determined with The Agilent Cary 60 UV-Vis spectrophotometer at 515 nm. The final scavenging activity was calculated as a percentage (AA%) and determined according to the following formula: AA% = [(A0 - AAT)/A0 x 100]; where A0 is the absorbance of the control reaction (DPPH radical); A1 is the absorbance of the tested sample.

#### *Bread making process*

The baking formula consisted of wheat flour T650 (250 g), water (150 mL), sucrose (2.5 g), salt (5 g), and yeast (5 g). All the ingredients were mixed in a spiral mixer for 6 min. The prepared dough was placed into an aluminum vessel and transferred to a fermentation cabinet at 32 °C and 85% relative humidity for 40 min. The loaves were baked in two stages: (i) at 180 °C for 17 min with the addition of 160 mL water and (ii) at 210 °C for 10 min in a steamy oven. Following the baking, the bread was left to stand at the laboratory temperature for 2 h and then analyzed.

#### *Water activity ($a_w$) and moisture content (MC) of bread*

Bread $a_w$ was measured using the Lab Master $a_w$ Standard. For this purpose, 2.0 g of crumbs was cut into a cube and placed into a sample pan. The value of $a_w$ was measured automatically at 25 °C for 15 – 20 min.

Bread MC was determined using a moisture analyzer Kern DBS 60-3. In this case, 1.0 g of crumbs was weighed on the sample plate and measurement was done at 120 °C for 10 – 15 min.

#### *Vapor-phase of the antifungal assay with bread*

The bread samples were cut into slices with a height of 150 mm and placed into 0.5 L sterile glass jars (Bormioli Rocco, Fidenza, Italy). A fungal spore of each strain was used for bread inoculation. Essential oil concentrations of 125, 250, and 500 $\mu L.L^{-1}$ (EOs + ethyl acetate) were evenly distributed on a sterile paper-filter disc (6 cm), which was inserted into the cover of the jar, except for the treatment of the control group. The jars were hermetically closed and kept at 25 °C for 14 days in the laboratory incubator. The colonies with visible mycelial growth and visible sporulation were evaluated. The antifungal activity was expressed as a percentage of mycelial growth inhibition (MGI) which was calculated using the formula: MGI = [(C – T) / C] * 100, where C = fungal growth in the control and T = fungal growth in the treatment group, as was

previously described **(Aman and Rai, 2015; Talibi et al., 2012)**.

**Description of the Experiment**

Sample preparation: 1
Number of samples analyzed: 3
Number of repeated analyses: 3
Number of experiment replication: 3

### Statistical analysis

MBIC50 and MBIC90 values (concentration causing 50% and 90% reduction of fungal growth) were estimated by the logit analysis for evaluation antifungal activity of EOs. In addition to MBIC50 and MBIC90 values, statistical evaluation 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

### Antioxidant activity of selected EOs

The scavenging ability of DPPH free radical is commonly used to evaluate the antioxidant potential of diverse plant extracts including EOs **(Fadel et al., 2020; Kačániová et al., 2020a; Kačániová et al., 2020b)**. Following the assay, the abilities of TTEO and HPEO to act as donors of hydrogen atoms or electrons in the transformation of DPPH radical into its reduced form DPPH-H were in our research investigated. As shown in Table 1, both EOs exhibited high free-radical scavenging activity through the used assay. Also, no significant difference ($p$ = 0.25) was observed between analyzed EOs.

In accordance with our study, the evident antioxidant capacity of TTEO (even stronger than vitamin E and α- tocopherol) was reported by **Zhang et al. (2018)**. In addition to this, **Kim et al. (2004)** have found that although terpinen-4-ol is the major bioactive chemical compound of TTEO, the majority of antioxidant activity in TTEO was attributed to the three terpenic compounds, i.e., α-terpinene, α-terpinolene, and γ-terpinene. The higher antioxidant activity of HPEO than TTEO determined in our research can be attributed to flavonoids and phenolic acids presented in HPEO molecular compositions. From them, hyperoside, rutin, quercitrin, and quercetin are the most abundant in *Hypericum perforatum* **(Orčić et al., 2011; Orhan and Kartal, 2015)**.

Generally, antioxidant properties of various bioactive substances including medicinal and aromatic plants play a key role in counteracting the deleterious impact of free radicals in biological systems **(Rajkapoor, Burkan and Senthilkumar, 2010; Pirbalouti et al., 2014)**. In foods (such as bread), poly-unsaturated fatty acids are particularly susceptible to oxidation by free radicals during the storage **(Donnelly and Robinson, 1995)**, manufacturing, distribution, and final preparation of foods. The oxidative changes can cause rancidity such as off-flavors, loss of color, altered nutrient value, and may produce toxic compounds with an adverse effect on the health of consumers **(Ahmed et al., 2016)**. We propose that the antioxidant capacity of TTEO and HPEO demonstrated in our study could have a positive effect on bread shelf life which could be attributed to their hydrogen donating ability consequently reducing the lipid oxidation in bread. However, to confirm the assumption, further analyses are needed.

### Moisture content and water activity of bread loaves

Generally, microscopic fungi can grow in conditions related to sufficient moisture and nutrition. Wheat bread is classified as intermediate moisture food (IMF). The product usually contains 20 – 50% of MC **(Vermeulen et al., 2015)**. In agreement with the findings, our bread as a substrate for the growth of *Penicillium* species had MC 43.01 ±0.341.

Water in food is a very important factor in the quality and safety of food products **(Al-Muhtaseb, McMinn and Magee, 2002)**. It is commonly observed that foods showing rapid degeneration due to chemical and biological changes are typically those with high MC **(Abdullah, Nawawi and Othman, 2000)**. Moreover, it has been recognized that the fungal growth of products is also significantly affected by $a_w$ **(Davey, 1989)**. Water activity in food is a term that expresses the availability of water to participate in physical, chemical, and microbial reactions **(Al-Muhtaseb, McMinn and Magee, 2002)**. According to **Labuza et al. (1972)**, $a_w$ above 0.7 is known to provide suitable conditions for fungal growth. Since an approximate value for $a_w$ in wheat bread is within the range of 0.94 to 0.97 **(Roos, Jouppila and Söderholm, 1999)**, which is in line with our finding ($a_w$ 0.947 ±0.006), the bread is susceptible to microbial spoilage with the main effect coming from the growth of various molds. Therefore, the shelf life of white bread is relatively short. Considering these facts, bread was chosen as a substrate for fungal growth in our experiment.

### In situ antifungal vapor contact assay

Essential oils and closely related components are well-known for their antimicrobial properties **(Chouhan, Sharma and Guleria, 2017; Wińska et al., 2019)**. As antimicrobial systems, EOs in the vapor phase be effective for many applications including the food industry **(Laird and Phillips, 2012)**. In our study, the antifungal activities of TTEO and HPEO against 3 *Penicillium* species artificially inoculated on bread were evaluated (Figure 1). The activities are presented as MGI in Table 2.

Our results revealed that the growing colony of *P. citrinum* was weakly reduced by all used concentrations of HPEO, and only slightly inhibited by high levels (≥250 $\mu L.L^{-1}$) of TTEO. The highest concentrations of both EOs are the only weak inhibitors of *P. crustosum* growth. On the other hand, the mycelial growth of *P. expansum* was stimulated by all concentrations of both EOs. In previous studies, we evaluated the antifungal effect of Coriander essential oil and Citrus aurantium essential oil against the same species of fungi (*P. citrinum, P. expansum, P. crustosum*). In contrast to current results, these essential oils have been found to have a strong inhibitory effect on fungal growth. These differences may be due to other types of essential oils used, which have different chemical compositions **(Kačániová et al., 2020a, Kačániová et al., 2020b)**.

**Table 1** Antioxidant activity of chosen EOs used in experimental design.

| EOs | Antioxidant activity (%) |
|---|---|
| **TTEO** | 64.94 ±7.34 |
| **HPEO** | 70.36 ±1.57 |

Note: The values are expressed mean ± standard deviation. Values in the same column are not significantly different.

**Table 2** Percentage of mycelial growth inhibition (MGI) of *Penicillium* strains expressing the antifungal potential of investigated EOs against the artificially inoculated fungi on bread.

| Fungi strains | MGI (%) | | | | | |
|---|---|---|---|---|---|---|
| | TTEO (µL.L$^{-1}$) | | | HPEO (µL.L$^{-1}$) | | |
| | **125** | **250** | **500** | **125** | **250** | **500** |
| *P.crustosum* | -6.72 | -6.11 | 3.05 | -28.11 | 0.00 | 4.68 |
| *P. citrinum* | -11.36 | 8.61 | 11.36 | 16.87 | 22.38 | 23.24 |
| *P. expansum* | -9.72 | -9.29 | -6.70 | -36.29 | -33.26 | -32.40 |

Note: The negative values indicate a profungal activity against *Penicillium* strains.

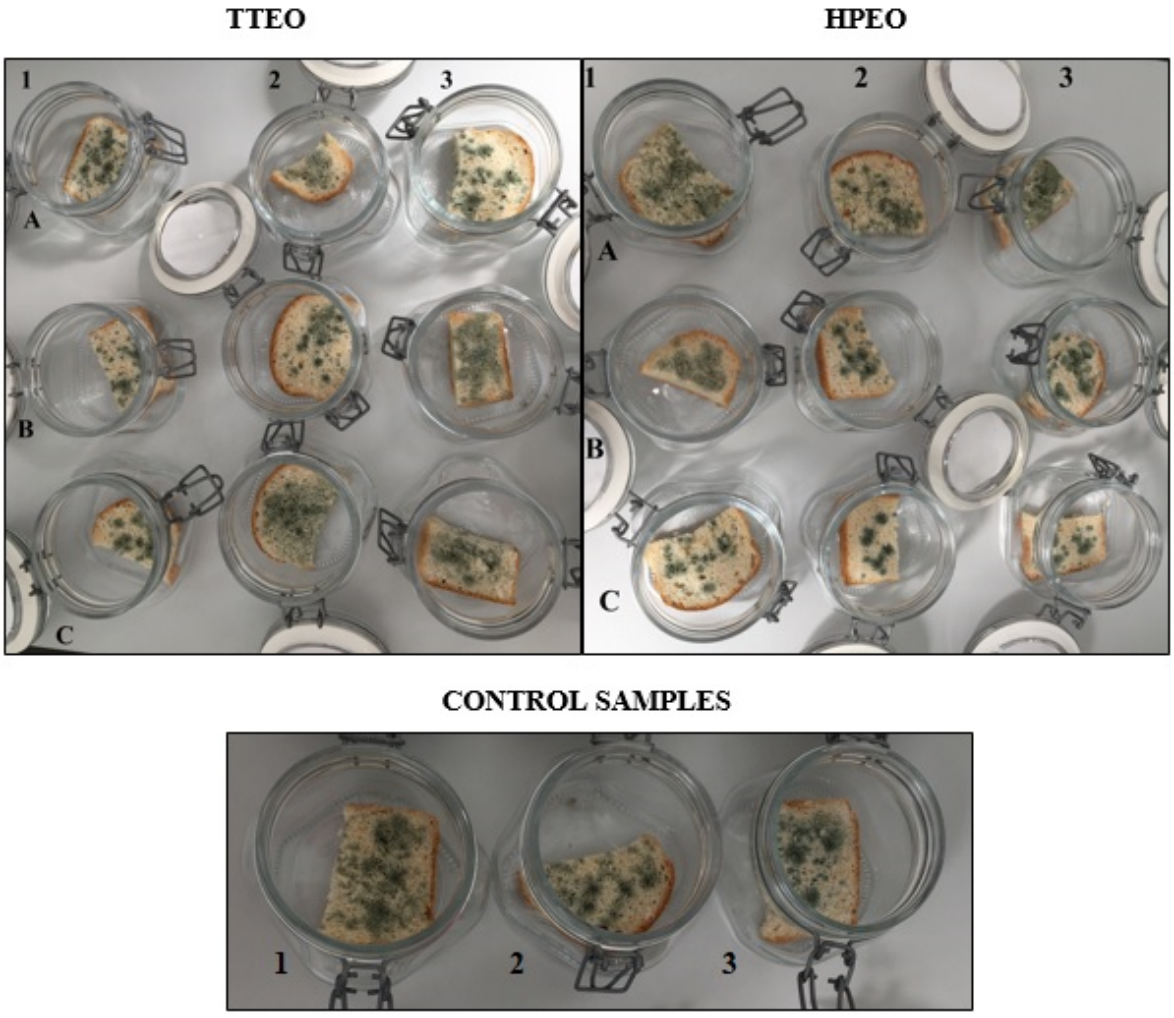


**Figure 1** *In situ* antifungal analyses of bread inoculated with *Penicillum* spp. (1 – *P. citrinum*; 2 – *P. expansum* and 3 – *P. crustosum*) after their treatment with different TTEO and HPEO concentrations (A – 125 µL.L$^{-1}$; B – 250 µL.L$^{-1}$ and C – 500 µL.L$^{-1}$).

Generally, EOs exert various mechanisms to inhibit the growth of pathogens. For instance, due to the hydrophobicity of their compounds (such as terpenoid derivatives), they can penetrate the lipid bilayer membrane of the cells leading to higher cell permeability and leakage of vital cell contents **(Burt, 2004)**. As a result, disruption of cellular functions such as inhibition of ionic transport and cellular respiration is observed in treated microorganisms **(Cox et al., 2001)**. Also, their entrance to the cell membrane may be associated with swelling and reducing membrane function inducing cell death **(Patel et al., 2005)**. The study by **Cox et al. (2000)** has demonstrated the ability of TTEO to disrupt the permeability barrier of microbial membrane structures as a mechanism of its action. The main compound responsible for the antifungal activity of TTEO was shown to be terpinen-4-ol **(Yu et al., 2015)**. In contrast to our study, the antifungal activity of *Melaleuca alternifolia* extract was displayed against *P. chrysogenum* **(Rogawansamy et al., 2015)**, *P. italicum Wehmer*, *P. digitatum Sacc.* **(Zhang et al., 2018)**, *P. verrucosum* and *P. funiculosum* **(Chidi, Bouhoudan and Khaddor, 2020)**. Taking into account all the aspects we propose that the slight degree of antifungal activity of TTEO against *P. citrinum* and *P. crustosum* investigated in our study could be connected with the weak capacity of the EO compounds to penetrate the cellular membranes and to inhibit the growth of the fungi. Moreover, the findings by **Li et al. (2017)** showed dose-dependent strong inhibition of mycelial growth of *P. expansum* (inoculated on cherry fruits) by TTEO which strongly contradicts our study. However, this discrepancy with our results can be linked to different chemical composition, used TTEO concentrations, as well as food substrates.

Against *P. citrinum*, higher antifungal activity of HPEO than TTEO (at all used levels) was found in our research. *Hypericum perforatum* is an antifungal inhibitor that was also demonstrated against *P. canescens* **(Milosevic, Solujic and Sukdolak, 2007)** and *P. funiculosum* **(Rančić et al., 2005)**. Besides, the antifungal activity of *Hypericum* species was reported also against *P. verrucosum* **(Grafakou et al., 2020)**. The antifungal potential of EOs from many *Hypericum* species is attributed to their dominant components, such as α- and β-pinene, as well as β-caryophyllene **(Crockett, 2010)**. The compounds could also contribute to slightly mycelial growth inhibition of *P. citrinum* in our experiment.

## CONCLUSION

Our study suggests that although EOs from Tea tree and St. John's wort exhibit strong antioxidant activity, they are in vapor phase-only slight inhibitors of *P. citrinum* growth in *in situ* conditions. Against other tested fungal strains (*P. crustosum* and *P. expansum*), their very low or even no growth inhibitory potentials were reported. Thus, our results allow for the conclusion that TTEO and HPEO in investigated concentrations are not suitable alternatives to the chemical inhibitors of fungal growth on bread substrate.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE POTENTIAL OF RED DRAGON FRUIT PEEL YOGURT TO IMPROVE PLATELET LEVELS IN HEPARIN-INDUCED THROMBOCYTOPENIA IN WISTAR RATS

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## ABSTRACT
Patients infected with the dengue virus will develop thrombocytopenia which can cause bleeding and complications. One of the materials that contain antioxidants and have potential as a functional food is red dragon fruit peel. This peel can be processed into yogurt as a way to increase antioxidant function which ultimately supports the immune system of its users. This study analyzed the effect of red dragon fruit peel yogurt on the platelet levels of thrombocytopenic Wistar rats. It used a pre-post-test control group design. Male Wistar rats were randomly assigned into seven groups: K-; K+; and five treatment groups that received dragon fruit peel yogurt at doses of 5% (K1); 10% (K2); 15% (K3); 20% (K4); and 25% (K5). Thrombocytopenia was induced by 0.1 mL.100g$^{-1}$ BW of heparin for 3 days. The intervention was carried out for 28 days. The result showed that all groups had significant differences before and after the intervention ($p$ <0.05). Tukey analysis showed that there were significant differences in all groups ($p$ <0.05). Yogurt containing 25% red dragon fruit peel provides an effective dose for improving platelet levels in thrombocytopenic rats.

**Keywords:** platelets; red dragon fruit peel; thrombocytopenia; Wistar; yogurt

## INTRODUCTION
Dengue virus infection is one of the infections caused by mosquitoes. It is spread over all geographic areas and causes 100 million dengue infections every year. Deaths due to infectious diseases decreased from 2005 to 2015, but deaths due to the dengue virus increased by 48% **(Malavige and Ogg, 2017)**. Infection caused by the dengue virus can cause dengue haemorrhagic fever (DHF). Phases in a patient with dengue include the febrile phase, the critical phase, and the recovery phase. The febrile phase occurs after an incubation process of 3 to 7 days and the patient can recover without experiencing complications. The febrile phase is an early phase characterized by an increase in body temperature to ≥38.5 °C, headache, vomiting, myalgia, joint pain, and transient macular rash. The critical phase is characterized by persistent vomiting, increased pain in the abdominal area, tender hepatomegaly, increased levels of haematocrit that coincide with a rapid decrease in platelet count, serous effusions, mucosal bleeding, and lethargy or restlessness. The recovery phase is characterized by changes in vascular permeability and occurs throughout 48 – 72 h **(Simmons et al., 2012)**.

Dengue virus infection (DENV) occurs through the transmission of the DENV serotype (DENV 1 – 4) by the *Aedes aegypti* mosquito. These four serotypes allow for asymptomatic infection or classic symptoms of dengue fever. Platelets are an important blood component in the coagulation process. Patients infected with DENV will develop thrombocytopenia which can cause bleeding and complications in the organs of the body. DENV will suppress bone marrow and decrease platelet production, and directly infect megakaryocytes **(Castilho et al., 2020)**.

Studies show that there are haematological changes in patients with dengue. These include thrombocytopenia, leukopenia, lymphocytosis, and lymphocytopenia **(de Azeredo, Monteiro and de Oliveira Pinto, 2015)**. Patients with dengue infection may develop iatrogenic complications. Patients who have severe dengue infection should routinely have haematocrit measurements including fluid monitoring. An alternative approach to control the *A. aegypti* vector is to use sterilized male mosquitoes to control the female mosquito population by reducing egg production so that the transmission of the dengue virus can be suppressed. This strategy involves the intracellular bacterium *Wolbachia* **(Candra, 2010)**.

Food intake plays a role in maintaining immunity for people with dengue infection. Food and fluids are non-pharmacological therapies to prevent and improve various risk factors associated with infectious manifestations due to dengue. Nutritional therapy related to changes in haematological conditions can be an alternative to prevent worsening of the condition of people with dengue infection.

A symbiotic product can be classed as a functional food due to its content of probiotic and prebiotic bacteria. Intestinal microflora can be affected by food products containing probiotics in appropriate doses so that they can act as antioxidants. Microbiota, both facultative and strict anaerobes including streptococci, bacteroides, lactobacilli, and yeasts, can be found from the mouth to the colon. Prebiotics can act as a growth stimulator and support probiotic activity such as by having biofunctionality in improving intestinal health, improving immunity, and even reducing the risk of inflammation **(Mofid et al., 2019)**.

One of the fruits that contain antioxidants and have potential as a functional food is red dragon fruit (*Hylocereus polyrhizus*) **(Joshi and Prabhakar, 2020)**, a variety of dragon fruit that has red flesh and peel, is oval in shape, and has a sour to sweet taste. It is rich in nutrients and minerals such as vitamin B1, B2, B3, C, carbohydrates, crude fibre, niacin, flavonoids, phenolics, betacyanins, lycopene, polyphenols, and phytoalbumin **(Choo et al., 2019)**. Red dragon fruit peel is very underutilized because it is only considered as waste. It accounts for up to 33% of the total weight of the fruit **(Nurliyana et al., 2010)**. The peel contains betacyanin compounds including betanin, isobetanin, phyllocactin, isophyllocactin, betanidin, isobetanidin, and hylocerenin. Betacyanins are identified as the main pigment in red dragon fruit peel which is purplish red. Pectin, triterpenoids, and steroids are also found in red dragon fruit peel **(Phongtongpasuk, 2016; Choo et al., 2018)**. The content of phenolic compounds, antioxidant activity and dietary fibre in red dragon fruit peel is higher than that in the flesh of the fruit **(Nurliyana et al., 2010).** Red dragon fruit peel has high antioxidant activity so that it has the potential to be developed as a natural antioxidant and even functional food.

Red dragon fruit peel can be processed into yogurt as a way to increase its economic value (Figure 1). Red dragon fruit peel yogurt is an alternative processed fermented drink to increase antioxidant function which ultimately supports the immune system of its users. Yogurt was chosen as the vehicle for processed red dragon fruit peel; because patients with dengue infection accompanied by fever need fluid intake to help restore homeostatic conditions. Also, yogurt is also easier to consume. This study aims to determine the potential of red dragon fruit peel yogurt as an alternative functional drink to improve haemoglobin, haematocrit, and platelet levels in heparin-induced thrombocytopenia in Wistar rats.

## Scientific Hypothesis

Red dragon fruit peel yogurt can improve haemoglobin, haematocrit, and platelet levels in heparin-induced thrombocytopenia (HIT) in Wistar rats.

## MATERIAL AND METHODOLOGY

### Samples

This study used male Wistar rats, weighing 160 – 200 g and aged 12 – 16 weeks. A total of 42 Wistar rats were randomly divided into seven groups.

### Chemicals

Thrombocytopenia in this study used Heparin ammonium salt, Merck, Sigma-Aldrich, form powder (biological source from porcine intestinal mucosa).

### Animals and Biological Material

This study used *Rattus norvegicus*, strain Wistar from House of Experimental Rats, Center for Food and Nutrition Studies (CFNS), Universitas Gadjah Mada, Yogyakarta, Indonesia.

The isolated culture for the red dragon fruit peel are *Lactobacillus bulgaricus* FNCC 0041 and *Streptococcus thermophillus* FNCC 0040 from CFNS, Universitas Gadjah Mada, Yogyakarta, Indonesia.

### Instruments

Hematology Analyzer, Sysmex KX-21, Kobe, Japan.

### Laboratory Methods

The automated hematology analyzer (AHA) was used to measure hemoglobin, hematocrit, and platelet levels **(Whitehead et al., 2019)**.

### Description of the Experiment

**Sample preparation:** Whole blood was obtained from each Wistar rats and stored with anticoagulant for the measurement of the haemoglobin, haematocrit, and total platelets.

**Number of samples analyzed:** Blood samples from 42 Wistar rats were collected from the retroorbital vein (before and after intervention).

**Number of repeated analyses:** duplo

**Number of experiment replication:** duplo

Facilities and handling of the experimental animals during the study were managed based on the Guidelines for Care and Use of Laboratory Animals of CFNS, Universitas Gadjah Mada, and were approved by the Committee on the Ethics of Health, Universitas Negeri Semarang (permission number: 077/KEPK/EC/2020). The experimental animals were obtained from the House of Experimental Rats, CFNS, Universitas Gadjah Mada, Yogyakarta, Indonesia. This study used male Wistar rats, weighing 160 – 200 g and aged 12 – 16 weeks. A total of 42 Wistar rats were randomly divided into seven groups consisting of six rats per group: negative control (K-); positive control (K+); and five treatment groups that received dragon fruit peel yogurt at doses of 5% (K1), 10% (K2), 15% (K3), 20% (K4) and 25% (K5) respectively. The cage environment had a light : dark cycle of 12 : 12 h, room temperature of 25 ±1 °C and humidity that was always maintained. Cage cleanliness and sanitation were also maintained to reduce stress on the experimental animals during treatment. Rats were placed in individual cages made of stainless steel. Each Wistar rat received 20 g per day standard feed and ad libitum water. The experimental animals were acclimatized for 7 days before the treatment.

**Induction of thrombocytopenia.** Thrombocytopenia was induced in the experimental animals by a single intraperitoneal injection of 0.1 mL.100g$^{-1}$ BW of heparin from Merck. Three days after the heparin injection, the rats experienced thrombocytopenia and received up to 200 mL yogurt containing dragon fruit peel at various concentrations for 28 days. Administration of the red dragon fruit peel yogurt was carried out in the morning.

**Experimental design.** This study used a randomized controlled pre-test post-test design in which a total of 42 Wistar rats were divided randomly into seven groups consisting of six rats each: K-, the negative control group (non-thrombocytopenic rats that did not receive any treatment); K+, the positive control group



**1st. Blend**
Red dragon fruit peel
Full cream milk
Sucrose

**2nd. Pasteurization**
Heating process
at 75 - 85° C for
15 minutes

**3rd. Cooling Process**
Lowers temperature
at 35-40° C



**4th. Fermentation**
**(42° C for 6-7 hours)**
Mix red dragon fruit peel
juice (step 1st) for added
isolated culture
(L. bulgaricus and
S. thermophillus)



**5th. Product**
Red Dragon Fruit
Peel Yogurt

**Figure 1** Red dragon fruit peel yogurt procedure.

(thrombocytopenic rats that did not receive any treatment); K1, thrombocytopenic rats that received 5% red dragon fruit peel yogurt; K2, thrombocytopenic rats that received 10% red dragon fruit peel yogurt; K3, thrombocytopenic rats that received 15% red dragon fruit peel yogurt; K4, thrombocytopenic rats that received 20% red dragon fruit peel yogurt; K5, thrombocytopenic rats that received 25% red dragon fruit peel yogurt.

The red dragon fruit peel yogurt; made based on the research conducted by Mardiana and Putriningtyas **(Mardiana Budiono and Putriningtyas, 2020; Putriningtyas and Wahyuningsih, 2017)** and, was administered orally through gastric intubation once daily for 28 days. The composition of red dragon fruit peel yogurt is full cream milk, fresh red dragon fruit peel, isolated culture (*L. bulgaricus* and *S. thermophillus*) and sucrose. After 7 hours of incubation at 42 °C, the yogurt formed was stored at 4 °C.

The rats' body weight was monitored weekly. The rats were euthanized by cervical decapitation after 28 days of intervention, then burned using an incinerator. Blood samples were collected from rats before and after the intervention.

Parameters analyzed. Blood samples were collected from the retroorbital vein and measurements of haemoglobin, haematocrit, and platelets were done using whole blood. Whole blood was obtained from each animal and stored with anticoagulant for the measurement of the haemoglobin, haematocrit, and total platelets. The parameters were measured using a automated haematology analyzer. The experiment was done in duplicate in two biological replication.

### Statistical Analysis

The mean ± standard deviation (SD) was calculated for each group of six rats. As all data were normally distributed, the significance of differences before and after treatments was determined using the pair t-test. The significance of differences between the groups were assessed by one-way analysis of variance (ANOVA), calculated by the SPSS version 20 program, with a significance level of $p < 0.05$ by the Tukey HSD test.

## RESULTS AND DISCUSSION

Table 1 shows the vitamin C and flavonoid content of red dragon fruit peel yogurt, measured using spectrophotometry. The analysis showed that the increase in vitamin C levels was not proportional to the increase in the amount of red dragon fruit peel. The flavonoid analysis also showed the same result as that for vitamin C. Flavonoids are bioactive compounds that act as antioxidants. Red dragon fruit peel is a source of phenolic antioxidants. Water-based dragon fruit peel extract has more attractive colour and better antioxidant activity **(Jamilah et al., 2011)**. Total phenolic content in dragon fruit peel extracted using ethanol and water solvents is in the range of 1.193 ±0.011 – 1.351 ±0.021 $\mu g.mL^{-1}$ **(Lourith and Kanlayavattanakul, 2013)**. Fermentation of milk by lactic acid bacteria can increase the nutritional content of yogurts, such as vitamins and phenols.

**Table 1** Nutritional content of red dragon fruit peel yogurt.

| Materials | Nutritional content | |
|---|---|---|
| | Vitamin C (mg.100g$^{-1}$) | Total Flavonoid (mg.100g$^{-1}$) |
| **5% red dragon fruit peel yogurt** | 22.379 | 38.519 |
| **10% red dragon fruit peel yogurt** | 10.612 | 48.485 |
| **15% red dragon fruit peel yogurt** | 6.515 | 22.209 |
| **20% red dragon fruit peel yogurt** | 11.024 | 42.349 |
| **25% red dragon fruit peel yogurt** | 20.072 | 43.277 |

**Table 2** Mean body weight of Wistar rats.

| Group | Body weight (g) | | | | |
|---|---|---|---|---|---|
| | Pre-injection | Pre | Post | Δ | *p** |
| **K-** | 184.17 ±4.49 | 189.83 ±4.58 | 214.67 ±4.97 | 24.83 ±0.75$^{b,c,d}$ | 0.001 |
| **K+** | 186.50 ±5.61 | 190.83 ±5.38 | 206.00 ±5.51 | 15.17 ±0.75$^{a,c,d,e,f,g}$ | 0.001 |
| **K1** | 190.00 ±4.90 | 194.17 ±4.71 | 214.17 ±4.79 | 20.00 ±0.63$^{a,b,e,f,g}$ | 0.001 |
| **K2** | 187.83 ±2.86 | 191.50 ±3.27 | 211.50 ±3.62 | 20.00 ±0.89$^{a,b,e,f,g}$ | 0.001 |
| **K3** | 184.17 ±6.97 | 188.17 ±7.06 | 212.50 ±7.31 | 24.33 ±0.82$^{b,c,d}$ | 0.001 |
| **K4** | 185.17 ±3.82 | 189.67 ±3.78 | 213.83 ±3.76 | 24.17 ±0.75$^{b,c,d}$ | 0.001 |
| **K5** | 186.00 ±4.78 | 190.17 ±4.96 | 214.33 ±5.24 | 24.17 ±0.98$^{b,c,d}$ | 0.001 |

Note: Sampling was done 3 days after induction of thrombocytopenia and 28 days after the start of the treatment. K-: negative control; K+: thrombocytopenia; K1: thrombocytopenia treated with 5% red dragon fruit peel yogurt; K2: thrombocytopenia treated with 10% red dragon fruit peel yogurt; K3: thrombocytopenia treated with 15% red dragon fruit peel yogurt; K4: thrombocytopenia treated with 20% red dragon fruit peel yogurt; K5: thrombocytopenia treated with 25% red dragon fruit peel yogurt. Values represent the mean ± SD for observations made on six rats in each group. Statistical analysis: *paired t-test, significant difference ($p$ <0.05); one-way analysis of variance (ANOVA); where significant, post hoc testing (least significant difference) was done for intergroup comparisons.
[a]Statistically significant difference ($p$ <0.05) when compared with K- values.
[b]Statistically significant difference ($p$ <0.05) when compared with K+ values.
[c]Statistically significant difference ($p$ <0.05) when compared with K1 values.
[d]Statistically significant difference ($p$ <0.05) when compared with K2 values.
[e]Statistically significant difference ($p$ <0.05) when compared with K3 values.
[f]Statistically significant difference ($p$ <0.05) when compared with K4 values.
[g]Statistically significant difference ($p$ <0.05) when compared with K5 values.

Lactic acid bacteria can grow and carry out fermentation activities maximally by utilizing sugars or carbohydrates in the media to form lactic acid and cause a decrease in pH **(Hanzen, Hastuti and Lukiati, 2016; Bintari et al., 2017)**.

The static bodyweight of the rats was assessed weekly (Biosep-In Vivo Research Instrument, USA) to determine rats' development during the study (Table 2).

The result of the data analysis shows that there were significant differences in each group before and after the intervention ($p$ <0.05). In all treatment groups, all Wistar rats gained weight; the group with the highest weight gain was the negative control group.

Weight gain in the treatment groups was probably due to the consumption of red dragon fruit peel yogurt. Fermentation using *Bacillus lactis*, *Streptococcus thermophillus*, *Lactobacillus bulgaricus,* and *Lactococcus lactis* can stimulate the production of metabolites such as butyrate, reducing pathobionts that cause inflammation and impair intestinal barrier function **(Veiga et al., 2014)**.

Thrombocytopenia is a complication caused by heparin, commonly referred to as HIT. HIT can be characterized by venous and arterial thrombosis, decreased platelets, and platelet aggregation. Heparin can function to bind antithrombin as the main inhibiting protein for the coagulation process **(Mulyadi and Soemarsono, 2007)**.

Table 3 shows that there were differences in haemoglobin, haematocrit, and platelet levels before and after treatment in all treatment groups: levels increased in the treatment groups that received red dragon fruit peel yogurt and decreased in the control group. The biggest increases were found in the group that received 25% red dragon fruit peel yogurt: 5.61 ±0.02, 13.71 ±0.10, and 192.96 ±4.53 mg.dL$^{-1}$ respectively. Results of the post hoc analysis showed that there were significant differences in all groups ($p$ <0.05).

Thrombocytopenia is one of the criteria used by the World Health Organization as a potential indicator for dengue infection. Thrombocytopenia is associated with pro-oxidant conditions in dengue infection.

**Table 3** Mean levels of haemoglobin, haematocrit and platelets in Wistar rats.

| Group | Parameters tested | | |
|---|---|---|---|
| | Hemoglobin (g.dL$^{-1}$) | Hematocrit (mL %) | Platelets (× 10$^3$.µL$^{-1}$) |
| **K-** | | | |
| **Pre** | 14.92 ±0.50 | 47.82 ±0.21 | 321.12 ±3.73 |
| **Post** | 14.73 ±0.47 | 46.93 ±0.26 | 312.54 ±3.45 |
| Δ | -0.19 ±0.10$^{b,c,d,e,f,g}$ | -0.88 ±0.06 | -8.58 ±0.64 |
| ***p*** | 0.001* | 0.005* | 0.001* |
| **K+** | | | |
| **Pre** | 9.07 ±0.20 | 30.32 ±0.72 | 97.13 ±3.96 |
| **Post** | 8.95 ±0.21 | 29.40 ±0.71 | 96.03 ±4.06 |
| Δ | -0.12 ±0.04$^{b,c,d,e,f,g}$ | -0.93 ±0.02 | -1.11 ±0.60 |
| ***p*** | 0.001* | 0.001* | 0.001* |
| **K1** | | | |
| **Pre** | 9.00 ±0.22 | 30.24 ±0.49 | 99.21 ±0.48 |
| **Post** | 10.87 ±0.15 | 36.05 ±0.49 | 133.37 ±0.90 |
| Δ | 1.87 ±0.07$^{a,b,c,d,e,f,g}$ | 5.82 ±0.01$^{a,b}$ | 34.16 ±0.46$^{a,b}$ |
| ***p*** | 0.001* | 0.001* | 0.001* |
| **K2** | | | |
| **Pre** | 9.13 ±0.36 | 29.20 ±0.90 | 97.95 ±0.41 |
| **Post** | 12.24 ±0.34 | 39.05 ±0.91 | 195.34 ±2.20 |
| Δ | 3.12 ±0.05$^{a,b,c}$ | 9.85 ±0.04 $^{a,b,c}$ | 97.39 ±2.14$^{a,b,c,e,f,g}$ |
| ***p*** | 0.001* | 0.001* | 0.001* |
| **K3** | | | |
| **Pre** | 8.67 ±0.13 | 29.81 ±0.66 | 98.12 ±0.45 |
| **Post** | 13.61 ±0.13 | 40.60 ±0.62 | 238.06 ±3.92 |
| Δ | 4.94 ±0.05$^{a,b,c,d}$ | 10.78 ±0.08$^{a,b,c,d}$ | 139.94 ±3.95$^{a,b,c,d,f,g}$ |
| ***p*** | 0.001* | 0.001* | 0.001* |
| **K4** | | | |
| **Pre** | 8.56 ±0.20 | 29.80 ±0.34 | 97.95 ±0.43 |
| **Post** | 13.92 ±0.20 | 42.61 ±0.44 | 279.24 ±5.08 |
| Δ | 5.36 ±0.27 | 12.81 ±0.52 | 181.29 ±4.87$^{a,b,c,d,e,g}$ |
| ***p*** | 0.001* | 0.001* | 0.001* |
| **K5** | | | |
| **Pre** | 8.63 ±0.19 | 29.90 ±0.41 | 98.03 ±0.43 |
| **Post** | 14.24 ±0.20 | 43.60 ±0.46 | 290.99 ±4.78 |
| Δ | 5.61 ±0.02 | 13.71 ±0.10 | 192.96 ±4.53$^{a,b,c,de,f}$ |
| ***p*** | 0.001* | 0.001* | 0.001* |

Note: Sampling was done 3 days after induction of thrombocytopenia and 28 days after the start of the treatment. K-: negative control; K+: thrombocytopenia; K1: thrombocytopenia treated with 5% red dragon fruit peel yogurt; K2: thrombocytopenia treated with 10% red dragon fruit peel yogurt; K3: thrombocytopenia treated with 15% red dragon fruit peel yogurt; K4: thrombocytopenia treated with 20% red dragon fruit peel yogurt; K5: thrombocytopenia treated with 25% red dragon fruit peel yogurt. Values represent the mean ± SD for observations made on six rats in each group. Statistical analysis: *paired t-test, significant difference ($p$ <0.05); one-way analysis of variance (ANOVA); where significant, post hoc testing (least significant difference) was done for intergroup comparisons.

[a]Statistically significant difference (p <0.05) when compared with K- values.
[b]Statistically significant difference (p <0.05) when compared with K+ values.
[c]Statistically significant difference (p <0.05) when compared with K1 values.
[d]Statistically significant difference (p <0.05) when compared with K2 values.
[e]Statistically significant difference (p <0.05) when compared with K3 values.
[f]Statistically significant difference (p <0.05) when compared with K4 values.
[g]Statistically significant difference (p <0.05) when compared with K5 values.

Oxidative stress can increase when there is an imbalance of oxidants and antioxidants due to internal or external exposure to stressors such as viruses **(Ahmed et al., 2014)**. Dengue virus can induce oxidative stress in humans due to its release of proinflammatory cytokines which also influence the immunopathogenic mechanism **(Chandra et al., 2013)**. Thrombocytopenia is also associated with lipid peroxidation.

Red dragon fruit peel yogurt contains vitamin C, a water-soluble vitamin also known as ascorbic acid which can stimulate immune function and has a role as an antioxidant **(Rammohan et al., 2019)**. Vitamin C can increase the platelet count by modulating the platelet oxidative state and because platelets have vitamin C transporters (SVCT2) which will influence the platelets to activate vitamin C intracellularly so that it can ultimately improve cells' lipid

peroxidation condition **(Safwan and Asar, 2017)**. Vitamin C can alter the oxidative state of thrombocytopenia by inhibiting the expression of CD40L, a transmembrane protein that is produced in both proinflammatory and prothrombotic conditions **(Mohammed et al., 2017)**. Vitamin C content is also related to haemoglobin status **(Ahmed et al., 2014)**. Previous studies have shown that the consumption of 25 – 1000 mg ascorbic acid added to a liquid formula containing 4.1 mg of nonheme iron can increase iron absorption by 0.8 – 7.1%, thereby improving haemoglobin status **(Safwan and Asar, 2017)**.

Endothelial damage in DENV is caused by the dengue virus itself. Thrombocytopenia is responsible for bleeding in patients. The condition of thrombocytopenia can be influenced by various factors such as reactive immune response and decreased platelet production, activation, and apoptosis **(Mutiara et al., 2019)**. Dengue virus can bind directly to prothrombin, thus inhibiting its conversion to thrombin and decreasing coagulation activation, thrombin formation, and associated bleeding complications.

Yogurt can release bioactive peptides and bacteriocins. β-Casein-derived peptides released from yogurt can trigger goblet cells to secrete mucin either in vitro or in vivo. The lactic acid bacteria found in yogurt can break down simple carbohydrates to produce lactate, acetate, or propionate **(El-Said et al., 2014)**. These bacteria can affect the breakdown of carbohydrates, alter the metabolic output, and provide an important substrate that supports the development of intestinal microbes **(Fernandez et al., 2017)**.

The increase in haemoglobin, haematocrit, and platelet levels after consumption of red dragon fruit peel yogurt could be due to its phenolic, flavonoid, and anthocyanin content. Phenolics, also called phenols, are found in various tropical plants **(Kapcum, Uriyapongson and Uriyapongson 2020)**. Flavonoids in dragon fruit peel have an antioxidant action that can affect immunity. Flavonoids also have antiviral activity against several RNA viruses **(Clain et al., 2019)**; this will probably not change the genotoxic effect on several mammalian cells, including human primary cells which are compatible with arboviral infection. Flavonoids also have a stimulant effect on blood cell production and may also trigger platelet production and platelet aggregation through arachidonate 12-lipoxygenase (ALOX 12), also known as platelet-type lipoxygenase as well as platelet activation factor receptor (PTAFR). Increased activity of this gene is required for platelet production and activation. The ALOX 12 gene is expressed in megakaryocytes and is responsible for the production of 12 hydroxyeicosatetraenoic acid (12-HETE) from platelets. The PTAFR gene is also a precursor for platelet production **(Agustina, 2019)**.

Red dragon fruit peel yogurt is made using *L. bulgaricus* and *S. thermophillus*. The fermentation process not only creates a distinctive aroma and taste but can increase the antioxidant potential of the drink. Research has shown that the level of betanin in fermented red dragon fruit peel drink (1.42%) is more than that of the flesh (0.23 – 0.39%) **(Stintzing, Schieber and Carle, 2002; Choo et al., 2018)**.

Compounds other than flavonoids contained in red dragon fruit peel are betacyanins and betalains, the content of which in red dragon fruit peel is higher than in the fruit flesh. Betalains are water-soluble polyphenolic pigments composed of yellow betaxanthins and purplish-red betacyanins. Betacyanins consist of betalamic acid and an acyclic amine group which is excellent electron donors which enables them to scavenge free radicals. The red-purple betacyanin pigments in red dragon fruit peel have recently been suggested as a potential betalain source **(Liaotrakoon et al., 2013)**.

The increases in haemoglobin, haematocrit, and platelet levels were directly proportional to the dose of dragon fruit peel given. This mechanism is possible because flavonoids are also known to be able to capture and neutralize free radicals such as ROS or RNS, which are associated with phenolic OH groups so that they can repair damaged tissue or inhibit the inflammatory process **(Schwiertz et al., 2010)**.

The flavonoids in red dragon fruit peel are catechin, epicatechin, quercetin, myricetin, and kaempferol, of which catechins have the highest concentration in red dragon fruit peel. Catechin is thought to inhibit DENV infection **(Simon, Sutherland and Pryzdial, 2015)**. The dragon fruit peel contains flavonoids in the form of catechin polyphenol active compounds: epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) **(Morais et al., 2019)**. The mechanism by which virus entry to the host is inhibited by EGCG begins in the early stages of the viral replication cycle. The EGCG in red dragon fruit peel yogurt can inhibit the virus adherence to the cell surface through direct interaction with the outer membrane of dengue virus particles **(Clain et al., 2019)**. Flavonoids are also thought to increase granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), stimulating megakaryopoiesis to increase platelet levels **(Wiyasihati, Wigati and Wardani, 2013)**.

## CONCLUSION

Red dragon fruit peel yogurt is effective for improving haemoglobin, haematocrit, and platelet levels in HIT in rats. Yogurt containing 25% red dragon fruit peel provides an effective dose for improving haemoglobin, haematocrit, and platelet levels in thrombocytopenic rats.

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# THE IMPACT OF CONSUMER PERSONALITY ON PURCHASING BEHAVIOR IN THE DAIRY MARKET IN TERMS OF PSYCHOLOGICAL FACTORS

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## ABSTRACT

Since the 1990s of the 20th century, the Slovak dairy industry has undergone many significant changes which have been related to the reduction of the herds of cattle and milk cows, the decrease of quantity of milked milk, low purchase prices of milk, the decline of a great number of milk producers and processors. The consumption of cow´s milk and dairy products in Slovakia is very low in comparison with the countries of the European Union (about 320 kg), Europe (293 kg), the Czech Republic (280 kg), and the rest of the world. It varies around 170 kg per capita per year, which is also considerably lower consumption than the dose of 220 – 240 kg per capita per year recommended by doctors. Since 1990 the production and consumption of dairy products and cheeses have decreased significantly. In Slovakia, the consumption is twice even three times less than in the developed countries of Europe. The reason for this situation is the behavior of a consumer and the factors having an impact on the consumption of cow`s milk and dairy products. The objective of the paper is to indicate the impact of the personal consumers` characteristics on the purchasing decisions on the market of the products of cow`s milk origin, based on the processed answers of the respondents participating in the questionnaire survey. The primary data were obtained via the questionnaire survey, which was conducted with 786 respondents. The questionnaire consisted of four parts – Eysenck`s personality test, the questions targeted at the detection of factors influencing the consumers` purchasing behavior and decision making, the questions related to expressing opinions about cow`s milk and dairy products, and the classification questions providing the information about the respondents. The data were processed by the methods of quantitative and qualitative statistics – Chi-square statistic $\chi 2$ test of square contingency, Chi-square $\chi 2$ goodness of fit test, two proportion test, Kolmogorov-Smirnov test, the test of hypotheses of relative proportion.

**Keywords:** consumer personality; consumer behavior; cow´s milk; dairy products; determinants of purchase; psychological factors

## INTRODUCTION

A consumer is every individual who buys products or services for his/her consumption to satisfy their own needs **(Biswas and Roy, 2015)**. **Richterová et al. (2015)** completed the definition by the idea that a consumer buys products which are aimed for the use in the family, the family members, or as a present for other persons. According to **Consumer Rights (2019)**, a consumer buys products or services for his/her consumption but not for the production or further sale. The purchasing decision of a consumer is influenced by their needs and wants **(Solomon et al., 2016)**.

**Golian (2017)** claims that consumer behavior reflects the competence of their decisions with the aspect of acquisition, consumption, and disposition of products and services, their activities, experience, consumer patterns in the surroundings, and their own or acquired processes from models in the micro or macro-environment. The behavior, which has an impact on purchasing decision-making, is different with each individual. The factors influencing some consumers do not have necessarily an impact on others **(Javornik, 2016)**. The internal factor is predominantly our conviction whether we need or do not need a product, and if we buy it or not **(Ramya and Ali, 2016)**. **Lypchuk and Larina (2018)** rank among the external determinants the price, packaging, advertisement, and all other marketing tools. Consumer decisions are also influenced by constantly changing food trends **(Santeramo et al., 2018)** and the habits of individual consumers **(Wallner et al., 2016)**. The globalization and internationalization of markets increase the interest in the knowledge of general and specific characteristics of consumers and their needs. It is connected with the traditional monitoring of trends of the development of lifestyle, consumption, and value orientation of consumers, (whose) the knowledge of which allowed many marketing organizations to achieve the competitive advantage **(Richterová et al., 2015)**. During the decision-making process, a consumer is influenced by many factors, which are divided by many authors into four or five essential groups – cultural, social, personal, psychological, and situational factors **(Armstrong et al., 2016; Horská et al.,**

**2009; Kotler and Keller, 2019; Koudelka, 2018; Nákonečný, 2009; Richterová, 2015; Sarma, 2018; Solomon et al., 2016; Vysekalová, 2014; Wänke, 2015)**.

A personality is perceived as a whole of the internal dispositions that along with the situation determine the content and procedures of the psychological processes which are understood as a reaction of an individual to a particular situation **(Nákonečný, 2009)**. A personality is associated with his/her internal characteristics, specific virtues, attributes, features, and manners, which differ consumers from each other. These characteristics have an impact not only on an individual`s product and trade choice but also on the way how a consumer reacts to the promotional tools of a seller **(Strydom, 2011)**. **Nákonečný (2009)** describes a personality as a whole of the internal dispositions that along with the situation determine the content and procedure of the psychological processes, which are perceived as a reaction of an individual to a particular situation. According to **Richterová et al. (2015)**, it is just a personality who decides about the way how an individual will achieve his/her objectives. Freud´s theory of personality, Neo-Freudian theory of personality, the theory of personality traits, and cognitive approach to the study of a personality by so-called Self-concept rank among the most significant and well-known theories. Freud´s theory of personality arises from the assumption that human behavior originates from the basic conflict between a human desire to satisfy the physical needs and the necessity to act as a responsible member of society. This struggle is carried out in mind between three systems – Id, Ego, and Superego **(Solomon et al., 2016; Vysekalová, 2011)**. Several followers of Sigmund Freud, among them Alfred Adler (theory of individual psychology), Carl Jung (theory of collective unconscious), Karen Horney (three types of personalities), and others enlarged Freud`s theory and created so-called Neo-Freudian school **(McLeod, 2018; Richterová et al., 2015)**. The British psychologist Hans Eysenck suggested the theory of personality based on biological factors. He states that individuals inherit the type of nervous system which has an impact on their ability to learn and adapt to the environment where they live **(Cherry, 2018)**. **Atkinson (2003)** describes two main factors – neuroticism (stable/unstable) and extraversion (extrovert/introvert). Their mutual combination forms different personal characteristics. According to **Hradiská and Letovancová (2007),** decision-making is the phase in a consumer´s behavior that results in a particular activity influenced by several factors. These factors are implicit in the individual characteristics of a consumer and they also have an impact on their motivation towards the consumer action. Emotions are closely related to motivation. From the aspect of consumer behavior, the result of the internal process is a feeling linked to a product. This emotion is not an irrational act. **Vysekalová (2011)** claims that emotions are an effective tool to persuade a consumer to buy a product. When watching an advertisement, each experiences an emotion-inducing need to buy or not to buy a given product. The sellers try to influence a customer via emotions and evoke the impression that their product is the best one. The interconnection of the positive emotions with a brand creates a positive image of the brand in the consumer´s mind. If an advertisement evokes the consumer´s positive and strong emotions, then there is a great probability that when shopping he/she will choose just the goods which stimulated the positive emotions. On the contrary, negative emotions are rarely used in adverts because the consumers are not interested in buying a product that is associated with the negative emotions.

The decision-making process is created by several phases – problem identification, searching for information, evaluation of alternatives, a decision about purchasing, and evaluation after purchase **(Kotler and Armstrong, 2004; Solomon et al., 2016)**.

When dealing with the consumption of milk and dairy products we often come across lactose intolerance or the different myths about these products **(Dairy Global, 2018)**. The collective of authors **Golian et al. (2019)** explain that milk produces sputum, it is diluted by water, it contains various preserving agents and also that the consumption of milk causes different health problems for some consumers. In the world, there are more than 7.5 milliard inhabitants, out of the 80% consume cow´s milk and dairy products **(Kapaj and Deci, 2017)**. The majority of consumers do not have any problems with their consumption. However, some consumers do not consume cow´s milk and dairy products for different reasons **(Elwood, Pickering and Fehily, 2007)**. Their negative attitude can originate from health problems, own conviction, new trends in nutrition, and recommendations. The significant aspect of the purchasing behavior can consist in the conclusions of the various researches and specialists“ opinions **(Kassin et al., 2018)**. Generally, milk is considered to be one of the principal structural parts of human nutrition **(Golian et al., 2019)**, therefore it is necessary to motivate the consumers to buy (it) and consume it more. The average annual consumption of cow´s milk and dairy products per capita in Slovakia is 176 kg, while the doctors recommend consuming 220 kg per capita **(Masár, 2018)**. According to **Herian (2019)**, since 1990 there has been a significant decrease in the production and consumption of dairy products and cheeses in Slovakia. The Slovaks consume twice even three times less acidophilus milk products, and mainly cheeses, than in the developed countries of Europe. *“A decreased consumption, and therefore also a decreased milk production, has the negative impact on agriculture, animal production, and human health. In 2017 – 696 milliard kg of milk were produced, out of it 28% in the EU,8% in other European countries, 30% in Asia, 18 % in North America, 9% in South America, 6% in Africa, and 5% in Oceania. For many Europeans, milk and dairy products are the essential sources of proteins and calcium, and cheese is necessary for the normal growth and development of bones and teeth. In 5 decagrams of hard cheese, there is about one-fifth of protein quantity, which is needed for an adult person, and approximately one-third of calcium quantity needed for a teenager. Cheeses contain also the vitamins A, B2, niacin, B12 and D, as well as the mineral substances like zinc and phosphorus"* **(Herian, 2019)**.

### Scientific hypothesis

Assumption No. 1: We assume that women think more rationally than men.

Assumption No. 2.: We assume that the quality of cow`s milk and dairy products have an impact on a consumer when buying these products.

Assumption No. 3: We assume that more than 70% of respondents purchase cow`s milk and its products.

Assumption No. 4: We assume that there exists a dependence between the economic status and the choice of a company.

## MATERIAL AND METHODOLOGY

The primary data for the elaboration of the paper was received by the questionnaire survey which was administered in the electronic form of Google Forms in the territory of the Slovak Republic. The data collection was pursued in 2018 – 2019. 786 respondents participated in the research. The questionnaire was divided into four independent parts. The first part provided information about the respondents, their gender, age, economic status, income, and place of residence. The second part consisted of the questions related to a consumer`s personality. The third part was aimed at the consumer behavior on the market of milk and dairy products. The last part focused on cow`s milk and dairy products.

The principal objective was to detect the impact of a personality and determinants on the consumer behavior on the market of cow`s milk and dairy products.

### Statistical Analysis

The formulated scientific hypotheses were tested by the methods of qualitative statistics – Chi-square of the goodness of fit test, the test of hypotheses of relative proportion, two proportion tests, and Kolmogorov-Smirnov test.

The set level of probability alfa ($\alpha = 0.05$) was compared with the significance level (*p*-value). If the *p*-value is lower than alfa (α), then we reject $H_0$. If the *p*-value is higher than alfa (α), then we accept $H_0$.

## RESULTS AND DISCUSSION

The first part of the questionnaire was formed by the classification questions related to the gender, age, completed education, economic status, monthly income, and place of residence of the respondents. The answers to the questions were recorded in the electronic form through Google form.

The research sample was represented by the consumers of cow`s milk and dairy products classified into four age categories. 786 respondents participated in the questionnaire survey, out of the 521 women (66.28%) and 265 men (33.72%). From the age aspect, the biggest representation was achieved by the group of people in the age category from 26 to 35 years (42.49%). The second biggest group were young people aged 25 years (28.63%). This group was followed by the age category from 36 to 50 years (17.18%). The lowest representation in the research – 92 respondents (11.70%) – was the group aged from 51 and more years.

The analysis of the economic status of the respondents participating in the research provided the information that the smallest number were employed people (39.31%), followed by the students (38.68%). The third position was taken by the self-employed people (38.68%). 34.10% of the respondents claimed that their monthly income is less than 400 €. The smallest group were the respondents (13.23%) whose income was higher than 1001€. 8.78% of respondents – students undergraduates stated that they do not have any own income. Out of the total number of respondents, 786 (58.65%) live in a town and 41.35 % live in the countryside.

The second part of the questionnaire survey was formed by Eysenck`s test of personality. Based on this test the respondents were identified from the aspects of extroversion/introversion and emotional stability/lability.

In the third part, the respondents answered the questions targeted at the determination of the factors of the purchasing behavior and decision making, which can influence them or not in the process of decision making and purchase of cow`s milk and dairy products. The answers were recorded on the evaluation scale of 1 – 5.

In the fourth part of the questionnaire, the questions were formulated in a way so that the respondents could express their opinion about the evaluated products from the viewpoint of their purchase, consumption, and preferred brands.

Based on the theory of personality, which is founded on two basic dimensions, the combination can create the different personality characteristics of each individual, thus Eysenck`s personality test was formed. This test allows detecting the level of extroversion and neuroticism of the respondents and classifying them into one of four types of temperament – sanguine (stable extrovert), choleric (unstable extrovert), phlegmatic (stable introvert), and melancholic (unstable introvert). The test consisted of 30 closed questions with the answers yes/no. The respondents` answers to the first 12 questions allowed to evaluate the scale of extraversion (granting one point for the answer "yes" with the questions 1, 3, 4, 6, 7, 8, 11, 12 and for the answer "no" with the questions 2, 5, 9, 10). If a respondent achieved more than 6 points, he/she was evaluated as an extrovert, in the case of fewer points as an introvert.

The results of the survey indicated the dominance of extroverts. Out of the total number of 337 extroverts, there were 240 women, men were mostly introverts. The group of "intertype" was formed by 126 respondents (ambiverts), who achieved the same number of 6 points for extroversion and introversion (Figure 1).

The responses to the second group of questions (13 – 24) allowed to determine the scale of neuroticism (emotional stability and lability of respondents). In this case, the same division of points was used as in the determination of the extroversion of respondents. If a respondent obtained more than 6 points, he/she was evaluated as emotionally labile, if less than 6 points, he/she was emotionally stable. If he achieved 6 points, he was classified in the group as "intertype". The results of the research pointed out the dominance of the emotionally stable respondents (total 256, 164 women and 92 men). In the group of emotionally labile, there were classified 201 respondents, 114 women and 87 men (Figure 2).

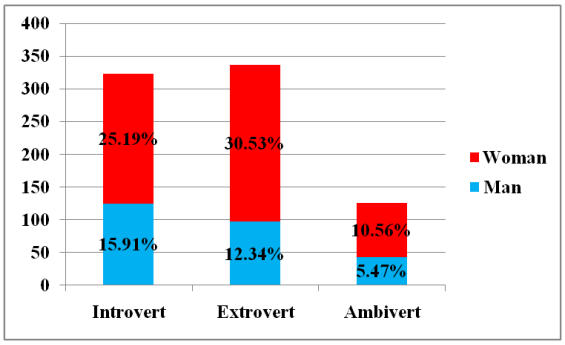


**Figure 1** Structure of respondents according to the scale of extroversion and gender.

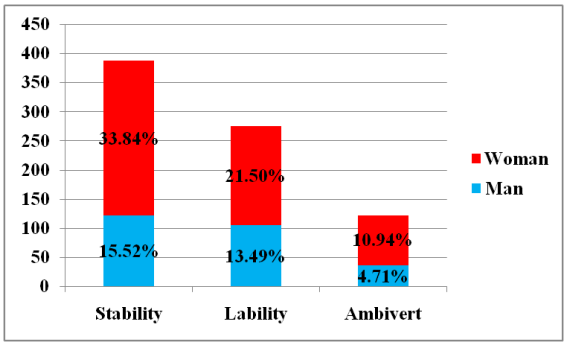


**Figure 2** Output from the statistical program.

Based on the results of the marketing survey we can claim that the majority of respondents are emotionally stable extroverts, who belong to the group of sanguines from the aspect of temperament, the second group is choleric – emotionally labile extroverts. The same number of respondents rank among phlegmatics (stable introverts) and melancholics (labile introverts). The respondents, who achieved the same amount of points in both scales and belonged to the group of "intertype", were not classified in any group according to temperament (Figure 3).

The third group of questions in the survey was targeted at consumer behavior and decision-making when purchasing milk and dairy products. The respondents should have chosen one out of 5 options of the submitted scale of responses (1 – strongly agree, 2 – agree, 3 – I do not know, 4 – disagree and 5 – strongly disagree).

The first question was aimed at purchase planning. The majority of respondents (26.59%) marked the answer 3 `I do not know`, which means that the respondents do not plan their shopping. Similar conclusions result from the survey of **Wand and Sahin (2018)**, who stated that the purchase planning depends on the purchased range of products. If a consumer goes shopping for products of daily consumption, then he/she hardly ever plans the purchase. The different situation occurs in the case of the purchase of clothes, electronics, or other durables. In this case, he/she plans the purchase and thinks over thoroughly the final decision. Then the options "disagree" followed (24.68%) and "agree" (24.30%). The reply `strongly agree` was indicated by 12.98% and `strongly disagree` by 11.45% of respondents.

The second question referred to the impulsive purchase. It is an unplanned purchase without any serious speculation about the effect/importance of this action. Also in this case it was affirmed that the majority of respondents could not express their opinion (32.57%). It means that the range of products and the way of purchasing can have an impact on their impulsive behavior. The research of **Ling and Yazdanifard (2015)** emphasized impulsive behavior with a particular range of products. The authors also declare that impulsive behavior is dependent on both internal and external factors.

The next option was `agree` (23.41%) and `disagree` (18.32%). 15.14% respondents marked `strongly agree` and 10.56% of respondents `strongly disagree`.

Hypothesis No. 1 was defined in the relation to purchasing planning:

*$H_0$: Women do not think more rationally than men.*

*$H_1$: Women think more rationally than men.*

The two proportion test was used to verify the hypothesis. In this case, the table value was 1.6449 and the test characteristics 2.4631, which means that we reject the null hypothesis. We accept the alternative hypothesis and affirm that women think more rationally than men. Based on the test results, we consider our assumption to be correct.

The responses to the question referred to advertising should provide the information to what extent the respondents agree with the statement that they buy foodstuff influenced by advertisements. The majority 37.02% of respondents strongly agreed. This statement only confirms that the advertisement still belongs to the significant marketing communication tools, having an impact on consumer behavior. This statement was approved also by the answers of respondents in the survey conducted by **Pechmann and Catlin (2016)**. The authors concluded that the consumers perceive subconsciously the advertisements of new products, and when shopping they recall repeatedly the information. According to **Boyland and Whalen (2015)**, the most influenceable groups of consumers by an advertisement are children and pensioners. The following option was `agree` (20.87%) and in the third place the answer `disagree` (19.47%) was placed. 10.69% of respondents strongly disagree, and 11.96% of respondents do not know how to evaluate this statement.

The last but one question referred to the influence of the decision-making of a consumer through quality. The reaction should have pointed out whether consumers decide to buy milk and dairy products also according to quality, and if they are willing to pay more. 25.70% of respondents strongly disagree. Recently still more attention is focused on the issue of food quality. Even in some cases, quality is considered to be the most important factor influencing the choice and purchase of food. The survey of **Jeong, Crompton and Hyun (2018)** was targeted at the quality versus the price of selected products. The results of this survey proved that the consumers are willing to pay more for the product of higher quality. Of course, the quality of the particular products must be declared. The next opinion was "disagree" (25.45%) and the answer "I do not know" (17.68%). 13.99% of respondents strongly agreed and 17.18% respondents agreed.

Concerning the impact of the quality of milk and dairy products on a consumer during shopping, hypothesis No. 2 was formulated:

*$H_0$: Quality does not have an impact on a consumer when purchasing milk and dairy products.*

*$H_1$: Quality has an impact on a consumer when purchasing milk and dairy products.*

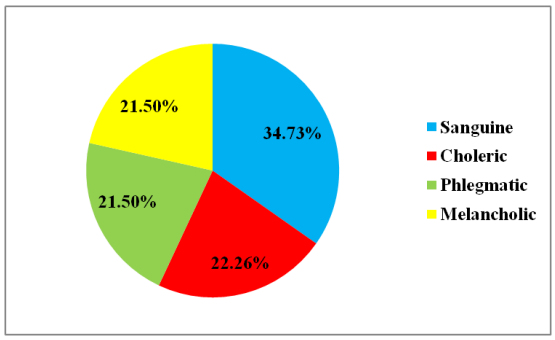


**Figure 3** Structure of respondents according to temperament.

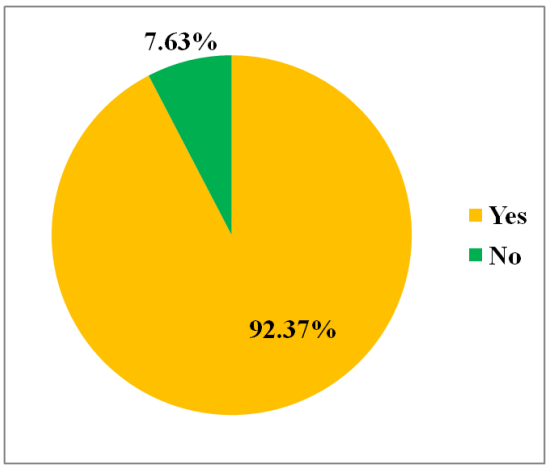


**Figure 4** Purchase of cow´s milk and dairy products.

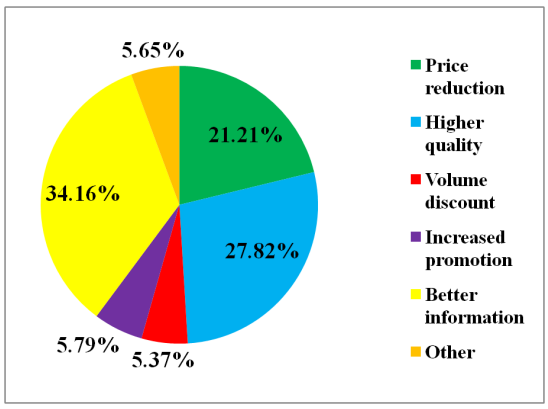


**Figure 5** Motivation to higher consumption of milk and dairy products.

We have used the Kolmogorov-Smirnov test to verify the hypotheses. In this case, the table value achieved the level 0.0736 and the testing characteristics were calculated at the level of 0.3776, which means that we reject the null hypothesis. We accept the alternative hypothesis and claim that the quality of milk and dairy products has an impact on a consumer´s purchase. Based on the test results we consider the assumption to be correct.

The last statement was aimed at the price – whether this factor is decisive in purchasing food. The majority of respondents marked the option "disagree" (25.32%). The survey of **Rödiger, Plaßmann, and Hamm (2016)** also detected that the price does not always rank among the decisive factors of purchase of a particular product. This survey determined that more than 60% of respondents bought a particular product at a higher price although the willingness to pay was lower than the price. The following was the option "I do not know" (23.54%) and the third place was taken by the answer "strongly disagree" (21.25%). 9.80% of respondents strongly agreed and 20.10% of respondents` agreed.

The last studied area was cow´s milk and dairy products. The respondents reactions should have provided the information if they buy cow´s milk and its products (Figure 4).

The graph indicates the positive relation towards the purchase of cow´s milk and dairy products (92.37%). The similar result, referred to the consumption of cow´s milk, has also been achieved in the survey of the collective of authors **Rybanská et al. (2019)** as well as in the survey of **Krivošíková et al. (2019)**. The negative answers can originate from several reasons (lactose intolerance, lifestyle, own conviction). The problem of lactose intolerance was studied by the team of researchers **Deng et al. (2015)** who concluded that the number of consumers suffering from this kind of intolerance is growing continuously. 7.63% of respondents, who stated in our survey not buying milk and dairy products, did not carry on the completion of the other questions in the questionnaire.

To test the number of respondents purchasing milk and dairy products we set hypothesis No. 3:

*$H_0$: More than 70% of respondents do not buy cow´s milk and its products.*

*$H_1$: More than 70% of respondents buy cow´s milk and its products.*

We used the test of hypotheses about the relative proportion to verify the hypotheses. In this case *p*-value = 0.9237 and the upper limit was calculated at the level 0.7001, which means that we reject the null hypothesis. We accept the alternative hypothesis and claim that more than 70% of respondents purchase cow´s milk and its products. Based on the test results, we consider our assumption to be correct.

The processed answers to the second question were related to the reason for purchasing milk and dairy products. The most frequent reason for the purchase of these products was their taste (50.41%), followed by healthy nutrition (28.24%) and as a raw material used for food preparation and baking (15.56%). 4.13% of respondents stated that they buy milk and dairy products predominantly for their children. The healthy nutrition as a reason for the purchase of milk and dairy products was also presented in the survey of **Kurajdová, Táborecká-Petrovičová and Kaščáková (2015)**. The following questions in the questionnaire survey were aimed at the motivation of respondents to higher consumption of milk and dairy products (Figure 5). The majority of the respondents stated that they would be motivated to the increased consumption by better informedness about the positive effects on their health (34.16%). This means that the respondents would be willing to buy milk if they had the adequate and sufficient amount of needed information and arguments. 27.82% of respondents consider a higher quality of these products as a reason for their purchase. The purpose of this request is the unexplained information related to the different myths which are spread about milk – the dilution of milk by water, insufficient content of vitamins and nutrients, the scarcity of information about the health benefits of milk, the excessive fat content, etc. The third reason for the insufficient interest in milk was the price, in particular, a lower price of milk (21.21%). The survey of **Paraffin, Zindove and Chimonyo (2017)** confirms that the consumers preferred the price to milk safety. The next factor was the increased promotion, bulk discounts, and others. 5.65% of respondents claimed that they are not motivated by anything to increase the consumption of cow´s milk and dairy products because they consider their consumption to be sufficient and do not need to increase it.

The following question referred to the factors which influence respondents mostly when buying cow´s milk and its products. The respondents had the opportunity to choose out of five factors. 25.48% of respondents think that the country of origin is the most significant factor, 24.66% price, 23.14% quality, 17.08% packaging, and 9.64% brand. The survey of **Elofsson et al. (2016)** emphasized the importance of brand and brand awareness to be significant also with milk and dairy products. The results of the survey referring to brand awareness declared the rise of milk purchase by 6 – 8%.

The answers to the question related to the most purchased dairy products allowed us to classify the order of the preferred purchased evaluated products made by the consumers participating in our survey. More than one-fifth of respondents prefer to buy milk, one-fifth prefers cheeses, followed by yogurts, butter, curds, and acidophilus milk products. Almost 2% of respondents stated milk puddings and creams in the category of the other products (Figure 6).

The survey pursued by the team of **Kubicová, Predanocyová and Kádeková (2019)** indicated almost the same results when the consumers preferred mainly milk, cheeses, and yogurts. The survey determined that the consumption of milk and dairy products is below the limit of the daily recommended consumption, which is caused predominantly by high prices of the dairy products

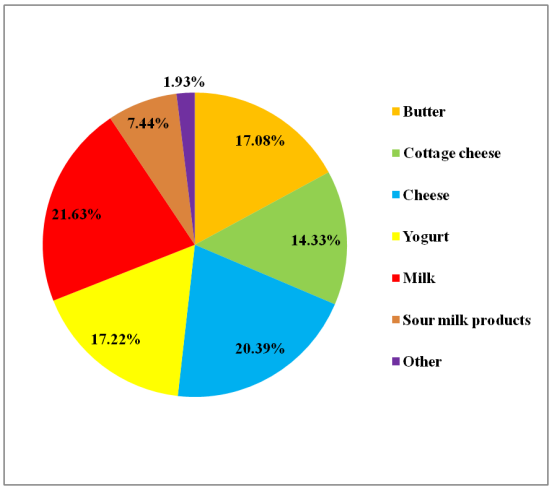


**Figure 6** Most frequently purchased dairy products.

(cheeses, butter, yogurts) in comparison with the incomes of households.

The last question of the survey was focused on the consumption of the Slovak traditional cheeses (steamed cheese "parenica", braided string cheese, cheese preserved by smoke curing "oštiepok" and so on). 685 (94.35%) respondents provided a positive answer to this question. 5.65% of participants expressed their negative relation to the Slovak types of cheeses. The respondents, who answered positively, were also asked which particular brand of the Slovak traditional cheeses is their favorite and preferred. The majority of respondents (58.69%) stated the brand Liptov. The next one was Agrofarma (21.75%), Milsy (15.47%), and others – Levmilk, Tami, Syrex, and the different local farms in their place of residence represented by 4.09% of respondents.

We detected the dependence of the economic status and the choice of a particular brand of the Slovak cheeses via the method of quantitative statistics.

*$H_0$: There does not exist any dependence between the economic status and the choice of a particular brand of Slovak cheeses.*

*$H_1$: There exists a dependence between the economic status and the choice of a particular brand of Slovak cheeses.*

We used the Chi-square test for more than two independent aggregates for the verification of hypothesis No. 4. In this case, the table value at the level 43.7730 and the test characteristics were calculated at the level 55.9116, which means that we reject the null hypothesis. We accept the alternative hypothesis and affirm that there is a dependence between the economic status and the choice of a particular brand of Slovak cheeses. Based on the test results we consider our assumption to be correct.

## CONCLUSION

Many producers are convinced that the decisions they make result from the rational analysis of the available sources. However, there exists another significant factor – emotions which can influence considerably their decisions, even change them. Nowadays, the promotion itself of these products via rational appeal is not sufficient, but emotional appeals are becoming still more important. Many companies utilize emotions in their advertising spots to induce some feelings and imagination of the target

group, which can have an impact on purchasing behavior and decision-making. A personality is a determinant that influences the model of consumption. Even if a consumer is not aware of it, he/she tends to buy and use the products which correspond with his/her personality. A personality reacts to the promotional interests of firms that have an impact on the consumer personality and his/her choice of a product and brand.

The results of the pursued questionnaire survey suggested that the consumer personality is influenced by the purchasing behavior, however, the personality itself is influenced by many factors – price, advertisement, opinion of other consumers. That very much depends on the consumer to what extent he/she is influenced by the external environment and which role is played by his/her judgment.

Based on the pursued marketing survey by Eyesenck`s personality questionnaire, the majority of respondents were ranked among the emotionally stable extroverts. According to temperament, these consumers belong to the group of sanguines (34.73%). The second biggest group were emotionally labile extroverts – cholerics (21.50%). From the viewpoint of consumer behavior, we can claim that the purchase planning depends on a particular kind of a purchased product. The impulsive behavior of respondents was not affirmed, therefore we assume that this type of purchasing behavior is influenced by a particular kind of dairy product and also the form of shopping. The survey has proved the increased interest in the purchase of the dairy products recommended by advertisements, where 37% of respondents expressed their positive attitude. In the quality versus decision-making factor about the purchase of the dairy products, we conclude that the consumers are not willing to buy more expensive products, although these commodities should be of a higher quality. 25% of respondents had a negative approach towards the price of milk and dairy products and their impact on the purchase of these products.

We can evaluate positively the significant interest in the consumption of milk and dairy products. More than 92% of respondents do not have any problems with the consumption of milk and dairy products. The principal reasons for the purchase of milk and dairy products were taste and health benefits for the human organism. The important fact is that the respondents would be motivated to the increased consumption of milk and dairy products by better informedness about the beneficial effects on human health (34.16%). The most remarkable factor, which has a positive impact on the purchase of cow`s milk, is its origin (more than 25% of respondents). The producers of the Slovak traditional cheeses can consider the consumption of the Slovak traditional cheeses to be a highly positive response (more than 94% of respondents).

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The authors declare no conflict of interest.

**Ethical Statement:**

All subjects that participated in our marketing research by providing information and filling out uniform questionnaires expressed their consent to the anonymous processing of the information provided.

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# SOCIAL DEMOGRAPHIC FACTORS INFLUENCING CONSUMER'S PREFERENCES ON RICE ATTRIBUTES IN INDONESIA: A MULTINOMIAL LOGISTIC APPROACH

*Julkhaidar Romadhon, Muhammad Yazid, Andy Mulyana, Yunita*

## ABSTRACT

This study assessed factors influencing consumer's preferences on rice attributes in Indonesia using data collected from a sample of 329 consumers in South Sumatra Province in Indonesia. This study used two variables such as independent variables and dependent variables. Independent variables as a predictor of attributes of consumer preferences include social-demographic variables. On another side, dependent variables include attributes of rice-based on the preferences of the consumer. Social demographic factors such as gender, age, number of families, occupation, education, and income are mentioned to influence consumer's preference for rice. Rice attributes such as small broken, chalky grains, higher broken, varieties, family reference, friend reference, suppliers, advertisement, foreign object, residue, packaging, brand, volume expansion, head rice, flavor, aroma soft texture, durability, and whiteness. This study employed the multinomial logistic regression analysis to examine the effects of these variables on rice preference. This study revealed that among household characteristics that influence consumers' preference for rice attributes were household income and the type of occupation of the household head.

**Keywords:** social-demographic; preferences; consumer; rice attributes; multinomial logistic regression

## INTRODUCTION

Rice is the most important commodity in Indonesia, especially for the poorest members of society **(Timmer, 2004; Widarjono, 2018)**. Rice is not only considered by consumers as a commodity but also as a product with certain criteria. Consumers' rice preferences differed greatly among nationalities. Rice trader's and the farmer have to understand these different preferences to offer the right products to their customers **(Suwannaporn and Linnemann, 2008)**. Product attributes are product elements that are considered important by consumers and are used as the basis for purchasing decisions **(Banović et al., 2010; Garvin, 1984)**. Product attributes are characteristics of a product that functions as evaluative attributes during decision making. Products have several characteristics that serve as indicators that represent quality for consumers **(Lancaster, 1966; Powel, Han and Chaloupka, 2010; Stávková, Stejskal and Toufarová, 2008; Zeithaml, 1988)**.

Along with the increase in income, there will gradually be a shift in spending patterns, in which expenditure on food will decrease and there will be an increase in the portion of expenditure for non-food items **(Engel, Blackwell, and Miniard, 1995; Grunert, 2005; Kotler and Amstrong, 2008)**. Consumer preference for the rice that will be purchased is also affected by several factors, namely marketing activities that are characterized by product variations in terms of packaging, brand and size, promotion, product quality, and customer testimonials **(Suwannaporn, Linnemann and Chaveesuk, 2008)**. The quality of rice is getting better with the existence of modern rice milling units, but the amount of rice consumption per capita has decreased, especially in high-income groups. The rice milling industry in Indonesia has been dominated by small-scale rice mills for a long time. This type of mill is incapable of producing good quality rice at low costs. The number of small-scale rice mills (SSRM) has continued to grow. This type of rice mills is currently presumed to face serious difficulty in obtaining grains, resulting in quite high idle capacity **(Sawit, 2019)**. High-income consumers who like premium rice from local varieties can push up the price of this type of rice, which is only available in small quantities in the market **(Damardjati et al., 1988; Unnevehr, Duff and Juliano, 1992)**.

The consumption of rice in South Sumatra from January to December 2018 was estimated to be around 824,290 tons, lower than the total rice production in the same year **(Central Bureau of Statistics, 2019)**. The rice surplus in South Sumatra in 2018 was estimated at around 687,690 tons. The per capita consumption was estimated using the average per capita consumption figure per province **(Central Bureau of Statistics, 2019)**. The

average rice consumption of the people in South Sumatra was 124 kg per capita per year while the average national consumption was 111.58 kg per capita per year. For the city of Palembang, with a population of 1.7 million people, the consumption of rice was estimated to achieve 210,000 tons. Palembang City contributes to rice production in South Sumatra Province in 2018 was only 24,470 tons **(Central Bureau of Statistics, 2017)**. The expenditure pattern of the Palembang City population during the last 4 years for non-food expenditure is greater than expenditure on food. This shows that the welfare of the population of Palembang City has increased so that the preference shift from prioritizing quantity to quality. In this case, the attributes of rice become important determinants of consumer preferences **(Central Bureau of Statistics, 2019)**.

South Sumatra Province is known as the center and is one of the biggest contributors to rice production in Indonesia. The estimated total rice production in South Sumatra in 2018 was 2.65 million tons which, if converted into rice was 1.5 million tons. The consumption of rice in South Sumatra from January to December 2018 was estimated at 824,290 tons **(BPS, 2019)**. Palembang has a diverse community structure where culture, lifestyle, education, and employment are reflected in everyday life. The diversity naturally affects the people in the city in consumption decision making of a product, including the consumption of rice. Sako, Kalidoni, and Ilir Timur III are districts that represent the most prevalent population of all districts in Palembang. The sub-district has a population with a background in social status that varies greatly from the lower, middle, and upper classes.

This article discusses the social demographic factors that influence consumer preferences for rice in Palembang based on the survey covering 3 districts in Palembang City.

## Scientific hypothesis

It is assumed that the social demographic characteristics of consumers in Palembang City that affect consumer preferences include age, gender, number of family members, education level, occupation, and income level.

# MATERIAL AND METHODOLOGY

## Study Area, Population, and Sample Size

Palembang City as the capital city of South Sumatra Province is located between $2^0$ 5' and $3^0$ 5' South latitude and between $104^0$ 37' and $104^0$ 52' East longitude. The area of Palembang City is 40,061 hectares or about 2.65 percent of the total land area of South Sumatra Province. In 2000 there were 14 sub-districts and 103 sub-districts and in 2017 there were 4 additional sub-districts, bringing the total to 18 sub-districts and 107 sub-districts.

This study was undertaken in the city of Palembang, South Sumatra Province, Indonesia (Figure 1). The districts selected were Sako District, Ilir Timur II District, and Kalidoni District. Sako District represents low-income consumers, Ilir Timur II District represents upper-middle-income consumers and Kalidoni District represents high-income consumers. The number of samples in this study was 329 respondents. This city was selected for: (1) residence of households consuming rice with quality reference, (2) place of various types of rice retailers from traditional to modern sellers. Data collection was conducted in May – September 2019. The distribution of the sample is presented in Table1. Figure 2 was given to show the distribution of respondent household income.

## Statistical Analysis

***Multinomial logistic regression analysis***

The multinomial logit regression (MNL) is commonly used in collision severity analysis, in which collisions can be categorized into more than two levels with one level as a reference category **(Guo et al., 2018)**. The multinomial or multivariate logit model, unlike the logit model, is rarely applied in analyzing consumer preferences for rice attributes. However, this model is more flexible since it can accommodate various choices faced by decision-makers. Not limited to just two options as in the logit model. Logistic regression does not assume a linear relationship between the independent and dependent variables but is non-linear so it does not require classical assumptions as in linear regression. The independent variables include gender, marital status, age of consumers, level of education, number of family members, occupation, income, and other rice attributes, while the dependent variable is the quality and physical attributes of rice, including crunchiness, taste, aroma, and grain.

The type of measurement used in this study is an ordinal measurement (stratified) with a Likert scale. The Likert scale is used to examine how strongly the subjects agree or disagree with statements on a 5-point scale, namely 1 = very dislike, 2 = dislike, 3 = neutral, 4 = like and 5 = very like **(Sugiyono, 2015)**.

The logit multinomial regression equation in this study is expressed in the form **(Hosmer and Lemeshow, 2000)**:

$$\text{Ln}\,(P / 1\text{-}P) = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + e \quad (1)$$

Where:
Ln = Natural logarithm
P = probability of consumer preference choosing an attribute
$b_0$ = Regression constant, or Intercept
b1,2,3 .... 6 = Age regression coefficient
$X_1$ = Age (years)
$X_2$ = gender (0 = female, 1 = male)
$X_3$ = Number of family members (people)
$X_4$ = Education level (0 = elementary-junior high school, 2 = high school, 3 = Bachelor degree)
$X_5$ = Occupation (0 = Housewife, 1 = private, 2 = PNS / BUMN)
$X_6$ = Income Level (Rupiah)

Rice Attribute:
$Y_1$ = Small broken, Chalky Grains, Higher Broken, Varieties
$Y_2$ = Family Reference, Friend Reference, Suppliers, Advertisement
$Y_3$ = Foreign object, Residue
$Y_4$ = Packaging, Brand
$Y_5$ = Volume Expansion, Head Rice, Flavor, Aroma
$Y_6$ = Soft texture, Durability
$Y_7$ = Whiteness
e = Confounding variance

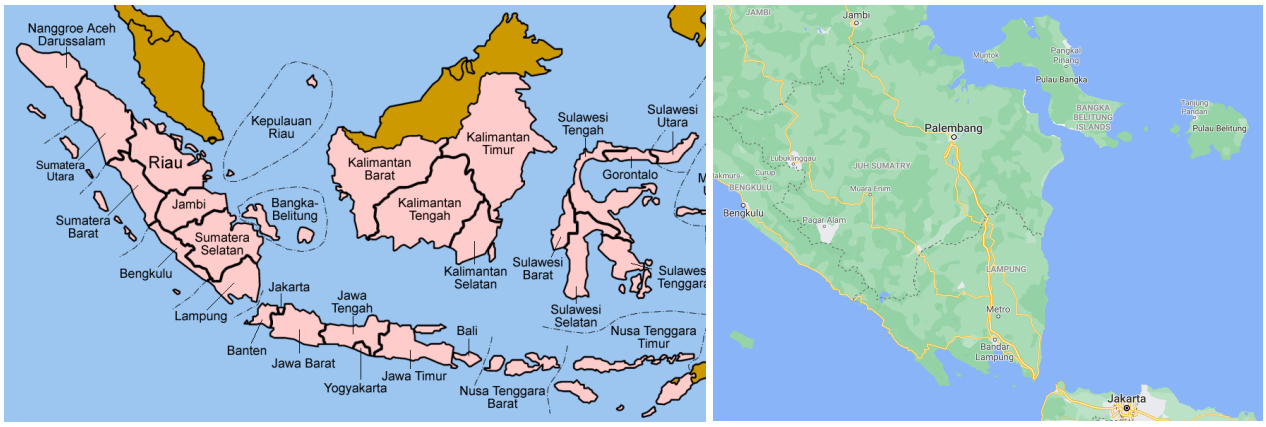


**Figure 1** Location of the study in Palembang, South Sumatra, Indonesia.

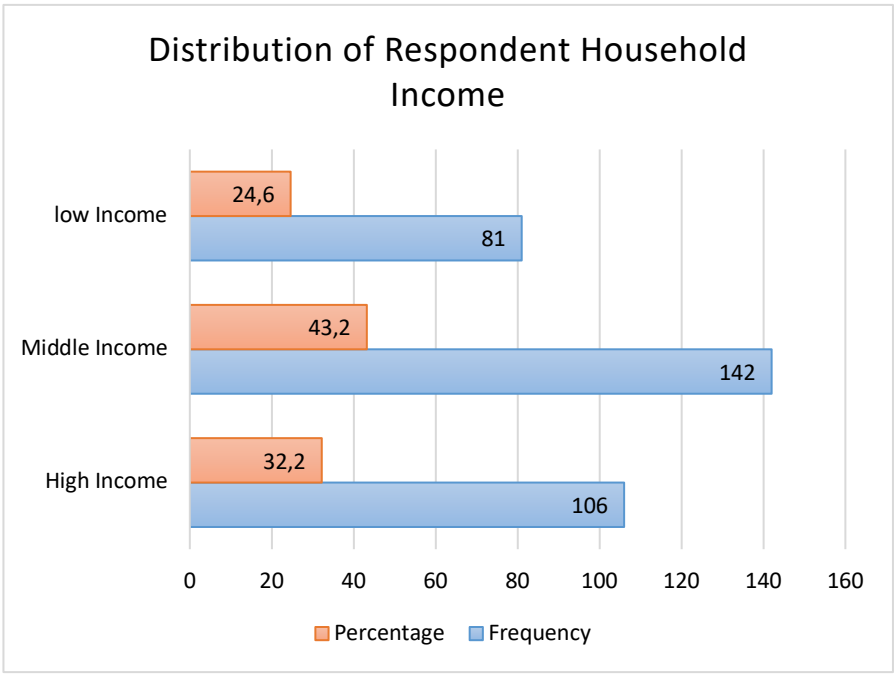


**Figure 2** Distribution of Respondent Household Income.

Logistic regression statistical testing is used to check the goodness of a model. The logistic regression method is expressed in a probability model, namely a model where the dependent variable is the logarithm of the probability that an attribute will apply in the presence of certain independent variables.

Multinomial logistic regression analysis is a logistic regression that is used when the dependent variable has a multinomial scale with a nominal scale response variable. Logistic regression analysis includes independent test, simultaneous testing, partial testing, model suitability, model goodness, and classification accuracy. The *p*-values used in the analysis were $p$ <0.01; 0.05 and 0.10.

***Model Significance Test***

This test is used to determine the effect of the independent variables on the dependent variable together (overall) in the logistic regression model. This test uses the Likelihood Ratio Test with the following hypothesis:

$H_0$: $\beta_1 = \beta_2 ..... = \beta_i = 0$ (there is no at least one independent variable that affects the dependent variable)
$H_1$: $\beta_i \neq 0$ (there is at least one independent variable that affects the dependent variable)
for i = 1,2,3, ..... n

The test statistics used in this test are:

$$G^2 = -2 \ln \frac{lo}{li} \qquad (2)$$

Where:
$l_0$= Maximum likelihood value of the reduction model (Reduced Model) or a model that only consists of constants (without explanatory variables)
$l_i$ = The maximum likelihood value of the full model (Full Model) or a model with all independent variables

The value of $G^2$ follows the Chi-squares distribution with degrees of freedom p, so the hypothesis is rejected if $G^2 > X^2$ (α, p) or *p*-value <α, which means that the independent variables (X) jointly affect the dependent variable (Y).

***Model Parameter Test***

This test is carried out after knowing that in the influential test result, there is at least one independent variable that affects the dependent variable. The purpose of this test is to determine the independent variables that significantly affect the dependent variable. This test is carried out through the Wald (W) test to test the meaning of the β coefficient partially with the following hypothesis:

$H_0$: $\beta_i = 0$ (the independent variable from i that has no significant effect on the dependent variable).

$H_1$: $\beta_i \neq 0$ (the independent variable from i that has a significant influence on the dependent variable).

for i = 1,2,3, .... n

The test statistics used are:

$$W_k = \frac{\beta_k}{SE(\beta_k)} \qquad (3)$$

Where:

$W_k$ = Wald value

$\beta_k$ = Coefficient vector associated with the estimator (coefficient X)

*SE* ($\beta_k$) = Error of $\beta_k$

$H_0$ will be rejected if $W > X^2$ (α, p) or *p*-value <α, which means the independent variable $X_i$ partially affects the dependent variable Y.

***Odds Ratio Test***

This test is a measure of risk, or the tendency to experience certain events from one category to another, where the category Xi = 1 against Xi = 0. The value of the odds ratio coefficient is expressed in exp (β), which states the risk, or the tendency of the effect of observations with category Xi = 1 is the number of times compared to the observation with the category Xi = 0.

## RESULTS AND DISCUSSION

### Social Demographic Characteristics of the Respondents

The distribution of the demographic profile of respondents is shown in Table 2. Social demographic characteristics of the respondents such as age, gender, education, income, and occupation were hypothesized to positively or negatively influence consumer preferences. The total sample comprises 217 males and 112 females. The age of respondents was grouped into 3 categories; 35 years or below comprises 45.3%, 36 to 49 years old 32.2%, and 50 years or more 22.5%. Respondent's educational background was grouped into 3, elementary to junior high school 7.6%, senior high school 18.9%, and university 73.5%. Respondent's household income was grouped into 3, high income 32.2%, middle income 43.2%, and low income 24.6% (Figure 2).

### Factors Affecting Consumer Preferences on Rice Attributes

Multinomial logistic regression analysis is a logistic regression that is used when the dependent variable has a multinomial scale. Logistic regression analysis includes independent test, simultaneous testing, partial testing, model suitability, model goodness, and classification accuracy (Table3).

***Relationship between predictor variables (Xs) and consumer preferences (Y)***

Based on the test statistics, Table 4 shows that the variables age and education has value $\chi^2$ *count* that is smaller than $\chi^2$ *table* and a *p*-value greater than 0.1, which means failure to reject $H_0$, so it can be concluded that with a confidence level of 90% there is no relationship between consumer preferences in Palembang City with age dan education level of consumers. So that in this simultaneous test only the variables of gender, family members, occupation, and income level will be further analyzed.

### Modeling of consumer preferences

The response variable in this study is consumer preferences based on the attributes which consist of seven groups, namely group 0 (small broken, chalky grains, broken grains, varieties), group 1 (family reference, friend reference, supplier, advertisement), group 2 (foreign object, residue), group 3 (packaging, brand), group 4 (volume expansion, head rice, flavor, aroma), group 5 (soft texture, durability) and group 6 (whiteness). Simultaneous testing is used to find out a predictor variable that has a significant effect on consumer preferences.

Table 5 shows that the G value of 802.378 is greater than the value 2 tables which are 79.386 and a *p*-value (0.10) of 0.000 which is smaller than 0.1, which means reject $H_0$, so it can be concluded that with a confidence level of 90% there is at least one predictor variable (social demographic factors) that has a significant effect on consumer preferences on rice attributes. Simultaneous testing of the consumer preference attribute group in the city of Palembang resulted in a decision to reject $H_0$ so that it could be continued on a partial test.

### Assessment of the feasibility of the regression model

Model feasibility testing is carried out to determine whether there is a difference in observation results and predictions. The model is said to be able to predict the value of the observation because it matches the observation data if the value is sig. Chi-square >0.10. The value of goodness of fit test in Table 6 is measured by the Chi-square value in the coefficient of deviation. In the table, it can be seen that the value of the statistical significance of Chi-square is 0.216 which is above 0.10.

Table 6 shows that the Pearsons value obtained was 1,274.793 smaller than the value $\chi 2$ table 1,300.131 and the *p*-value of 0.216 are greater than 0.10 (*p*-value >α; (0.216 >0.10), so it can be concluded that with a confidence level of 90% the resulting regression model is fit for further analysis, namely Pseudo $R^2$ and Classification Test.

**Table 1** The distribution of the sample.

| Districts | Population (People) | Sub Sample Size (People) |
|---|---|---|
| Sako | 95,104 | 113 |
| Ilir Timur II | 167,491 | 116 |
| Kalidoni | 122,672 | 100 |
| Total | 385,267 | 329 |

Note: Source*:* Field survey results (2019); **Central Bureau of Statistics (2019)**.

**Table 2** Socio-demographic profile of respondents.

| Characteristics | Attributes | Number (N = 329) | % |
|---|---|---|---|
| Age | ≤35 years | 149 | 45.3 |
| | 36 – 49 | 106 | 32.2 |
| | 50 up | 74 | 22.5 |
| Gender | Male | 217 | 66.0 |
| | Female | 112 | 34.0 |
| Occupation | Government officers | 168 | 51.1 |
| | Private sector workers | 118 | 35.9 |
| | Housewives | 43 | 13.1 |
| Educational background | Elementary – Junior High | 25 | 7.6 |
| | Senior High School | 62 | 18.9 |
| | Bachelor | 242 | 73.5 |
| Monthly income | Below Rp 2.999.999 | 122 | 37.1 |
| | Rp 3.000.000 – 9.999.999 | 176 | 53.5 |
| | Rp 10.000.000 or higher | 31 | 9.4 |
| Number of family members | 3 | 130 | 39.5 |
| | 4 – 5 | 151 | 45.9 |
| | >5 | 48 | 14.6 |

Note: Source: Field survey results (2019).

**Table 3** The variable sused in the study.

| Variable | Type | Category |
|---|---|---|
| Consumer Preference (Y) | Multinomial | 1 = Small broken, Chalky Grains, Higher Broken, Varieties (Reference category)<br>2 = Family Reference, Friend Reference, Suppliers, Advertisement<br>3 = Foreign object, Residue<br>4 = Packaging, Brand<br>5 = Volume expansion, Head Rice, Flavor, Aroma<br>6 = Soft texture, Durability<br>7 = Whiteness |
| Age ($X_1$) | Ratio | Year |
| Gender ($X_2$) | Nominal | 0 = Female<br>1 = Male (Reference category) |
| Family members ($X_3$) | Ratio | Person |
| Education level ($X_4$) | Ordinal | 0 = Elementary – secondary school<br>1 = High school<br>2 = University (Reference category) |
| Occupation ($X_5$) | Nominal | 0 = Housewife<br>1 = Private<br>2 = Government Official (Reference category) |
| Income ($X_6$) | Ratio | Rupiah |

Note: Source: Results of data analysis.

**Table 4** Independence test results.

| Variable | Df | $\chi^2$ count | $\chi^2$ table | *p*-value | Decision |
|---|---|---|---|---|---|
| Age | 12 | 21,680 | 21,026 | 0.179 | Failed to reject H0 |
| Gender | 6 | 30,375 | 12,591 | 0.000 | Reject H0 |
| Family members | 12 | 9,737 | 21,026 | 0.022 | Reject H0 |
| Education | 12 | 10,888 | 21,026 | 0.539 | Failed to reject H0 |
| Occupation | 18 | 33,475 | 28,869 | 0.005 | Reject H0 |
| Income | 12 | 33,865 | 21,026 | 0.002 | Reject H0 |

Note: Source: Results of data analysis.

**Table 5** Concurrent test results.

| | Likelihood Ratio Test | | | |
|---|---|---|---|---|
| Model | G. | Df | $\chi^2$ table | *p*-value |
| Final | 802,378 | 30 | 79,386 | 0.000 |

Note: Source: Results of data analysis.

**Table 6** Model suitability test results.

| | Likelihood Ratio Test | | | |
|---|---|---|---|---|
| | Chi-Square | Df | $\chi^2$ table | *p*-value |
| Pearsons | 1,274.793 | 1,236 | 1,300.131 | 0.126 |
| Deviance | 691.133 | 1,236 | 1,300.131 | 1.000 |

Note: Source: Results of data analysis.

**Table 7** Pseudo R-square Value.

| Type | Score |
|---|---|
| Cox and Snell | .214 |
| Na gelkerke | .224 |
| McFadden | .076 |

Note: Source: Results of data analysis.

**Table 8** Accuracy of model classification.

| Observed | Predicted | | | | | | | Percent correct (%) |
|---|---|---|---|---|---|---|---|---|
| | F I | F II | F III | F IV | F V | F VI | F VII | |
| F I | 0 | 0 | 3 | 0 | 0 | 5 | 0 | 0.0 |
| F II | 0 | 0 | 3 | 0 | 0 | 29 | 0 | 0.0 |
| F III | 0 | 0 | 25 | 0 | 0 | 38 | 0 | 39.7 |
| F IV | 0 | 0 | 9 | 0 | 0 | 16 | 0 | 0.0 |
| F V | 0 | 0 | 1 | 1 | 0 | 36 | 0 | 0.0 |
| F VI | 0 | 0 | 9 | 0 | 0 | 137 | 0 | 93.8 |
| F VII | 0 | 0 | 2 | 0 | 0 | 16 | 0 | 0.0 |
| Overall Percentage (%) | 0 | 0 | 15.8 | 0 | 0 | 84.2 | 0 | 49.2 |

Note: F (factor). Source: Results of data analysis.

Pseudo $R^2$ values were measured using the Nagelkerke R Square **(Agresti, 2011)**. Nagelkerke R Square is a modification of the Cox and Snell's coefficients to ensure that the value varies from 0 to 1. This is done by dividing Cox and Snell's $R^2$ values by their maximum values. The Nagelkerke $R^2$ value can be interpreted as the $R^2$ value for multiple regression. The results of the Nagelkerke value can be seen in Table 7.

The Nagelkerke R Square value of 0.224 indicates that the variability of the dependent variable which can be explained by the variability of the independent variable is 22.4%, while the remaining 77.6% is explained by other variables not used in this study.

Model feasibility can also be predicted using a classification matrix that calculates the correct and incorrect estimation values on the dependent variable. The

classification matrix shows the predictive power of the regression model. The classification accuracy obtained by the model can be seen in Table 8.

Table 8 shows the classification accuracy of the model, which is 49.2%, which means the model's ability to predict accurately according to observations (real conditions) is 49.2%, while the resulting classification error is 50.8%.

## Partial Analysis of the Effect of Predictor Variables on Consumer Preferences

To determine the significance of the influence of the predictor variables on individual consumer preferences, a parameter test was carried out individually using the Wald Test. The test results using the attribute group of small broken, chalky grains, higher broken and varieties as a comparison category for parameter estimates between the attribute groups of rice with age, sex, type of work, and income level can be seen in Table 9.

Table 9 shows that the variables that have a significant effect on consumer preferences are consumer age, gender, occupation and income level, which can be expressed in the six multinomial logistic regression functions as follows:

g1 (X) = 2.545 + 0.778x2 (0) - 0.138x3 - 1.786x5 (0) - 0.006x5 (1) - 0.190x6
g2 (X) = 3.142 - 0.543x2 (0) - 0.094x3 - 2.826x5 (0) - 0.043x5 (1) - 0.021x6
g3 (X) = 2.128 + 0.029x2 (0) - 0.063x3 - 2.361x5 (0) - 0.183x5 (1) - 0.068x6
g4 (X) = 2.487 + 0.437x2 (0) - 0.229x3 + 0.437x5 (0) + 0.919x5 (1) - 0.096x6
g5 (X) = 3.203 + 0.795x2 (0) - 0.067x3 - 0.613x5 (0) + 0.568x5 (1) - 0.128x6
g6 (X) = 0.364 + 0.389x2 (0) + 0.248x3 - 2.464x5 (0) + 0.096x5 (1) - 0.127x6

The first logit covers attributes of family references, friend references, suppliers, and advertisements. Explanatory variables that have a significant effect on the decision choosing this attribute is the level of income with a *p*-value of 0.007, while gender, number of family members, and type of work do not have a significant effect with a *p*-value >0.10 at the significance level of $\alpha = 10\%$. In the logit equation 1, the coefficient of the income variable is -0.190 with an odds ratio of 0.827 and the Wald test is significant at the 10% level. This shows if the variables of gender, family member, type of work are constant, then every IDR 1 million increase in terms of income level, the opportunity to choose attributes of family references, friends references, suppliers, and advertisements compared to choosing attributes of whole grains, broken items, groats, and shapes is 0.827. This means that the higher the income of consumers, the more likely it is to prefer the attributes of small broken, chalky grains, higher broken, varieties compared to attributes of family references, friend references, suppliers, and advertisements.

The analysis result that household characteristics that influence consumers' preference for rice attributes were household income and the type of occupation of the household head. This finding is in line with studies **(Wahyudi et al., 2019)** which found that consumers' incomes lead to increased demand for quality.

Preferences for rice attributes are found influenced by gender, education levels, household size and income, rice consumption, expenditure share, and purchase frequency **(Anang, Adjei Adjetey and Abiriwe, 2011)**. Increased Consumers' personal food choices are changing due to a greater variety of food products higher incomes, and better choices of food retailers **(Brečić, Mesić and Cerjak, 2017)**.

Consumers with high household income choosing attributes of whole grains, broken items, groats, and shapes. The physical appearance of the rice is an attraction for consumers to buy. Another support of preference consumers is presented by **(Tomlins et al., 2007)**. Visual characteristics of rice grains are important to search attributes that affect consumers' purchasing decisions and hence are used as some of the first selection criteria in varietal improvement programs. The appearance of a product can influence consumer choice in different ways. A product's appearance can have aesthetic and symbolic value for consumers, communicate functional characteristics and give a quality impression (functional value), and communicate the ease of use (ergonomic value) **(Creusen and Schoormans, 2005)**.

High-income consumers have the largest variability in rice grain quality attributes and concurrently appear to have the most pronounced preferences among consumers **(Cuevas et al., 2016)**. Quality attributes of rice are different in the other countries. Guatemala consumers were willing to pay premiums for those desirable qualities.

Specific attributes such are taste, cooking quality, cooking time, and aroma were the quality characteristics that most consumers preferred **(Anang, Adjei Adjetey and Abiriwe, 2011)**. In New Zealand, the top three attributes supported for community, freshness, and seasonality **(Hiroki, Garnevska and McLaren, 2016)**. In Sri Lanka, attributes of rice like rice production, processing, marketing, and value addition **(Walisinghe and Gunaratne, 2012)**.

Food acceptability, choice, and consumption are complex processes influenced by many factors as intrinsic, e.g. color, aroma, flavor, and texture, as well as extrinsic to the product. The extrinsic factors have been included in several types of research aiming at having a better understanding of consumer behavior **(Iop, Teixeira and Deliza, 2006)**. Consumers' tastes are known as factors that affect product demand such as rice. Branded packaged rice produced by local agro-industries is expected to meet the rice multi-attribute **(Widayanti et al., 2020)**.

Family members and age are not significantly influencing consumers' preference for rice attributes in this analysis. A large number of families means a lot of rice is consumed so that the attribute factor is ignored. At this point, the price factor becomes the main determining factor considered by consumers. It's inline with finding studies **(Supriana and Pane, 2018)** that characteristics of rice consumers have a positive and significant effect on the amount of rice consumed are age and the number of family members. In other studies **(Wahyudi et al., 2019)** which analyzed socio-demographic factors that economic and demographic changes such as education, urbanization rates, and female labor force participation rates, along with current transportation and communications advances, influence consumer preferences

**Table 9** The variables that have a significant effect on consumer preferences.

| Logit Equation | Predictor Variable | B | Wald | *p*-value | Odds Ratio |
|---|---|---|---|---|---|
| Logit 1 (family reference, friend reference, supplier, advertisement) | Constant | 2.545 | 3.648 | 0.056 | |
| | Gender (0) | 0.778 | 0.718 | 0.397 | 2.176 |
| | Aug Family | -0.138 | 0.302 | 0.582 | 0.871 |
| | Occupation (0) | -1.786 | 2.445 | 0.118 | 0.168 |
| | Occupation (1) | -0.006 | 0.000 | 0.995 | 0.994 |
| | Income | -0.190 | 7.240 | 0.007 *** | 0.827 |
| Logit 2 (foreign object, residue) | Constant | 3.142 | 6.602 | 0.010 | |
| | Gender (0) | -0.543 | 0.407 | 0.524 | 0.581 |
| | Family Members | -0.094 | 0.163 | 0.686 | 0.910 |
| | Occupation (0) | -2.826 | 4.142 | 0.042 ** | 0.059 |
| | Occupation (1) | -0.043 | 0.002 | 0.963 | 0.968 |
| | Income | -0.021 | 0.207 | 0.649 | 0.980 |
| Logit 3 (packaging, brand) | Constant | 2.128 | 2.536 | 0.111 | |
| | Gender (0) | 0.229 | 0.001 | 0.974 | 1.030 |
| | Family Members | -0.063 | 0.060 | 0.806 | 0.939 |
| | Occupation (0) | -2.361 | 2.800 | 0.094 * | 0.094 |
| | Occupation (1) | -0.183 | 0.034 | 0.854 | 0.853 |
| | Income | -0.068 | 1.313 | 0.252 | 0.934 |
| Logit 4 (volume expansion, head rice, flavor, aroma) | Constant | 2.487 | 3.623 | 0.057 | |
| | Gender (0) | 0.437 | 0.240 | 0.625 | 1.549 |
| | Family Members | -0.229 | 0.855 | 0.355 | 0.795 |
| | Occupation (0) | 0.437 | 0.240 | 0.625 | 1.549 |
| | Occupation (1) | 0.919 | 0.919 | 0.338 | 2.507 |
| | Income | -0.096 | 2.467 | 0.116 | 0.909 |
| Logit 5 (soft texture, durability) | Constant | 3.203 | 7.157 | 0.007 | |
| | Gender (0) | 0.795 | 0.895 | 0.344 | 2.214 |
| | Family Members | -0.067 | 0.091 | 0.763 | 0.935 |
| | Occupation (0) | -0.613 | 0.394 | 0.530 | 0.542 |
| | Occupation (1) | 0.568 | 0.390 | 0.533 | 1.764 |
| | Income | -0.128 | 5.955 | 0.015 ** | 0.880 |
| | | 0.364 | 0.066 | 0.797 | |

Note: *** = Significant at $p$ <0.01; ** = Significant at $p$ <0.05; * = Significant at $p$ <0.10.

## CONCLUSION

Household characteristic factors that significantly influence the preference attributes of rice, family reference, friend reference, supplier, advertisement, soft texture, durability, volume expansion, head rice, taste and degree of whiteness are the level of consumer income. Meanwhile, the household characteristic factors that significantly influence the preference attributes of foreign matter rice, residue, packaging, brand, and degree of whiteness are the types of consumer occupation.

The reference group is an important factor in increasing the demand for rice. Thus, the rice should be marketed in organizational groups. The development of the rice market should be carried out in collaboration with employee cooperatives in public and private institutions. This group is a very potential target market, because it is relatively well educated, has a steady income, and has a high intensity of communication among members.

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This article does not contain any studies that would require an ethical statement.

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# COMPARATIVE CHARACTERISTICS OF EGGS OF CHICKENS OF DOMESTIC AND FOREIGN SELECTION IN THEIR DIVERSE AGE

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## ABSTRACT
At this juncture, more and more attention is drawn to the preservation of the gene pool of local breeds of poultry, which have a set of valuable features, including high-quality eggs. In this study, the quality parameters of eggs (egg weight, weight, and proportion of yolk, weight, and proportion of albumen, yolk-albumen ratio, energy value, lipid and fatty acid content in the yolk) of chickens of Ukrainian selection(such hybrids as Plymouth white, Poltava Clay, Birkivska Barvysta and their interbreed hybrids) and foreign commercial crosses (Tetra SL, Tetra H, Super Harko, Lohmann Brown, Highsex White and Brown, High Line W36), at different ages (26, 34, 44 weeks of age) were studied in the comparative aspect. The average egg weight of Ukrainian breeds and hybrids was 51.9 g, 56.5 g, and 61.2 g at the age of 26, 34, and 44 weeks, respectively, in foreign crosses it was much higher – 59 g, 61.2 g, and 64.5 g, respectively ($p$ <0.01). At the same time, there was a probable advantage of the Ukrainian selection bird over the foreign selection bird in the proportion of yolk (by 4.3%, 2.9%, and 4.1% at the age of 26.34 and 44 weeks, respectively), the yolk-albumen ratio (by 0.12, 0.07 and 0.08, respectively) and the energy value of the egg (76, 48 and 60 kJ, respectively). Higher lipid content was found in the egg yolks of domestic chickens in comparison with the foreign hybrid Tetra SL. The total content of saturated and unsaturated fatty acids in the egg yolks in hens of local breeds is higher than in foreign cross. High-quality characteristics of eggs of local breeds and populations are evidence of the presence of a valuable set of genes that require comprehensive study, preservation, and use in the creation of new selectively significant forms.

**Keywords:** egg; line; physical quality; morphological quality; lipid

## INTRODUCTION
The egg composition, especially the content of amino acids and fatty acids, is crucial for embryo development **(Hammershøj and Johansen, 2016)**. Energy and nutrients needed for the development of chicken embryos are mainly provided by nutrients that are stored in eggs. Egg yolk provides 90% of the energy needed for embryonic development in the form of fatty acids **(Haron et al., 2017; Zhu et al., 2020)**. The main part of the dry matter of egg yolk consists of lipids (over 60%), which are the main source of energy for embryos **(Yadgary et al., 2013)**.

Lipid metabolism during the embryonic development of birds is characterized by certain features. In particular, a chicken embryo, which is contained in the yolk of a fertilized egg for 21 days, develops in a closed environment **(Surai, Fisinin and Karadas, 2016)**. During embryonic development, complex physiological and biochemical processes take place in the egg between the embryo, yolk, protein, and shell. In the early stages of development, the embryo assimilates yolk substances, including lipids, and uses them as a flexible material and energy source **(Duh, Vovk and Martsynovskyi, 2017)**.

It is known that the energy value of lipids is twice as high as proteins and carbohydrates, so lipids are one of the main sources of energy. The lipids in egg yolk are mainly presented by phospholipids and triacylglycerols, after their hydrolysis by lipolytic enzymes they are used as a structural and energy material. Fatty acids mainly linoleic, oleic, stearic, and palmitic ones are present in the composition of egg yolk. The first two are necessary for the initial stages of embryo formation because they are closest to them and are used earlier. Also, unsaturated fatty acids are precursors of eicosanoids, prostaglandins, prostacyclins, leukotrienes, and other substances. Lipid nutrition affects the metabolic processes in the embryo's body, the intensity of their development, productivity, and resistance of birds **(Orishchuk et al., 2014)**.

Besides, the embryo needs fatty acids to synthesize the phospholipids, which are the components of cell membranes, as well as to synthesize the triglycerides to store energy. There is data that egg yolk lipids contain significantly more linoleic acid than lipids of other tissues. Poultry`s need for polyunsaturated fatty acids (PUFAs) at an early age depends on their use in embryonic

development), which are involved in the regulation of several physiological processes **(Tkach et al., 2018)**.
Almost all lipids are fat-soluble vitamins. More than half of proteins and most parts of the water-soluble egg's vitamins are in the yolk **(Yadgary et al., 2013)**. The yolk influence on the chicken embryo becomes obvious mainly after full use of the protein by the embryo. It is at the end of embryogenesis and starts on the 16-17$^{th}$ day and lasts during the early postpartum period when the chick uses nutrients from the vestigial yolk **(Duh, Vovk and Martsynovskyi, 2017)**.

The need for the chicken embryo in the protein of the yolk varies depending on the level of development of the hatching stage. There are 119 proteins were found in chicken egg yolk. Among them, serum albumin, the breakdown products of vitellogenin (VTG), apovetelenin, immunoglobulin Y (IgY), and ovalbumin have the highest amounts in egg yolk plasma **(Zhu et al., 2020)**.
Therefore, changes in egg composition can significantly affect embryonic development. According to many scholars **(Bhattacharyya et al., 2018; Kermanshahi et al., 2017)**, the full value of eggs primarily depends on the rational bird diet. Restriction in the feeding of laying hens reduces the egg weight and significantly reduces egg production, egg shape index, and content of cholesterol in the yolk **(Li et al., 2019a)**.

Studies **(Li et al., 2019b)** have shown that low food, which laying hens intake, affects the process of embryonic development, changes the expression of myostatin and myogenin genes, and reduces the weight of the offspring at birth. Also, the laying hen's diet affects the amino acids and fatty acids of eggs, which are important nutrients for embryonic development during hatching.
However, significant changes in the ratio of egg components can occur as a result of intensive selection to increase egg production. In highly productive foreign crosses (Lohmann, Highsex, Iza, High Line) the increase in egg mass at the beginning of egg-laying (the first 5 – 6 months) is mainly due to the mass of protein. The percentage of it in the egg reaches 67% **(Orishchuk et al., 2014; Singh, Cheng and Silversides, 2009; Zita, Tůmová and Štolc, 2009)** As a result, the protein-yolk ratio is at the level of 2.5 – 2.7. At the same time, it was found that the most nutritious properties have the eggs with a protein-yolk ratio of 1.9 – 2.0.

Aboriginal poultry (local breeds), which, although less productive, but well adapted to local conditions and is a carrier of a set of valuable features such as resistance to several diseases and adverse environmental conditions, also, has high-quality eggs and meat and is valued socially-cultural life of local communities. In this regard, at the global level, there is a problem to study the quality of bird product of different genetic origin to widely use genotypes which are capable of high egg production and high product quality.

Thus, the purpose of our work was to compare the physical and chemical quality of eggs of different lines and breeds of chickens of domestic and foreign selection.

### Scientific hypothesis

This study is based on the hypothesis that genotype is one of the most important factors which influence not only egg-laying and weight, but also the egg quality characteristics. This is especially true in the difference between highly productive foreign crosses (Lohmann, Haysex, Iza, High Line, etc.), which are widely used for egg production, and aboriginal (local) breeds, which are valued in the socio-cultural life of local communities and, although less productive but well adapted to local conditions. Comparative analysis of product quality in poultry of different genetic origin would draw attention to the preservation of the gene pool of local poultry breeds, which are carriers of a set of valuable features such as resistance to several diseases and unfriendly environmental conditions, high-quality eggs, and meat and creation of new selectively significant forms.

## MATERIAL AND METHODOLOGY

### Samples

The State Poultry Research Station of the National Academy of Agrarian Sciences of Ukraine has been working on the conservation of genetic resources of poultry and its use for many years to create hybrids with high egg production and product quality. Both the autochthonous breeds and populations (Poltava Clay) and the breeds and lines of the Ukrainian selection, (factory line H2) of the Plymouth White breed, Birkivska barvysta) created on their basis, are preserved in the gene pool.

Experimental studies involving poultry were approved and endorsed by the Commission on Bioethics of the State Poultry Research Station NAAS (Ukraine) and conducted following the standards of breeding, keeping, and feeding, as well as the recommendations of the European Convention for the protection of vertebrate animals used for experiments or other scientific purposes.

The aboriginal breed of hens of the egg-meat direction of productivity Poltava clay (line 14) is approved as a new selection achievement by the Order of the Ministry of Agrarian Policy of Ukraine No 781/111 from 6.11.07. The bird is characterized by excellent adaptation to local breeding conditions, increased resistance to neoplastic diseases, high preservation (94.4%) at laying ability 123 – 132 eggs per average laying hen at the age 52 weeks, and egg weight 53.6 – 54.7 g at 30 weeks.

Meat and egg chickens of the factory line H2 of the Plymouth Rock breed approved as a selection achievement by the Order of the Ministry of Agrarian Policy and Food of Ukraine No 146 of April 11, 2016, are better known in Ukraine as Hercules. The bird has a well-defined double type of productivity and high adaptive qualities and are characterized by a high live weight of 3.14 kg (chicks) – 4.1 kg (roosters) and egg weight (65.5 – 68.1 g) at 52 weeks of age at egg-laying per average laying hen 114 – 126 pcs.
Birds of egg production Birkivska barvysta (line A) are of synthetic origin, they were bred based on crossing chickens White Leghorn and roosters of the local population of colored leghorn. The breed is in the final stages of creation, autosexual, sorting accuracy at the age of up to 98%. The bird lays 250 – 265 eggs per year with an average weight of 50 – 51 g at 30 weeks age, 58 – 59 g – at 52 weeks.

These genetic resources are lower in productivity to foreign commercial hybrids, which are represented in the Ukrainian market but are characterized by extremely valuable features, such as high adaptation to local climatic conditions, resistance to several diseases, unpretentiousness to feed, high product quality. That is, local breeds remain

the carriers of valuable hereditary qualities and gene complexes, without which the further breed-forming process would be one-sided.

**Animals and Biological Material:**

Physico-morphological qualities of eggs of hens of Ukrainian selection - three initial parental forms (H2 - Plymouth White, 14 - Poltava Clay, A - Birkivska barvysta) and two hybrids (H2x14, 14xA) in different age periods (26, 34, 44 weeks of life) were studied. Adult chickens of domestic selection were kept in two-tier group cage batteries at a planting density of 9.5 – 9.8 species per $m^2$, feeding and keeping conditions of all groups met the standards. The feeding ratio during the egg-laying phase was isoprotein (17% crude protein) and isoenergetic (2650 kcal/kg) and met the norms for hens in terms of basic nutritional values. Dietary ingredients: corn – 49.4%, wheat – 10.0%, sunflower cake – 12.3%, soybean meal – 14.1%, meat and bone meal – 5.0%, bone meal – 1.15%, lysine – 0.08%, methionine – 0.11%, chalk – 7.5%, salt – 0.16%, vitamin premix – 0.2%. A randomized sample of eggs (60 pieces) was evaluated from each experimental group. A total of 900 Ukrainian poultry eggs were evaluated.

At the same time, a comparative analysis of the studied indexes of local poultry with the indexes of imported commercial crosses used in Ukraine (Tetra SL, Tetra H, Super Harko, Lohmann Brown, Haysex White and Brown, High Line) was made. The physical and morphological qualities of eggs from both poultry and final hybrids were studied. Keeping industrial poultry is the group in multilevel cage batteries. Randomized samples of eggs from hens of foreign crosses (60 pieces from each cross) were obtained directly from producers in the. Kharkiv oblast (Ukraine). A total of 720 eggs of the poultry of foreign selection were evaluated.

**Laboratory Methods**

Freshly laid eggs were examined in the laboratory following such indexes as egg weight (g), shell weight (g), yolk weight (g), albumen weight (g), yolk content, albumen content (%), yolk- albumen ratio, energy value in terms of per 100 g of egg mass (kJ), the content of total lipids in the yolk (%), the fatty acid composition of the yolks (mg/yolk).

Firstly, the eggs were individually weighed with the help of laboratory quadrant scales VLKT-500 (Gosmetr, Russia), after breaking the egg carefully manually separated the yolk from the albumen. Eggshell, including membranes, and yolk were weighed with laboratory quadrant VLKT-500 scales (Gosmetr, Russia). The yolk was carefully rolled out on a paper towel to remove the rest of the albumen before weighing, and the chalazes were carefully removed with tweezers. All masses of egg components were determined within accuracy to 0.1 g.

The mass of the albumen was calculated by subtracting the mass of the shell and yolk from the initial mass of the egg. Using these indicators, the proportion of yolk and protein was calculated as a percentage of total egg weight, and the yolk-albumen ratio was calculated as the ratio of yolk mass to albumen mass.

The energy value was calculated according to the formula (1) offered by Ostryakova and others. **(Ostryakova, Podstreshnyi and Breslavets, 2003)**.

$$E = 100 \cdot (16 \cdot Myo + 2 \cdot Malb) / (Me - Msh) \quad (1)$$

Where:

E is the energy value of eggs (per 100 g of egg mass), kJ; Me, Msh, Myo, Malb – a mass of eggs, shells, yolks, albumen, g; 16 and 2 are constants of energy content in 1 g of yolk and albumen.

**Description of the Experiment**

The total lipid content in the yolk and assessment of their fatty acid composition was carried out on eggs of domestic chickens (H2 - Plymouth White, 14 – Poltava clay, A – Birkivska barvysta) and foreign cross Tetra SL at the age of 44 weeks. Lipid extraction was carried out by the Soxhlet method. Lipids from 4 g of yolk were extracted with 150 mL of petroleum ether using a Soxhlet extraction device. The extraction had being lasted for 2 hours at a temperature of 150 °C. The fat content was determined by the gravimetric (weight) method: the residue after extraction was dried and weighed.

To determine the fatty acid composition of the yolks there were selected 5 eggs from each group. The yolks were isolated, weighed, and stored at minus 12 °C for subsequent chromatographic analysis according to the method described in **GOST 30418 (1996)** "Vegetable oils. The approach to determining the fatty acid composition". Chromatographic analysis was performed using laboratory gas chromatograph "Crystallux-4000M" with a flame ionization detector and temperature programming, a thermostat for temperatures not lower than 200 °C, with an evaporator for temperatures not lower than 300 °C. Glass gas chromatographic column 2 m long, 2 – 4 mm inner diameter. The following analysis conditions were set on the chromatography: column thermostat temperature – 180 – 190 °C; evaporator temperature – 250 °C; detector oven temperature – 200 °C; carrier gas flow rate – 30 – 40 $cm.min^{-1}$; sample volume – about 1 mm of a solution of methyl esters of acids in hexane.

## Statistical Analysis

Calculation of calculated egg quality indexes (albumen weight, albumen. and yolk content, egg-yolk ratio, the energy value of eggs) and statistical processing of the material obtained in the experiment (average values and their standard errors) were carried out with the help of statistical analysis of Microsoft Office spreadsheet Excel. The average values were compared using the Tukey test. The significance level for all tests was $p < 0.01$.

The data of physical and morphological qualities of eggs were analyzed using a two-factor dispersion complex (ANOVA) based on breed and age of chickens, using the general procedure of linear models. The data of egg components were analyzed according to the following model (2):

$$Yijk = \mu + Bi + Aj + (BA)\,ij + eijk \quad (2)$$

Where:

μ is the total average value; Bi – the effect of the I – ïbreed, cross; Aj – the effect of the j`s- age; (BA) ij – the interaction of the i-ï breed, cross with the j`s- age; eijk – accidental error.

## RESULTS AND DISCUSSION

The physical and morphological qualities of eggs (egg weight, protein, yolk, and shell) were studied to give the comparative assessment of hybrids, lines and breeds of Ukrainian selection and parental forms and final hybrids of some imported crosses used in Ukraine (Tetra SL, Tetra H, Super Harko, Lohmann Brown, Highsex White and Brown, High Line W36) at different age periods (26, 34 and 44 weeks of life). Several index indicators, based on the obtained data, were calculated: the proportion of albumen and yolk, energy value indicators, and the yolk-albumen ratio.

Most crosses of foreign selection are characterized by high precocity and rapid increase in egg weight, especially in the first months of egg-laying. The birds of local selection, which are mainly a breed of the egg-meat type of productivity, have a lower precocity and slightly lower egg weight **(Rizzi and Chiericato, 2005; Di Rosa et al., 2020; Franco et al., 2020)**.

In our research, the average egg weight of Ukrainian breeds and hybrids was 51.9 g at 26 weeks age and 61.2 g – at 44 weeks, while in foreign crosses it was significantly higher in the respective age periods – 59 g and 64.5 g, respectively.

Parental forms of crosses in the age aspect have been studied taking into account different paces of weight gain of poultry eggs of foreign and local selection, several indicators characterizing the internal qualities of eggs for different breeds, hybrid.

Already at the age of 26 weeks, significant differences ($p$ <0.01) were found between chickens of different origins in such indicators as yolk weight, albumen, and their ratio and energy value of eggs.

Thus, the weight of the yolk in poultry of local and foreign selection at the age of 26 weeks was almost the same and amounted to 14.2 – 14.4 g, although the weight of eggs differed significantly – more than 7.1 g (13.7%). This difference was reflected in the share of yolk, which in local chickens was higher by 4.3% on average and amounted to 28.5% against 24.2% in foreign crosses. In terms of mass and proportion of albumen, the opposite picture was observed. There was a rapid increase in egg mass from the beginning of egg-laying occurred primarily due to an increase in albumen mass in hens of foreign selection. With the same mass of yolk between groups of birds, the mass of albumen in foreign crosses was much higher – by 8 g, as well as its share in the egg – by 5.7%. At this age, there is a significant difference ($p$ <0.01) in the ratio of yolk-albumen – 0.5 in local breeds and hybrids against 0.38 in foreign crosses. Due to the increased yolk content in the egg, the energy value of eggs of the poultry of local selection exceeded the foreign average by an average of 76 kJ ($p$ <0.01).

Similar results were obtained by **Lordelo et al. (2020)**, which at a higher egg weight in a commercial hybrid compared to chickens of some local breeds by 13% recorded a higher proportion of albumen in it by 7.2% and a lower proportion of yolk – by 7.8%.

According to the results of **Danchuk and Trach (2017)**, in highly productive commercial crosses the yolk mass increases with age more than the albumen mass.

In our studies, with the increasing of birds` age, the yolk mass in foreign chicken selection is increased by 17%, in local breeds and hybrids – by almost 30%. At the same time, albumen mass increased by 6% and 22%, respectively. As for the proportion of yolk, for 18 weeks of productivity (from the 26$^{th}$ to the 44$^{th}$ week of the life of the bird) there was a slight increase in this indicator in both local and foreign laying hens - in an average of 1.4-1.6%, but there is a predominance of 4.1% for birds of Ukrainian selection, which eggs are characterized by a large yolk.

At the same time, there is a tendency to a slight decrease in the share of egg albumen with the age of poultry in foreign crosses from 62.8% at 26 weeks to 61 – 61.1% at 34 – 44 weeks. These data are relevant to the results of studies by **Rizzi and Chiericato (2005)**, where it was found a decrease in the proportion of protein from 30 to 42 weeks by 0.9 – 2.7% in chickens cross High Line white and High Line brown.

In chickens of local breeds and hybrids from the 26$^{th}$ to the 34$^{th}$ week of life there was a slight increase in the proportion of albumen (by 1.6%), but then there was a decrease in this figure by 0.6%.

**Table 1** The share of influence of various factors on the expression of indicators of morphological qualities of eggs,%.

| Factor | Indices | | | | |
|---|---|---|---|---|---|
| | ME | MY | PY | RYA | EV |
| Poultry age | 45.5[a] | 49.2[a] | 5.4 | 2.7 | 2.4 |
| Genotype (breed , hybrid) | 23.3 | 13.4 | 38.7 | 32.5 | 35.2 |
| Correlation of age and genotype | 8.2 | 13.5 | 12.1 | 12.2 | 10.9 |
| Random error | 23.0 | 23.9 | 43.8 | 52.6 | 51.5 |

Notes: [1]ME – egg mass, g; MY – yolk mass, g; PY – the proportion of yolk,%; RYA – the ratio of yolk-albumen; EV – energy value, kJ; a – probably at $p$ <0.01.

**Table 2** The number of total lipids in the egg yolk of chickens of domestic and foreign selection.

| Index | A | 14 | H2 | TSL (FH) |
|---|---|---|---|---|
| The amount of total lipids in the yolk, g | 5.8 ±0.02[a] | 6.0 ±0.03[b] | 6.1 ±0.02[b] | 5.1 ±0.02[c] |
| The amount of total lipids in the yolk,% (in dry matter) | 61.7 ±0.5[a] | 62.0 ±0.5[a] | 62.5 ±0.7[a] | 57.3 ±0.6[b] |
| The amount of total lipids in the yolk,% (in natural substance) | 31.6 ±0.3[a] | 31.9 ±0.3[a] | 32.0 ±0.3[a] | 29.8 ±0.2[b] |

Notes: A – Birkivska barvysta, 14 – Poltava Clay, H2 – Plymouth White, foreign cross Tetra SL (Fnal Hybrid (FH)); a, b – averages with different upper scripts within one age differ significantly in $p$ <0.01.

**Table 3** The composition of fatty acids in the yolk of eggs of chickens of domestic and foreign selection, mg/yolk.

| Fatty Acid | The amount of fatty acid in the yolk of chicken eggs, mg/yolk | | | |
|---|---|---|---|---|
| | A | 14 | H2 | TSL (FH) |
| Saturated fatty acids | | | | |
| Miristinova (C14: 0) | 17.4 ±0.9 | 14.6 ±1.0[a] | - | 18.9 ±0.8[b] |
| Palmitinova (C16: 0) | 1.288 ±8.4[a] | 1.379 ±9.7[c] | 1.443 ±8.1[d] | 978 ±8.3[b] |
| Margarinova (C17: 0) | 11.6 ±1.2 | - | - | 12.7 ±1.2 |
| Stearinova (C18: 0) | 441 ±3.4[a] | 445 ±4.1[a] | 462 ±3.8[d] | 341 ±4.2[b] |
| Total | **1.758 ±11.6[a]** | **1.839 ±12.1[c]** | **1.904 ±11.9[d]** | **1.350 ±11.7[b]** |
| **Monounsaturated fatty acids** | | | | |
| Palmitoleic (C16: 1) | 157 ±2.4[a] | 155 ±3.1[a] | 163 ±3.0[d] | 132 ±2.6[b] |
| Heptadecene (C17: 1) | 5.8 ±0.4[a] | 3.7 ±0.6[c] | - | - |
| Oleinova (C18: 1) | 2.321 ±12.7[a] | 1.978 ±12.4[c] | 2.240 ±11.9[a] | 1,729 ±12.5[b] |
| Gadolein (C20: 1) | 11.6 ±1.2[b] | 45.7 ±3.5[c] | 207 ±7.4[d] | 12.4 ±2.1[b] |
| Total | **2.495 ±16.4[a]** | **2.182 ±14.7[c]** | **2.610 ±15.4[d]** | **1.873 ±13.9[b]** |
| **Polyunsaturated fatty acids** | | | | |
| Linoleum (C18: 2) n-6 | 1.143 ±8.2[a] | 1.051 ±9.6[c] | 846 ±8.5[d] | 759 ±8.2[b] |
| Peanut (C20: 4) n-6 | 112 ±3.2[a] | 206 ±4.1[c] | 239 ±4.6[d] | 194 ±3.8[b] |
| Linolenic (C18: 3) n-3 | 23.2 ±2.7[a] | 28.4 ±2.9[a] | 41.2 ±3.3[d] | 28.4 ±2.8[a] |
| Total | **1.278 ±12.4[a]** | **1.285 ±13.1[a]** | **1.126 ±12.6[d]** | **0.981 ±12.9[b]** |

Note: A – Birkivska barvysta, 14 – Poltava Clay, H2 – Plymouth White, foreign cross Tetra SL (Final Hybrid (FH)); a, b – averages with different upper scripts within one age differ significantly in $p < 0.01$.

**Table 4** The composition of fatty acids in the yolk of eggs of domestic and foreign chickens (in% to the total fatty acid content).

| Fatty Acid | The amount of fatty acid in the yolk of chicken eggs,% to the total fatty acid content | | | |
|---|---|---|---|---|
| | A | 14 | H2 | TSL(FH) |
| Saturated fatty acids | | | | |
| Miristinova (C14: 0) | 0.31 ±0.02 | 0.28 ±0.02 | - | 0.45 ±0.02 |
| Palmitinova (C16: 0) | 23.29 ±0.15 | 25.99 ±0.18 | 25.58 ±0.14 | 23.26 ±0.2 |
| Margarinova (C17: 0) | 0.21 ±0.02 | - | - | 0.3 ±0.03 |
| Stearinova (C18: 0) | 7.97 ±0.06 | 8.39 ±0.08 | 8.18 ±0.07 | 8.11 ±0.1 |
| Total | **31.78 ±0.21** | **34.66 ±0.23** | **33.76 ±0.21** | **32.12 ±0.28** |
| **Monounsaturated fatty acids** | | | | |
| Palmitoleic (C16: 1) | 2.84 ±0.04 | 2.92 ±0.06 | 2.89 ±0.05 | 3.13 ±0.06 |
| Heptadecene (C17: 1) | 0.1 ±0.01 | 0.07 ±0.01 | - | - |
| Oleinova (C18: 1) | 41.96 ±0.23 | 37.27 ±0.23 | 39.72 ±0.21 | 41.13 ±0.3 |
| Gadolein (C20: 1) | 0.21 ±0.02 | 0.86 ±0.07 | 3.67 ±0.13 | 0.29 ±0.05 |
| Total | **45.11 ±0.82** | **41.12 ±0.28** | **46.28 ±0.27** | **44.55 ±0.33** |
| **Polyunsaturated fatty acids** | | | | |
| Linoleum (C18: 2) n-6 | 20.67 ±0.15 | 19.81 ±0.18 | 15.0 ±0.15 | 18.05 ±0.2 |
| Peanut (C20: 4) n-6 | 2.02 ±0.06 | 3.88 ±0.08 | 4.23 ±0.08 | 4.61 ±0.09 |
| Linolenic (C18: 3) n-3 | 0.42 ±0.05 | 0.54 ±0.05 | 0.73 ±0.06 | 0.67 ±0.07 |
| n-6: n-3 | 54.02 ±0.31 | 43.87 ±0.33 | 26.34 ±0.29 | 33.82 ±0.31 |
| Total | **23.11 ±0.22** | **24.22 ±0.25** | **19.96 ±0.22** | **23.33 ±0.3** |

Note: A – Birkivska barvysta, 14 – Poltava Clay, H2 – Plymouth White, foreign cross Tetra SL (Final Hybrid (FH)).

This difference in the age dependence of the protein content between poultry of local and foreign selection, in our opinion, is explained by the later start of egg-laying in hens of local breeds.

The age-related change in the components of the egg (albumen and yolk) was reflected in its energy value, which directly depends on its content. There was an increase in the energy value of 100 g of egg mass in poultry of local breeds and populations from 665 kJ at the age of 26 weeks to 676.0 kJ at the age of 44 weeks, in foreign birds from 589.0 kJ to 616 kJ, respectively.

According to the results of the evaluation of the influence of various factors on some studied indicators of chicken egg quality (egg weight, yolk weight, yolk content, yolk-albumen ratio, the energy value of eggs), shown in Table 1, the largest and statistically significant contribution to variability of indicators such as weight eggs and yolk weight, had a factor of "bird`s age" 45.5 – 49.2%. The obtained data are also confirmed by the fact that during the experiment (18 weeks) changes in egg weight ranged from 9.3% in foreign birds to 17.9% in local, and by weight of the yolk from 16.9% to 2.1%, respectively.

**Table 5** Quality indicators of eggs of breeds, parental flocks and hybrids of poultry of local and foreign selection at different ages (M ± m; n = 60).

| Origin | Breed[2] | Indices | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ME | MY | PY | MA | PA | RYA | EV |
| **26 weeks** | | | | | | | | |
| LS | A | 49.2 ±0.40 | 14.2 ±0.13 | 28.9 ±0.24 | 28.0 ±0.30 | 56.8 ±0.36 | 0.51 ±0.007 | 672 ±4.1 |
| | 14 | 51.3 ±0.44 | 14.6 ±0.19 | 28.6 ±0.33 | 29.4 ±0.40 | 57.2 ±0.50 | 0.50 ±0.009 | 667 ±5.9 |
| | H2 | 51.3 ±0.58 | 14.1 ±0.20 | 27.5 ±0.29 | 29.1 ±0.45 | 56.6 ±0.42 | 0.49 ±0.008 | 658 ±5.3 |
| | Hyb1 | 51.4 ±0.52 | 14.8 ±0.15 | 28.8 ±0.28 | 29.2 ±0.42 | 56.8 ±0.36 | 0.51 ±0.008 | 671 ±5.0 |
| | Hyb2 | 51.1 ±0.52 | 14.4 ±0.12 | 28.2 ±0.26 | 29.7 ±0.43 | 58.0 ±0.31 | 0.49 ±0.007 | 658 ±5.6 |
| | **average** | **51.9 ±0.57[a]** | **14.4 ±0.15** | **28.5 ±0.29[b]** | **29.1 ±0.32[a]** | **57.1 ±0.28 [a]** | **0.5 ±0.005[b]** | **665 ±3.4[b]** |
| FS | HL (PF[4]) | 58.3 ±0.30 | 13.1 ±0.10 | 22.5 ±0.16 | 37.3 ±0.27 | 63.9 ±0.22 | 0.35 ±0.004 | 565 ±2.8 |
| | TSL(PF) | 58.7 ±0.47 | 14.8 ±0.14 | 25.2 ±0.20 | 36.7 ±0.37 | 62.5 ±0.27 | 0.40 ±0.005 | 603 ±3.4 |
| | TH (PF) | 59.9 ±0.43 | 14.8 ±0.13 | 24.7 ±0.24 | 37.7 ±0.39 | 62.8 ±0.28 | 0.39 ±0.006 | 595 ±3.9 |
| | SKh(PF) | 56.0 ±0.49 | 13.8 ±0.13 | 24.7 ±0.20 | 35.6 ±0.39 | 63.6 ±0.23 | 0.39 ±0.004 | 591 ±3.2 |
| | TSL(FH[4]) | 62.5 ±0.30 | 14.0 ±0.16 | 22.4 ±0.26 | 40.9 ±0.33 | 65.4 ±0.34 | 0.34 ±0.006 | 557 ±4.4 |
| | HB(FH) | 58.1 ±0.47 | 14.0 ±0.13 | 24.2 ±0.27 | 35.9 ±0.45 | 61.7 ±0.34 | 0.39 ±0.006 | 595 ±4.6 |
| | LB (FH) | 59.4 ±0.57 | 15.1 ±0.14 | 25.4 ±0.12 | 35.6. ±0.42 | 60.0 ±0.33 | 0.42 ±0.004 | 617 ±4.5 |
| | **average** | **59.0 ±0.81[b]** | **14.2 ±0.29** | **24.2 ±0.50[a]** | **37.1 ±0.76[b]** | **62.8 ±0.70[b]** | **0.38 ±0.011[a]** | **589 ±8.6[a]** |
| **34 weeks** | | | | | | | | |
| LS | A | 55.6 ±0.46 | 16.3 ±0.13 | 29.4 ±0.25 | 32.0 ±0.38 | 57.4 ±0.30 | 0.51 ±0.007 | 674 ±4.2 |
| | 14 | 56.3 ±0.55 | 15.8 ±0.15 | 28.1 ±0.25 | 33.6 ±0.45 | 59.5 ±0.34 | 0.47 ±0.007 | 649 ±4.4 |
| | H2 | 58.4 ±0.50 | 16.8 ±0.16 | 28.8 ±0.29 | 34.3 ±0.44 | 58.7 ±0.34 | 0.49 ±0.008 | 661 ±4.9 |
| | Hyb1 | 56.3 ±0.56 | 16.2 ±0.15 | 29.0 ±0.28 | 32.3 ±0.47 | 58.4 ±0.35 | 0.50 ±0.008 | 663 ±4.9 |
| | Hyb2 | 56.1 ±0.61 | 15.5 ±0.14 | 27.7 ±0.25 | 33.5 ±0.50 | 59.4 ±0.33 | 0.47 ±0.006 | 647 ±4.2 |
| | **average** | **56.5 ±0.53[a]** | **16.1 ±0.25** | **28.6 ±0.34[b]** | **33.1 ±0.48[a]** | **58.7 ±0.43[a]** | **0.49 ±0.009[a]** | **659 ±5.5[a]** |
| FS | HL (PF[4]) | 61.5 ±0.27 | 15.5 ±0.07 | 25.2 ±0.11 | 37.9 ±0.23 | 61.6 ±0.16 | 0.41 ±0.003 | 607 ±1.8 |
| | HB (FH) | 62.6 ±0.54 | 16.8 ±0.16 | 26.8 ±0.27 | 37.8 ±0.47 | 60.3 ±0.31 | 0.45 ±0.007 | 631 ±4.5 |
| | LB (FH) | 59.6 ±0.58 | 15.0 ±0.12 | 25.1 ±0,11 | 36.4 ±0.40 | 61.1 ±0.15 | 0.41 ±0.004 | 594 ±3.9 |
| | **average** | **61.2 ±1.07[b]** | **15.8 ±0.66** | **25.7 ±0.67[a]** | **37.4 ±0.59[b]** | **61.0 ±0.49[b]** | **0.42 ±0.016[b]** | **611 ±13.3[b]** |
| **44 weeks** | | | | | | | | |
| LS | A | 60.6 ±0.43 | 18.2 ±0.15 | 30.0 ±0.21 | 34.7 ±0.35 | 57.2 ±0.26 | 0.53 ±0.006 | 682 ±3.6 |
| | 14 | 60.5 ±0.52 | 17.4 ±0.21 | 28.7 ±0.29 | 36.3 ±0.40 | 59.9 ±0.30 | 0.48 ±0.007 | 654 ±4.6 |
| | H2 | 64.0 ±0.63 | 19.2 ±0.25 | 30.0 ±0.34 | 37.3 ±0.51 | 58.1 ±0.36 | 0.52 ±0.009 | 677 ±5.3 |
| | Hyb1 | 61.1 ±0.52 | 19.1 ±0.20 | 31.4 ±0.27 | 34.9 ±0.42 | 57.1 ±0.29 | 0.53 ±0.015 | 696 ±4.3 |
| | Hyb2 | 59.7 ±0.66 | 17.4 ±0.20 | 29.3 ±0.32 | 34.8 ±0.54 | 58.2 ±0.35 | 0.51 ±0.009 | 669 ±5.3 |
| | **average** | **61.2 ±0.83[a]** | **18.3 ±0.44[b]** | **29.9 ±0.50[b]** | **35.6 ±0.58** | **58.1 ±0.56** | **0.51 ±0.010[b]** | **676 ±7.8[b]** |
| FS | HL (PF[4]) | 63.8 ±0.55 | 17.3 ±0.15 | 27.1 ±0.24 | 38.2 ±0.48 | 59.8 ±0.29 | 0.46 ±0.006 | 637 ±4.0 |
| | LB (FH) | 65.2 ±0.61 | 15.8 ±0.12 | 24.4 ±0.22 | 40.6 ±0.56 | 62.3 ±0.37 | 0.39 ±0.005 | 594 ±3.9 |
| | **average** | **64.5 ±0.40[b]** | **16.6 ±0.43[a]** | **25.8 ±0.78[a]** | **39.4 ±0.69[b]** | **61.1 ±0.72[b]** | **0.43 ±0.020[a]** | **616 ±12.4** |

Note: [1]LS – local selection, FS – foreign selection;[2]A –Birkivska barvysta, 14 – Poltava Clay, H2 – Plymouth White, Hyb1 – hybrid H2 × 14, Hyb2 – hybrid 14 × A, HL – High Line W36, TSL – Tetra SL, TN – Tetra H, SKh – Super Kharko, HB – Highsex Brown, LB – Lohmann brown;[4]BF – parental Flock; FH – the final hybrid; [3]ME – egg weight, g; MY – yolk mass, g; PY – the proportion of yolk,%; MA –albumen mass, g; PA – the proportion of albumen,%; RYA – the ratio of yolk-albumen; EV – energy value, kJ; a, b – averages with different upper indexes within the same age differ significantly in $p < 0.01$.

At the same time, such a relative indicator as the proportion of yolk is the most (38.7%) influenced by the genetic origin of birds. The same high share influence of genotype on the calculated indicators of the yolk- albumen ratio and energy value is 32.5 and 35.2%, respectively. Also, there was a significant impact of unaccounted random factors on these features, which results in a significant increase in random error (43.8 – 52.6%).

Summing up the results of a comparative study of physical and morphological parameters of eggs of Ukrainian and foreign chickens, we can conclude that the genotype has a significant impact on egg quality.

In all studied periods of the experiment (26, 34, and 44 weeks of the birds` life) there were eggs with higher weight from poultry of foreign selection with an advantage of an average of 5.1 – 12.0% depending on the age of chickens, which significantly affected this indicator. However, even though the eggs of local breeds and hybrids had less weight, they had a higher percentage of yolk – 28.5 – 29.9% against 24.2 – 25.8% in imported crosses. Due to the larger share of yolk in the eggs of hens of Ukrainian selection, their energy value (per 100 g of egg mass) also had an advantage over foreign crosses and amounted to 659.0 – 676.0 kJ against 589.0 – 616.0 kJ. Therefore, eggs from chickens of local breeds, which are valued in the socio-cultural life of local communities, may be more popular among consumers who prefer eggs with large yolks. Besides, the high-quality characteristics of eggs of local breeds and populations are evidence of their value set of genes that require comprehensive study, preservation, and use in the creation of new selectively significant forms.

In the next stage of the study, we studied the total lipid content in the egg yolk of chickens of local selection in comparison with foreign cross Tetra SL (final hybrid (FH)). Higher content of lipids in the egg yolks of domestic chickens was found in comparison with the foreign hybrid Tetra SL (Table 2). Also, the lipid content in eggs of the poultry of egg productivity (line A) is by 0.3 g lower than in eggs of hens of meat-egg productivity (H2).

The study of the fatty acid composition of egg yolks showed that palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids have the highest content in yolks: their total amount is more than 80% of the total content (Table 3 and Table 4).

In yolks of eggs of hens of local breeds the general maintenance of saturated and unsaturated fatty acids is higher, than at foreign cross, namely: the maintenance of saturated, monounsaturated, and polyunsaturated fatty acids in eggs of hens of domestic selection was within 1.758 – 1.904, 2.495 – 2.610, 1.126 – 1.278 g/yolk, while in the tested eggs of the final hybrid their content was 1.350, 1.873 and 0.981 g/yolk, respectively. It is necessary to pay attention to the lower content of palmitic acid in eggs of a bird of a foreign cross – 978.0 mg/yolk (Table 3). While in chickens of local breeds this indicator was in the range of 1.288 – 1.443 mg/yolk.

The results of studies by **Bunea et al. (2017)**, **Antova et al. (2019)** and **Mi (2019)**, **Xiao et al. (2020)**, and **Ko et al. (2021)** support our findings. They proved that the main fatty acids in the lipids were oleic (39.1 – 47.3%) and palmitic (26.0 – 35.5%) acids.

Palmitic acid is known to be a factor in hypercholesterolemia, a disease in which blood cholesterol levels rise to abnormal levels. Cholesterol ensures the stability of cell membranes, is involved in the synthesis of vitamins, bile acids, hormones. But with hypercholesterolemia, is deposited on the walls of blood vessels, affects metabolic processes, and leads to the development of serious complications, including heart attack, stroke, hypertension, and others.

Analyzing the content of palmitic acid in TSL eggs as a percentage of the total content of fatty acid in the yolk, we can find the difference in 2.3% and 2.7% compared to eggs H2 and Poltava Clay, respectively, which does not seem significant (Table 4). The total content of saturated fatty acids (SFA) in the eggs of domestic chickens was at the level of 1.758 – 1.904 mg/yolk (31.78 – 34.66% of the total fatty acids), in the eggs of the Tetra SL hybrid – 1.350 mg/yolk (32.12%). As for polyunsaturated fatty acids (PUFA), in the eggs of domestic breeds, their content was determined in the range of 1.126 – 1.285 mg/yolk (19.96 – 24.22%), in the eggs of foreign hybrids – 0.981 mg/yolk %), which indicates a lower nutritional value of the latter (Table 3 and Table 4).

The total content of PUFA n-6 in eggs of chickens of domestic selection in comparison with the foreign cross was estimated. The amount of PUFA n-6 in eggs of local breeds was at the level of 1085.0 – 1255.0 mg/yolk, foreign cross – 953.0 mg/yolk. The content of PUFA n-3 was 1.5 times higher in H2 eggs compared to TSL – 41.2 mg/yolk, which is more than 16% of the minimum daily intake (Table 3).

In percentage of the total fatty acid content in the eggs of the studied breeds of chickens, the largest number are monounsaturated fatty acids, due to the high content of oleic acid in the range of 37.27 – 41.96% (Table 4). The percentage of fatty acids in the eggs of both domestic and foreign birds was almost at the same level. In general, the fatty acid composition of eggs reported in our studies differs slightly from those reported in other studies **(Lordelo et al., 2020; Danchuk and Trach, 2017; Rakonjac et al., 2017; Cherian and Quezada, 2016)**.

## CONCLUSION

It was found that the average egg weight of hens of the Ukrainian gene pool (H2, 14, A) and hybrids obtained by crossing them (H2x14, 14xA) was 51.9 g, 56.5 g, and 61.2 g at the age of 26, 34, and 44 weeks, respectively, while in chickens of foreign selection, it was significantly higher in these age periods – 59 g, 61.2 g, and 64.5 g, respectively ($p$ <0.01).

Despite the lower weight of the egg, there is a probable advantage of purebred and hybrid birds of Ukrainian selection over birds of foreign selection in such important components of the internal content of eggs as the proportion of yolk (4.3%, 2.9%, and 4.1% at the age of 26, 34 and 44 weeks, respectively) the yolk-albumen ratio (0.12, 0.07 and 0.08, respectively) and the estimated energy value of eggs (76.0, 48.0 and 60.0 kJ, respectively).

Analysis of variance of the studied quality indicators of eggs showed that the most significant (45.5 – 49.2%) on the weight of the egg and the weight of the yolk is influenced by the age of the bird, and on such calculated indicators as yolk content, yolk-protein ratio, and energy value, significant influence has the genetic origin of poultry (38.7%, 32.5%, and 35.2%, respectively).

It was found that the total lipid content in the egg yolk of domestic chickens is higher by 0.7 – 0.9 g compared to foreign cross Tetra SL (final hybrid). In the egg yolks of domestic chickens, the total content of saturated and unsaturated fatty acids is higher (in the range of 1.758 – 1.904, 2.495 – 2.610, 1.126 – 1.278 g/yolk), while in the studied eggs of foreign cross their content was 1.350, 1.873 and 0.981 g/yolk, respectively. The amount of PUFA n-6 in eggs of local breeds was at the level of 1085 – 1255 mg/yolk, and foreign cross – 953.0 mg/yolk. The content of PUFA n-3 was 1.5 times higher in H2 eggs compared to TSL – 41.2 mg/yolk against 28.4 mg/yolk, which is more than 16% of the minimum daily intake.

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# AMINO ACIDS IN ENTERAL FORMULA BASED ON LOCAL FERMENTED FOOD FOR CHILDREN WITH PROTEIN ENERGY MALNUTRITION

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## ABSTRACT

Protein-energy malnutrition still becomes a problem in the world and Indonesia. The enteral formula is needed in the process of fulfilling overall nutrition in the form of a liquid diet in malnourished patients to digest and absorb nutrients without any difficulties. Amino acid lysine, methionine, cysteine, threonine, and tryptophan are often deficits in children's food; even, malnourished children (stunting, wasting, or protein-energy malnutrition) have decreased amino acids. *Tempeh gembus*, fermented local food, is used as the main ingredient for the enteral formula, and the hydrolysate process is carried out and made into flour, resulting in more amino acids due to the process of protein breakdown. The making of enteral formula from local food fermentation aims to enable the community to optimize local food into more nutritious food so that it can be produced at the household scale. Other supplementary ingredients are isolated soy protein, pumpkin flour, maltodextrin, sugar, and soybean oil. The enteral formula is isocaloric and isoprotein; it is divided into two with different compositions of the hydrolysate of *Tempeh gembus* flour. This study aims to determine the content of protein, amino acids, and limiting amino acids in the enteral formula. The amino acid analysis was performed using the HPLC method. Amino acid glutamate has the highest content in enteral formulas A and B (2,080 mg.100g$^{-1}$and 1,950 mg.100g$^{-1}$). The total amino acid content of enteral formula A is higher than that of enteral formula B with a difference of 210 mg.100g$^{-1}$. Enteral formula A has a higher average amino acid content (1,400 mg.100g$^{-1}$) than enteral formula B (1,378 mg.100g$^{-1}$), and there is no significant difference ($p$ = 0.812) between them. The enteral formula A has a higher amino acid content, but the highest protein content is in enteral formula B. The limiting amino acids in enteral formulas A and B are the methionine amino acids. Enteral formulas A and B can fulfill the needs of amino acids and be an alternative formula for Children with PEM.

**Keywords:** *Tempeh gembus* flour hydrolysate; amino acid; enteral formula; toddler; PEM

## INTRODUCTION

Protein-energy malnutrition (PEM) indicates a health problem caused by an imbalanced consumption of macronutrients such as protein, thus causing the occurrence of kwashiorkor as a form of protein-energy malnutrition and even death **(Ngo and Serra-Majem, 2018; Anggraeny et al., 2016)**. The prevalence of protein-energy malnutrition in the world as of March 2020 is 5 – 7% or around 40,000 – 47,000 children, a decrease from year to year, and in Asia in 2016 and 2017 decreased while in 2018 it had not changed. However, in 2019, it had increased to be categorized as the highest prevalence of more than 15% **(UNICEF, WHO, World Bank Group, 2019; UNICEF, WHO, World Bank Group, 2020)**.

Risk factors for malnutrition include Low Birth Weight (LBW), non-exclusive breastfeeding, inappropriate complementary feeding, and recurrent infectious diseases **(Bhutta and Salam, 2012)**. The problem of food insecurity, low access to quality and quantity of food, and low sanitation also affect the growth and development of children **(Ngo and Serra-Majem, 2018; Cooper, 2010)**. Malnutrition has synergism in infectious diseases and influences morbidity and mortality **(Ashworth, 2017)**.

One of the treatments for children with PEM is the administration of enteral formulas to fulfill their nutritional needs **(Pratiwi and Noer, 2014)** in the form of liquid diet foods **(Ariani et al., 2013)**. The enteral formula has a liquid to viscous texture because children with PEM experience disorders including chewing and swallowing disorders and keep the gastrointestinal work physiologically **(Damayanti et al., 2012)**. The administration of enteral formula has been shown to increase body weight quickly in children with PEM **(Chusnatayaini et al., 2018)** and maintain normal nutritional status **(Sousa et al., 2014)**. Modified Dried Skimmed Milk Coconut Oil (modisco) enteral formula is commonly used for suboptimal therapy in compensating for protein deficiency. This is due to the occurrence of protein

malabsorption due to protease enzyme deficiency **(Kamalia and Sulistyaningsih, 2014)**.

The enteral formulas made from *Tempeh gembus* with other components are isolated soy protein, pumpkin flour, maltodextrin, granulated sugar, and soybean oil. *Tempeh gembus* is a common fermented product in Indonesia and is made from tofu dregs with the *Rhizopus oligosporus* microorganism **(Sulchan and EndangNur, 2007)**. Food fermentation has a benefit as a preservation technique to improve sensory properties and nutritional value **(Bujang and Taib, 2014)**. *Tempeh gembus* has proteolytic activity **(Afifah et al., 2014)** if bromelain enzyme is added can break peptide bonds to proteins into amino acids quickly **(Widjaja et al., 2013; Wijaya and Yunianta, 2015; Kurniasari et al., 2017),** and the function of bromelain enzyme to biocatalysts in protein breakdown **(Kumaunang and Kamu, 2010)**. *Tempeh gembus* is processed into flour to increase the shelf life so that it inhibits the growth of microorganisms and chemical reactions **(Karyadi et al., 2012; Astawan et al., 2015)**. The soy protein isolated flour is added to increase nutritional value because it has high protein content **(Rauf and Utami, 2020)** and children with PEM needs more protein; on the other hand, isolated soy protein is one component of protein in enteral formula **(Brown et al., 2014)**. Pumpkin flour has high energy and protein content **(Nakon et al., 2017; USDA., 2018)**. The function of adding maltodextrin to stabilize the enteral formula because the characteristic is soluble so influences the value of viscosity **(Rauf and Utami, 2020)**. The addition of sugar can increase carbohydrate intake while the addition of soybean oil in the enteral formula can fulfill the intake of protein and fat **(Dietitians Association of Australia, 2018)**.

Protein contains structural and functional components in living systems derived from food consumption. Protein is found in skeletal muscle by 40%, in body organs by more than 25%, and in the skin and blood **(Gropper and Smith, 2013)**. Food proteins contain essential amino acids for the body **(Boye et al., 2012)**. Amino acids have a very important component to see the quality of protein and physiology in the body. Amino acids are related to the body because they are needed in a variety of reactions and biochemical mechanisms, so the function of an organism runs normally and maintains its health status. Essential amino acids can only be found in food. *Tempeh gembus* (netto) has the highest amino acid composition (glutamate of 0.29%) while the lowest amino acid is methionine (0.01%). The highest amino acid content in dried *Tempeh gembus* is threonine (0.95%) and the lowest is serine (0.07%). Amino acid levels in *Tempeh gembus* are smaller than in soybean *Tempeh* **(Sulchan and EndangNur, 2007)**.

The first limiting amino acid is an amino acid with the smallest score used as a limitation on the biological value of a food protein. Lysine, tryptophan, and sulfuric amino acids are limiting amino acids in food products based on soybean **(Ribarova, 2018)**. Methionine and cysteine or sulfuric amino acids are limiting essential amino acids in soybeans **(Serna-saldivar et al., 2019)**. This study is aimed at investigating whether enteral formulas A and B have high protein and amino acid content that can fulfill the needs and be an alternative formula for Children with PEM.

### Scientific hypothesis

Enteral formulas A and B based on local fermented food can fulfill the amino acids needed by children with PEM.

## MATERIAL AND METHODOLOGY

### Samples

The samples of this study are enteral formulas A and B, which have the composition of a hydrolysate of *Tempeh gembus* flour, isolated soy protein, pumpkin flour, maltodextrin, sugar, and soybean oil.

### Chemicals

Bromelain enzyme brand Pinecaps (powder) used to hydrolysate of *Tempeh gembus*. Bromelain enzyme from Rumah Sehat Yogyakarta City, Indonesia. Isolated soy protein brand Marksoy was obtained from an online shop in Subang Regency, West Java, Indonesia. Maltodextrin food grade (10 – 12 DE) brand Lansida was obtained from an online shop in Yogyakarta City, Indonesia.

### Instruments

The formula was based on the recommendation of ASPEN (American Society for Parenteral and Enteral Nutrition). Enteral formulas A and B are isocaloric, 200kcal/200mL consists of 20 kcal protein, 60 kcal fat, and 120 kcal carbohydrate. Furthermore, the need for each ingredient was determined using Algebra equation, as follow **(Knapp, 2016)**:

$$(AxWxCp)+(BxWxCp)+(CxWxCp) = 20 \text{ kcal protein}$$

Where :
A = Hydrolysate of *Tempeh gembus* (%); B = Isolated soy protein (%); C = Pumpkin flour (%); W = Weight of enteral formula; Cp = calorie of protein ($kcal.g^{-1}$).

$$(AxWxCp)+(CxWxCp)+(ExWxCp) = 60 \text{ kcal fat}$$

Where :
A= Hydrolysate of *Tempeh gembus* (%); C = Pumpkin flour (%); E = Soybean oil (%); W = Weight of enteral formula
Cp = calorie of fat ($kcal.g^{-1}$).

$$(AxWxCp)+(CxWxCp)+(DxWxCp)+(FxWxCp)= 120 \text{ kcal carbohydrate}$$

Where :
A= Hydrolysate of *Tempeh gembus* (%); C = Pumpkin flour (%); D = Maltodextrin (%); F = Sugar (%); W = Weight of enteral formula; Cp = calorie of carbohydrate ($kcal.g^{-1}$).

In this study, it was estimated that 50 g of the enteral formula A and 60 g of the enteral formula B. Food ingredients contain the protein are included in the protein equation, as well as fats and carbohydrates.

Based on Table 1, the weight of each ingredient from the calculation of algebraic equation from protein, fat, and carbohydrate. Enteral formula A has the lowest composition of the *Tempeh gembus* flour, but the highest composition of other components compared to enteral formula B. On the other hand, enteral formula B has the highest composition of *Tempeh gembus* flour, but the lowest composition of other components compared to enteral formula A.

**Table 1** Calculation of enteral formulas A and B.

| Materials | Enteral Formula A (g) | Enteral Formula B (g) |
|---|---|---|
| Hydrolysate *Tempeh gembus* Flour | 25 | 36 |
| Isolated Soy Protein | 2.211 | 1.962 |
| Pumpkin Flour | 5.942 | 5.809 |
| Maltodextrin | 5 | 5 |
| Soybean Oil | 3.305 | 3.292 |
| Sugar | 8.813 | 8.29 |
| Total | 50.269 | 60.353 |

Other components in enteral formulas A and B have a very small difference compared to the composition of the hydrolysate *Tempeh gembus* flour.

The calculation is based on the protein requirements of malnourished toddlers who have entered the rehabilitation phase. The nutritional needs of malnourished children in the rehabilitation phase are 150 – 220 kcal/kgBW/day, protein 4 – 6 g/kgBW/day, and liquid 150 – 200 mL/kgBW/day **(WHO, 2009)**.

**Laboratory Methods**

High-Performance Liquid Chromatography (HPLC) technique (IK.LP-04.7-LT-1.0) is used as the amino acid testing standard in the Integrated Laboratory of the Bogor Institute of Agriculture. HPLC consists of 4 steps, that are making protein hydrolysate, drying, derivatization, injection and amino acid analysis. Calculation of Amino Acid Score (AAS) in mg of limiting amino acid in 1 g test protein (product) divided with mg amino acid in the required pattern for a child (1–2 years)**(FAO Food and Nutrition, 2013)**. Amino acid scores were truncated to 1.00 if the score greater than 1.00 **(Hughes et al., 2011)**.

**Description of the Experiment**

**Samples preparation:** The main component of the enteral formula is *Tempeh gembus* made from local soybean pulp fermented by *Rhizopus* sp. *Tempeh gembus* is obtained from a *Tempeh* maker in the Semarang Indah area, Semarang City, Indonesia. The deposition of *Tempeh gembus* was conducted in the laboratory of the Faculty of Agricultural Technology of GadjahMada University, Yogyakarta City, Indonesia. Pumpkin flour was obtained from the Center for Food and Nutrition Studies Laboratory of GadjahMada University, Yogyakarta City, Indonesia. Sugar brand Gulaku and soybean oil brand Mazola were obtained from a supermarket in Semarang City, Indonesia. The process of making hydrolysate of *Tempeh gembus* is bromelain enzyme brand Pinecaps 500 ppm (1 capsule) added in 100 gram *Tempeh gembus* and wait for 30 minutes. Enteral formula A (50 grams) served for one-time consumption and 60 grams of enteral formula B served for one-time consumption. The enteral formula is served by mixing all the ingredients then added 200 mL of hot water at 90 ºC, and stirred until dissolved.

**Number of samples analyzed:** Enteral formulas A and B.

**Number of repeated analyses:** Enteral formulas A and B in duplicate.

**Statistical analysis**

The data were analyzed using SPSS version 20.0 Amino acid testing in duplicate. Shapiro-Wilk test was used to know the distributed data. The Mann Whitney test was used for statistical analysis because the data were not normally distributed($p$ <0.05).

## RESULTS AND DISCUSSION

Enteral formula based on *Tempeh gembus* flour is a modified form of enteral formula with high protein content. This formula is given to children with PEM entering the rehabilitation phase, a recovery phase that aims to increase children's growth. Therefore, enteral formula based on local food with high nutritional value can be an alternative food given to children with PEM **(Sholihah and Noer, 2014)**.

The resulting enteral formula is high in energy and protein values. Enteral formula A has a protein content (%) of 15.215 ±0.021 and enteral formula B has a higher protein content (%) of 16.668 ±0.064. In addition to its nutritional content, the advantage of enteral formula based on local food is its affordable price because it uses the basic ingredients of *Tempeh gembus* which has a low selling price on the market.

In this study, eight essential amino acids analyzed include isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, and histidine, and nine non-essential amino acids include aspartic, glutamate, glycine, proline, serine, tyrosine, alanine, cystine, and arginine supplemented with total amino acids. Table 2 displays the amino acid content and Table 3 displays the average amino acid content in both formulas.

Enteral formulas A and B have different weights (a difference of 11 grams from enteral formula A). This happens because the weight obtained is based on the algebraic equation. The nutritional content and caloric value of each ingredient must be known to produce the calculations needed for enteral formulas, namely protein 20 kcal (10%), fat 60 kcal (30%), and carbohydrate 120 kcal (60%). This formula is based on recommendations from ASPEN (American Society for Parenteral and Enteral Nutrition) **(Brown et al., 2014)**. Trials were performed to get two appropriate enteral formulas. The suitability of enteral formulas is seen from the presence of sediment between oil and solids.

Overall, the results show that enteral formula A has a total amino acid of 12,610 mg.100g$^{-1}$ ±0.339 higher than that of enteral formula B of 12,400 mg.100g$^{-1}$ ±0.435. Meanwhile, the average amino acid content of enteral formula A of 1,401 mg.100g$^{-1}$ ±2.844 is also higher than of formula enteral B of 1,378 mg.100g$^{-1}$ ±2.792. Enteral formula A contains 2,080 mg.100g$^{-1}$ ±0.0849 glutamate acid followed by aspartic acid 1,505 mg.100g$^{-1}$ ±0.021, leucine 1,495 mg.100g$^{-1}$ ±0.035. Enteral formula B contains 1,950 mg.100g$^{-1}$ ±0.099glutamic acids followed by leucine 1,500 mg.100g$^{-1}$ ±0.099 and aspartic acid 1,360 mg.100g$^{-1}$±0.057.

**Table 2** Amino acid content in enteral formulas A and B in dry weight.

| Amino Acid Content | Enteral Formula | |
|---|---|---|
| | A (mg.100g$^{-1}$±*SD*) | B (mg.100g$^{-1}$±*SD*) |
| **Aspartic** | 1,505 ±0.021 | 1,360 ±0.057 |
| **Serine** | 650 ±0.028 | 615 ±0.021 |
| **Glutamate** | 2,080 ±0.0849 | 1,950 ±0.099 |
| **Proline** | 555 ±0.021 | 545 ±0.021 |
| **Glycine** | 640 ±0.028 | 625 ±0.035 |
| **Alanine** | 705 ±0.021 | 675 ±0.035 |
| **Cystine** | 60 ±0.000 | 65 ±0.007 |
| **Tyrosine** | 75 ±0.007 | 180 ±0.000 |
| **Arginine** | 850 ±0.071 | 1,040 ±0.14 |
| **Valine** | 710 ±0.014 | 685 ±0.035 |
| **Methionine** | 115 ±0.007 | 120 ±0.014 |
| **Dileucine** | 625 ±0.007 | 600 ±0.028 |
| **Leucine** | 1,495 ±0.035 | 1,500 ±0.099 |
| **Phenylalanine** | 670 ±0.028 | 650 ±0.028 |
| **Histidine** | 465 ±0.021 | 475 ±0.021 |
| **Lysine** | 840 ±0.057 | 770 ±0.071 |
| **Threonine** | 575 ±0.007 | 555 ±0.021 |
| **Total Amino Acid** | 12,610 ±0.339 | 12,400 ±0.435 |

Note: Data mean ±*SD* with n = 2.

**Table 3** Average amino acid content in enteral formulas A and B in dry weight.

| Formula Group | Mean (mg.100g$^{-1}$) | *p** |
|---|---|---|
| **A** | 1,401 ±2.844 | 0.812 |
| **B** | 1,378 ±2.792 | |

Note: Data mean ±*SD* with n = 2;*Mann Whitney Test.

Based on all essential amino acids, the highest content in enteral formulas A and B is leucine while the lowest are threonine and histidine. Leucine contributes 27 – 28% of the total essential amino acids in both formulas. The amount of six other essential amino acids were found to vary in each enteral formula.

In enteral formulas A and B, the decrease in the order of essential amino acids after leucine is lysine > valine > phenylalanine > isoleucine > threonine > histidine > methionine. Likewise, the lowest amount of amino acids in enteral formulas A and B is cysteine. This occurred because of the difference in the weight of the composition in the two formulas.

Based on the data normality test using the Shapiro-Wilk test, the data on the average amino acid content of each formula were not normally distributed with $p$ (0.001) < 0.05; it was then tested using Mann Whitney test with the result that the enteral formulas A and B did not have a significant difference with $p$ (0.812) >0.05. This is because the difference in the *Tempeh gembus* flour in each formula is only 11 grams or a very small difference.

*Tempeh gembus* in this study was hydrolysate with bromelain. Bromelain can break down proteins at the ends of the carbonyl lysine, alanine, tyrosine, and glycine in amino acids. Protein hydrolysate produced with bromelain contains a mixture of bioactive peptides and non-bioactive peptides **(Kusumaningtyas et al., 2015)**, as well as *Tempeh gembus* added with bromelain or hydrolysate enzymes containing 5.12% of protein, 67.16% protein digestibility, and 13.99% acid amino **(Afifah et al., 2019)**. This is consistent with the results of proximate analysis of protein content in enteral formula B which has a higher protein content of 1.47% compared to enteral formula A.

The greater weight of the hydrolysate of *Tempeh gembus* flour in the enteral formula is the higher the amino acid content will be. However, enteral formula A has a higher average amino acid content compared to enteral formula B. Enteral formula A has 210 mg.100g$^{-1}$ of amino acid higher than formula B.

The main difference in enteral formulas A and B is found in the hydrolysate of *Tempeh gembus* flour. Other compositions of the two formulas have different weights, but the difference in weight of other compositions is not more than 1 gram. The difference between the enteral formulas A and B is related to the higher supporting composition or other composition. Isolated soy protein that has a high protein content has a large influence in increasing amino acid content **(Brown et al., 2014)**; besides, isolated soy protein also has good digestibility **(Thrane et al., 2017)**. Isolated soy protein has to limit amino acids namely methionine, lysine, and less BCAA (Branched Chain Amino Acid) **(Luiking et al., 2005)**. Another composition that contains protein is pumpkin flour. The mixture of the composition of a hydrolysate of *Tempeh gembus* flour, isolated soy protein, and pumpkin flour can increase the amino acid content in the enteral formula.

The quality of a protein is influenced by digestibility and its amino acid content, which is related to the percentage and proportion of the amino acid itself. Amino acids are fulfilled from the consumption of protein in food, protein breakdown in the body, and synthesis of nonessential amino acids. In infants and toddlers, the role of protein and amino acids occurs during growth through the synthesis of protein in muscles and increased growth. Lysine, methionine, cysteine, threonine, and tryptophan are amino acids that are often deficit in children's food.

**Table 4** Comparison of the conversion of essential amino acids in enteral formulas A and B with amino acid score patterns based on the age of 1 – 2 years.

| Amino Acid Content | Enteral Formula A ($mg.g^{-1}$) | Enteral Formula B ($mg.g^{-1}$) | Amino Acid Pattern (mg)* | Amino Acid Score A | Amino Acid Score B |
|---|---|---|---|---|---|
| **Valine** | 7.1 | 6.85 | 41 | 17.32 | 16.71 |
| **SAA** | 1.15 | 1.2 | 25 | 4.6 | 4.8 |
| **Ileucine** | 6.25 | 6.0 | 31 | 20.16 | 19.35 |
| **Leucine** | 14.95 | 15.0 | 63 | 23.73 | 23.81 |
| **AAA** | 6.7 | 6.5 | 46 | 14.57 | 14.13 |
| **Histidine** | 4.65 | 4.75 | 18 | 25.83 | 26.39 |
| **Lysine** | 8.4 | 7.7 | 52 | 16.15 | 14.81 |
| **Threonine** | 5.75 | 5.55 | 27 | 21.29 | 20.56 |

Note: *FAO Expert Consultation, 2013; * Amino Acid Score were truncated to 1.00, if the score more than 1.00; * SAA (Sulfuric Amino Acid consist of methionine) and AAA (Aromatic Amino Acid consist of phenylalanine).

Children aged 1 – 2 years need amino acids containing sulfur (methionine), lysine, threonine, and tryptophan by 25, 52, 27, and 7 mg **(FAO Food and Nutrition, 2013)**. In this study, sulfur amino acid, lysine, and threonine have a higher amino acid than the amino acid pattern in children aged 1 – 2.

Lysine supplementation in children can reduce the risk of diarrheal disease that can cause malnutrition and increase body weight significantly. Glutamine amino acids have a significant role in the body to improve the immune system, and glutamine supplementation can improve intestinal mucosal function and increase body weight. The function of glutamate in the digestive system is to facilitate efficient protein digestion because glutamate gives a sweet taste and has physiological and metabolic functions in terms of tissue protection and maintenance **(Ghosh and Uauy, 2016)**. So, the amino acid contained in enteral formulas has good benefits for children with PEM.

Based on Table 4, enteral formulas A and B contain amino acids which can meet daily amino acid in children aged 1 – 2 years. The amino acid listed by WHO is the lowest level amino acid score from protein intake because overall protein intake is higher. The importance of an optimal amino acid profile and amino acid absorption is related to the response to the digestible protein because the protein requirement comes from nitrogen balance so that protein can be used under any circumstances **(FAO Food and Nutrition, 2013)**. The benefit of consuming protein intake greater than the minimum is increasing muscle mass, muscle strength, and functional results as an increase in health outcomes **(Wolfe, 2012)**. Increased arginine amino acids can synthesize proteins and enhance immune function **(Jonker et al., 2012)**.

The function of assessing the amino acid score is the fulfillment of protein requirements for essential amino acids that are absorbed in the body **(Dewi et al., 2010; WHO/FAO/UNU Expert Consultation, 2007)**. The value of AAS was truncated to 1.00 if the value greater than 1.00 **(Hughes et al., 2011)**. The value amino acid score of methionine (sulfuric amino acid) is a limiting amino acid in enteral formulas A and B. According to **Serna-saldivar et al. (2019)** and **Thrane et al. (2017)**, methionine and cysteine or sulfuric amino acids are limiting amino acids in soybeans. Likewise, in this study, amino acids containing sulfur were found in low quantities.

Malnutrition problems include stunting, wasting, and PEM; stunted children have essential amino acids (tryptophan, isoleucine, leucine, valine, methionine, threonine, histidine, phenylalanine, and lysine), conditionally essential amino acids (arginine, glycine, and glutamine), and nonessential amino acids (asparagine, glutamate, and serine) are lower than normal children. The decreased amino acids are caused by children not receiving sufficient amino acid food intake **(Semba et al., 2016)**, so the requirements of essential amino acids must be fulfilled because it affects the metabolic processes in the body **(Wu, 2009)**.

The total protein and amino acid contents in enteral formulas A and B are different. This is mainly due to the protein testing method used. Protein extraction is also a factor that contributes to the inaccuracy of protein values. Foods that consist of various or heterogeneous nutrients are one cause of the differences in protein values **(Wilson and Walker, 2010)**. The protein content was analyzed using the Kjeldahl method while the amino acid content was analyzed using the High-Performance Liquid Chromatography (HPLC) method. The protein content analysis with the Kjeldahl method based on AOAC 1995 has the principle of determining the nitrogen content of the material as a whole and determining the crude protein. The protein value is obtained from the nitrogen content obtained multiplied by the conversion rate. The calculation error in the Kjeldahl method is because the analyzed nitrogen is sourced from protein, whereas nitrogen also comes from nitrogen-containing compounds such as nucleic acids, alkaloids, chlorophyll, and non-protein additives and contaminants **(Utami et al., 2016; Mæhre et al., 2018; Jiang et al., 2014)**. The amount of nitrogen contained in most proteins is 16% with a conversion factor of 6.25 **(Jiang et al., 2014; Mariotti et al., 2008)**. Overestimation of the 6.25 nitrogen correction factor also causes an increase in protein content **(Mæhre et al., 2018; Hall and Schönfeldt, 2013)**.

The Kjeldahl method also has three analysis stages namely destruction, distillation, and titration **(Utami et al., 2016; Mæhre et al., 2018)** while the HPLC method consists of four stages of making protein hydrolysate, drying, derivatization, and the injection, and acid amino analysis **(Utami et al., 2016)**. The protein content analyzed by the Kjeldahl method can be smaller than the actual amount due to broken peptide bonds. Besides, amino acids can be

reduced or even destroyed due to the use of strong acids and the heating process that occurs in the hydrolysis process of the HPLC method **(Widya et al., 2019; Hall and Schönfeldt, 2013)**.

Based on the current study, the enteral formula based on local fermented food can be produced at the household scale. The content of protein and amino acids found in the enteral formula can improve the nutritional status of children because the addition of the bromelain enzyme in *Tempeh gembus* can break down amino acids into a simpler form.

## CONCLUSION

Enteral formulas A and B that are isocaloric can fulfill the daily needs of amino acids in children aged 1 – 2 years. Enteral formula A has a higher amino acid content of 210 mg.100g$^{-1}$than enteral formula B, but formula B has a higher protein content of 1.453 grams compared to formula A. The highest amino acid in formulas A and B is glutamate while the limiting amino acid is methionine. Enteral formulas A and B based on local food can be an alternative formula for Children with PEM.

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# RESEARCH OF HOP POLYPHENOLS IMPACT ON MALT HOPPED WORT AROMA FORMATION MODEL EXPERIMENTS

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## ABSTRACT

Currently, a lot of research is being done on the flavoring compounds of hops. However, much less attention has been paid to the aroma formation considering the hop polyphenol different groups by various methods at the wort hopping stage. Therefore, the main goal of the research is an impact of hopping conditions on the polyphenolic compounds, when the hop is extracted variously into both wort and water to better understand extraction conditions, mechanisms, and factors as well as aroma formation considering various groups of polyphenols. As shown the hop variety, boiling time, and treatment type affect the hop polyphenols amount extracted into the wort. Aromatics varied upon a hop variety and wort boiling time accompanying a positive softening effect regarding aroma formated from malt wort compounds comparing to hopped aqueous extracts. The research proved the most noticeable pH (7 and above) impact on aroma formation caused by the polyphenol conversion. As also shown the first time, a temperature and acidic pH doubled the rutin amount, as well as the best extraction of the prenylflavanoid isoxanthohumol, was achieved by boiling at an alkaline medium pH. The results obtained indicate that various hydrophilic amino acids containing in the wort can stabilize polyphenols affecting the quality indicators of beer produced from different grain raw materials.

**Keywords:** hop polyphenol compounds; essential oils; beer aroma; polyphenol stabilizers; terpene hydrocarbon transformation

## INTRODUCTION

It has been known that the hops use in brewing is very important from many different angles. The article concentrates on the hop polyphenol′s impact on the beer aroma formation and taste starting from the malt wort obtaining stage.

As researchers say, hop polyphenol′s constitute up to 30% of all raw polyphenols **(Wannenmacher, Gastl and Becker, 2018)**. A wide range of phenolic compounds presenting in beer is extracted from vegetable raw materials (malted and non-malted grain and hop). It is believed that polyphenolic substances mainly provide the colloidal stability and head retention of a beer.

Hop phenolic substances consist of monomeric and oligomeric polyphenol′s, including phenolic acids, coumarins, prenylated chalcones, flavonoids, catechins, and proanthocyanidins **(De Keukeleire et al., 2003; Dostálek, Karabín and Jelínek, 2017; Stevens et al., 1997)**. 20% of the total polyphenols are low molecular weight compounds esterified with sugar-like compounds. Such substances include catechin or proanthocyanidins, phenolic carbon acids and quercetin, kaempferol **(Biendl and Pinzl, 2009)**. Prenylflavanoids and chalcones account for 80% of the high molecular weight compounds of hop polyphenols.

Not all the hop polyphenol′s are present in beer. Thus, the research has indicated that proanthocyanidin B, catechin, epicatechin, quercetin glycoside, kaempferol, isoxanthohumol, caffeine acid are present in hop beer **(Forster, Gar and Gar, 2013)**. Which is caused by biochemical processes occurring during beer fermentation and maturation. Researchers note that 10 – 20% flavan-3-ols quantity in beer decreases after 3 weeks of maturation meanwhile isoxanthohumol as a phenylpropanoid decreases its content by 50% after 1 week of maturation **(Mikyška, Dušek and Slabý, 2019)**.

However, the impact of polyphenol′s on the beer taste has been noted. Taste tones such as astringency, hotness, oiliness, "mouthpiece", stickiness are attributed to polyphenols **(Meilgaard, 2001)**. Scientists note that catechin and epicatechin causing the astringent taste in beer should be contained at levels up to 20 $mg.L^{-1}$ **(Dadic and Belleau, 1973)**.

Simple phenolic acids (ferulic, p-coumaric, vanillic, hydrocinnamic acid, etc.) transformations during the brewing process along with their impact on the finished beer aroma and taste are known and examined **(McMurrough et al., 1996; Meilgaard, 1975; Wackerbauer, Kramer and Siepert, 1982)**.

Polyphenol′s physiologically activate tongue TAS2R bitterness receptors in the mouth and thus the research has indicated the polyphenols overlap causing the beer

bitterness **(McLaughlin, Lederer and Shellhammer, 2008; Roland et al., 2013)**.

Polyphenolic compounds interact with salivary secretion nitrogen-containing compounds to form complexes causing an astringent sensation **(Condelli et al., 2006)**. The polyphenol′s molecular weight affects the astringent taste formation **(El Gharras, 2009)**. When even in minor concentrations certain polyphenols cause a bitter taste **(Soares et al., 2013)**.

The polyphenol′s impact on taste can be not direct only but also indirect due to some polyphenol′s effect (catechin and ferulic acid) on the suppression of the carbonyl compounds formation **(Walters, Heasman and Hughes, 1997)**.

When processed the polyphenol′s resistance to non-enzymatic degradation depends upon numerous factors such as molecular structure, pH, temperature, oxygen, light, processing, interaction conditions, and/or presence of other compounds and components **(Boussetta et al., 2011; Ioannou et al., 2012; Iacobucci and Sweeney, 1983)**. Phenolic acids and flavonol glycosides are reported to be more stable and less susceptible to degradation at higher temperatures **(Teleszko, Nowicka and Wojdyło, 2016; Zorić et al., 2014)**. This can explain the presence of low molecular weight polyphenol′s from hops and significant losses of isoxanthohumulone in beer.

To define exactly the hop polyphenol′s impact on the wort aroma formation, we fix the research goal to examine the hopping effect of both traditionally boiled and dry-hopped on the polyphenol content as wells as the hopped wort and water aroma in model experiments using two different types of hops (bitter Magnum and aromatic Tetnanger).

## Scientific Hypothesis

When researching several hypotheses were examined:

a. The wort prolongs boiling with hops causing the release of more polyphenol′s, regardless of the hop type;

b. The liquid medium composition affects extraction and aroma formation bound to hop polyphenolic compounds;

c. The pH medium and different stabilizers affect the aroma formation and polyphenol dissolution.

## MATERIAL AND METHODOLOGY

### Samples

The hops pellets Hallertaur Magnum and Tettnanger varieties are of the 2019 harvest containing the following amount of oil and α-acids respectively: Magnum (1.6 – 2.6 mL.100g$^{-1}$, 12.0%) (Figure 1) and Tettnanger (0.5 – 0.9 mL.100g$^{-1}$, 3.8%) (Figure 2). The precise oil and α-acid content were determined by the producer.

### Chemicals

All reagents and standards were of analytical grade. Quercetin, rutin, isoxantogumol, and phenolic acids standards were from Sigma-Aldrich with a purity ≥ of 99%. Potassium dihydrogen phosphate ($KH_2PO_4$), acetonitrile, acetic acid, orthophosphoric acid ($H_3PO_4$) were purchased from Galachem (Moscow, Russia).

1-butanol, hydrochloric acid (HCl), iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), methanol, carboxymethylcellulose, ethylenediaminetetraacetic acid (EDTA), ferric ammonium citrate ($(NH_4)_5[Fe(C_6H_4O_7)_2]$), ammonium hydroxide ($NH_4OH$) were purchased from Limited liability company “Reatorg” (Moscow, Russia).

### Instruments

This investigation was used centrifuge Armed 80-2 (Russia), photoelectric colorimeter Apel AP-101 (Japan), pH meter Milwaukee (USA), chromatographic equipment "Agilent Technologies 1200" ("Agilent", USA) with diode array detector. HPLC equipment was fitted column Hypersil 5 u C18 250 x 4.6 mm 5 μm (Thermo, USA) with wavelength 270 and 310 nm, and fitted column Luna 5 u C18 (2) 250 x 4.6 mm 5 μm (Phenomenex, USA) with wavelength 290 nm and column Kromasil C18 150 x 4.6 mm 5 μm (Supelco, USA) with wavelength 290 nm.

### Animals and Biological Material:

The hops selected for the research represented by T90 pellets of the Hallertaur Magnum and Tettnanger varieties purchased from Joh.Barth & Sohn Company (Freiligratstrasse, 7-9, 90482, Nuremberg, Germany).

### Laboratory Methods

***Total polyphenol content***

The total hop polyphenol content and wort samples were determined according to method 7.14; Analytica-EBC, 2010. Beer sample (10 mL) was mixed with a preparation of carboxymethylcellulose (CMC, 1%) and ethylenediaminetetraacetic acid (EDTA, 0.2%) (8 mL) in a 25 mL volumetric flask; then ferric ammonium citrate (3.5%, 0.5 mL) was added and then followed by ammonium hydroxide solution (33.3%, 0.5 mL) being mixed after each addition. The solution was made with Reverse Osmosis (RO) water up to mark and left at room temperature for 10 min. The absorbance of the solution was taken at 600 nm and multiplied by 820 to give the total polyphenol content in beer (mg.L$^{-1}$) **(EBC, 2010)**.

***Anthocyanogens content of hop, water extracts and wort samples***

Anthocyanogens (Method 2.17.2; MEBAK, 2011): Anthocyanogens determination to Harris and Ricketts (photometry at 550 nm) **(MEBAK, 2011)**.

***High-performance liquid chromatography (HPLC) analysis***

A high-performance liquid chromatography method using "Agilent Technologies 1200" ("Agilent", USA) diode array detector was applied to determine the phenol acids mass concentration. HPLC equipment was fitted Hypersil 5u C18 250 x 4.6 mm 5 μm (Thermo, USA) column with 270 and 310 nm wavelength. The samples and all standards solutions at a volume of 20 μL were injected into a reversed-phase column at 25 °C. The mobile phase was 0.025 mol.L$^{-1}$ potassium dihydrogen phosphate solution (A) (pH 2.5) and acetonitrile solution (B) with the ratio (A:B – 87:13). The eluent flow rate was 1.3 mL.min$^{-1}$.

A high-performance liquid chromatography method using "Agilent Technologies 1200" ("Agilent", USA) diode array detector was applied to determine the quercetin and rutin mass concentration. HPLC equipment was fitted Luna 5 u C18 (2) 250 x 4.6 mm 5 μm (Phenomenex, USA) column with 290 nm wavelength. The samples and all standards solutions at a volume of 20 μL were injected into a reversed-phase column at 25 °C. The mobile phase was 2% acetic acid solution (A) and acetonitrile solution (B) with the ratio (A:B – 70:30). The eluent flow rate was 1.5 mL.min$^{-1}$.

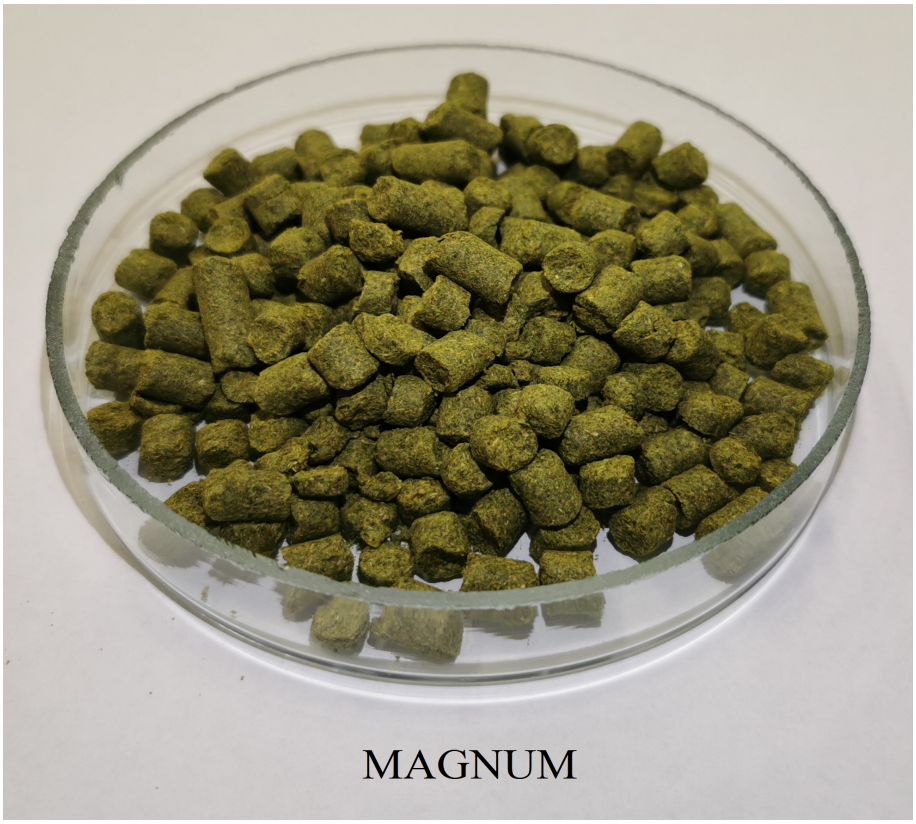


**Figure 1** The hop pellets Magnum variety.




**Figure 2** The hop pellets Tettnanger variety.

***Determination of isoxantogumol concentration in hoped samples***

***High-performance liquid chromatography (HPLC) analysis***

A high-performance liquid chromatography method using "Agilent Technologies 1200" ("Agilent", USA) diode array detector was applied to determine the isoxantogumol mass concentration. HPLC equipment was fitted Kromasil C18 150 x 4.6 mm 5 μm (Supelco, USA) column with 290 nm wavelength. The samples and all standards solutions at a volume of 10 μL were injected into a reversed-phase column at 25 °C. The mobile phase was acetonitrile solution (A), water (B), and orthophosphoric acid solution (C) with the ratio (A:B:C – 40:60:0.1). The eluent flow rate was 1 $mL.min^{-1}$.

***Determination of pH samples***

Sample pH was determined with a pH meter. All measurements were made in triplicate.

***Organoleptic evaluation of hopped samples by descriptors***

The organoleptic analysis was done by a professional group of researchers, consisting of 10 people on a 5-point scale according to the characteristic taste descriptors selected. 5 points mean a strong descriptor shade, 4 points – a well-developed descriptor shade, 2 points – a slightly visible descriptor shade, 1 – a subtle descriptor shade. The results obtained were summarized and the average score was recorded.

**Description of the Experiment**

**Sample preparation: Wort production and dry hopping**

The wort was produced in a pilot brewery (Bavaria, Germany) of the All-Russian Research Institute of Brewing, Non-alcoholic, and Wine-Making Industry. The original wort density produced from 100% malt was 12 °P. The wort was produced according to the conventional infusion mash procedure with temperature breaks at 52 °C, 63 °C, and 72 °C during 20 – 30 min each.

The wort was hopped with two Hallertaur Magnum and Tettnanger hop varieties to achieve a target of 12 bitterness units (BU). Hop samples were added at 15, 30 and 45 minutes after the wort boiling. The boiling time was 60 min and the post-boiling density was 1.048 (12 °P). Also, hop samples were placed to the whirlpool applying dry-hopping technology. The data is shown in Table 1.

T90 pellet hops at a rate of 4 $g.L^{-1}$ (Hallertaur Magnum and Tettnanger variety) were added to the wort current pumped into the whirlpool at a 35 $m.sec^{-1}$ flow rate. The cold hopping stage lasted 25 minutes;

**Model hop water solution preparation**

The boiling time for the preparation of model water hopped solutions was 15 minutes at different pH values applying the same hop doses as for the wort. The pH was adjusted by adding a 0.1% citric acid solution.

**Number of samples analyzed:** The number of one experiment analyzed samples in one replication was 16.

**Number of repeated analyses:** The analysis was done in triplicate.

**Number of experiment replication:** The experiment replication was done two times.

**Statistical Analysis**

Descriptive statistics performed and values expressed as mean ± standard deviation. The Student-Fischer method was used in the studies. The obtained data reliability limit ($p$ <0.05) was considered to assess the various factors that impact the polyphenols content in all studies; the statistical data was processed by the Statistics program (Microsoft Corporation, Redmond, WA, USA, 2006).

## RESULTS AND DISCUSSION

### Boiling time and liquid medium composition effect studies on extraction and aroma formation bound to hop polyphenolic compounds

It is known that the finished beer organoleptic profile depends upon the hops types used in the technology and forms due to various organic compounds – bitter acids, ether compounds, polyphenolic substances **(Praet, 2016; Van Opstaele et al., 2010)**. Sometimes the sharp shade in the hop compounds taste becomes leveled and changes also due to the presence of organic malt substances (β-glucan, starch dextrins, etc.) in the wort **(Fox, 2018; Langstaff and Lewis, 1993)**. In our terms the boiling time of the wort with hops is of great importance.

Therefore, and at the first research stage, comparative studies of the environmental impact on the various hop polyphenol′s dissolution (polyphenoles (POL) and anthocianogence (ANTH) in malt wort (MW) and aqueous solutions (AS) with pH = 5.2) were presented in Table 2.

Table 2 data indicates that the hop type, hopping procedure (kettle or dry-hopping, boiling time), and all together affects the polyphenolic compounds amount transferred. When comparing the data in Table 2, one can see that the hop variety affects the extraction of polyphenol′s – use of bitter Magnum provides a lower polyphenols release comparing to the finely aromatic Tetnanger which is consistent with the quantitative polyphenolic compounds composition in the original hop varieties **(Callemien and Collin, 2009)**. However, the data indicates that the amount of the polyphenolic compound in the boiled wort hop-free is higher than hopped with Magnum regardless of the boiling time. The polyphenol content is reduced by 33% in the thermal interaction of malt protein substances with polyphenol substances and by 40 – 60% in the thermal interaction of malt protein substances and Magnum hop polyphenol′s as well as by 8 – 40% in the case of Tetnanger. One can note that 15 minutes less prolonged boiling with Tetnanger provides the polyphenol′s dissolution by 78% more than their amount in the boiled wort indicating incomplete protein-polyphenol associates formation due to a short interaction time. In our terms, this may be due to the higher intensity of the association of protein and polyphenolic compounds of malt and hops in the wort **(Mikyška et al., 2002)**. The use of fine-flavored Tetnanger hops provides greater polyphenolic substances extraction both in comparison with Magnum in the hopping dynamics and with the amount in the boiled wort. Cold hopping revealed that the use of the fine-flavored hops provides 4% greater polyphenol′s release comparing to the use of bitter Magnum.

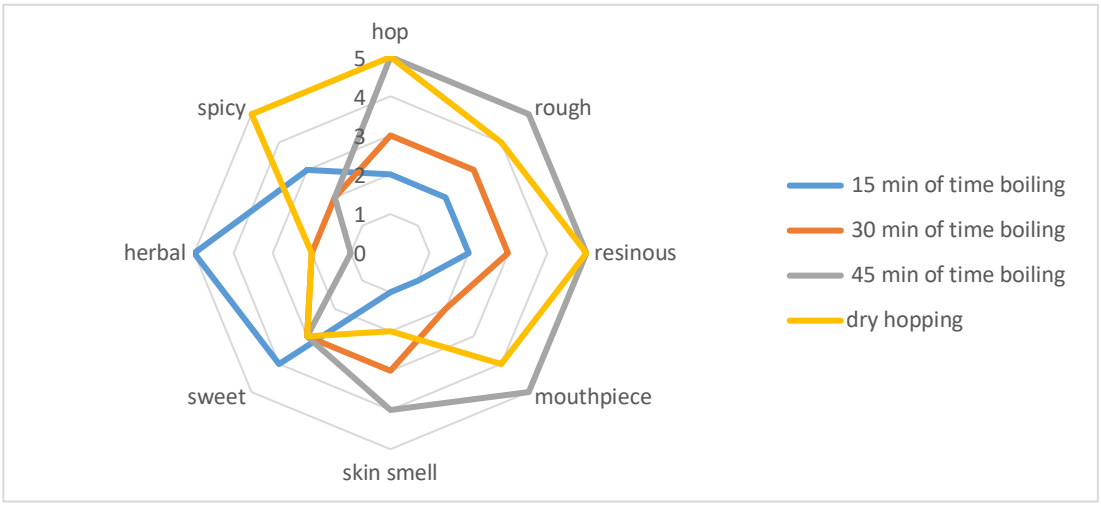


**Figure 3** The hopped wort organoleptic profile using Magnum hop variety depending on the application and hopping duration method.

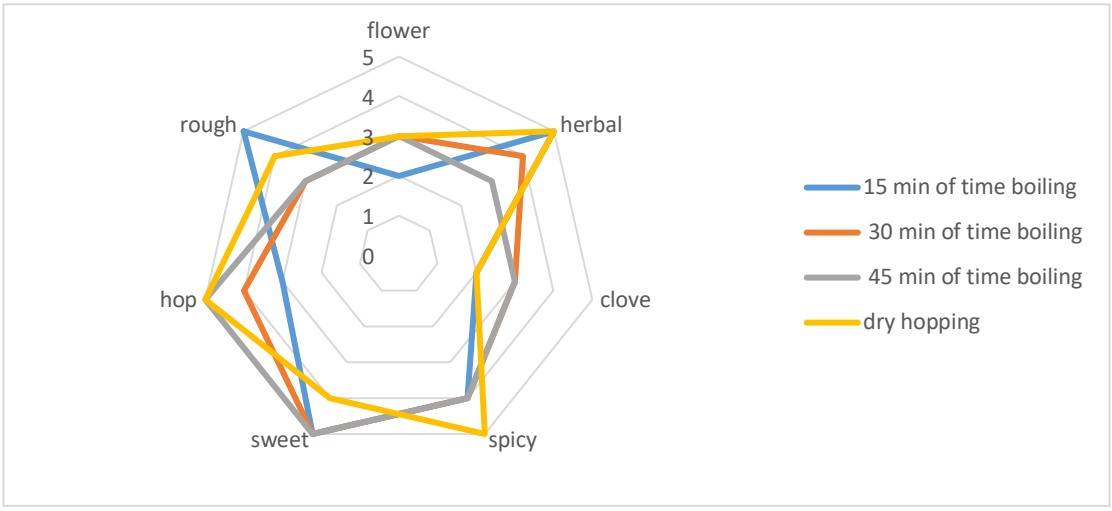


**Figure 4** The hoped wort organoleptic profile using Tettnanger hop variety depending on the application and hopping duration method.

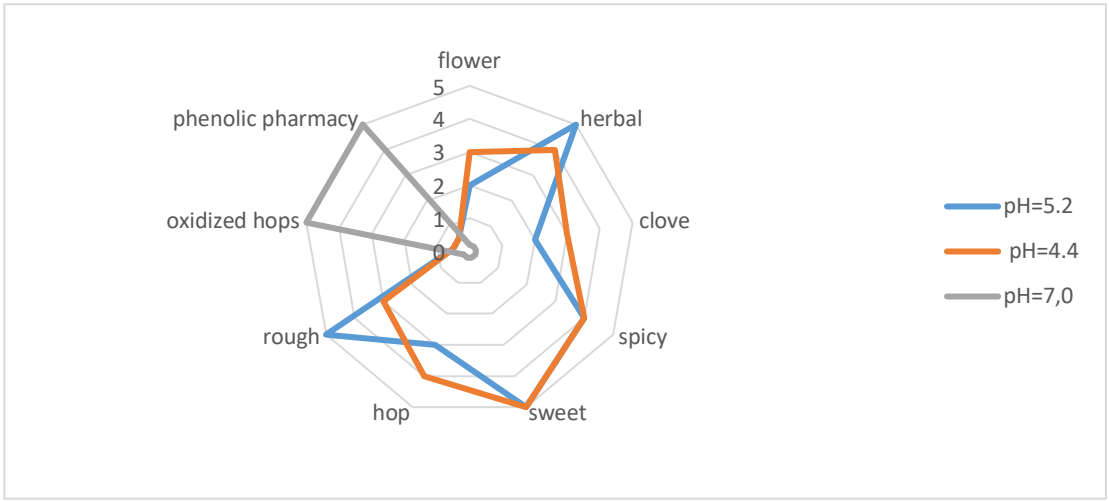


**Figure 5** The hoped water organoleptic profile using Tettnanger hop variety depending on the pH medium.

When comparing the intensity of the polyphenol dissolution in wort (MW) and water (AS), the level of polyphenols decreased by 0.5 – 16 times when using Magnum and 2 – 6 times when using Tetnanger relatively to the content in the hopped wort under the same conditions. This indicates that the most complete hop polyphenol′s dissolution requires not only the appropriate pH of the medium (5.2) but also other compounds′ presence allowing their possible release from the hop plant matrix.

As shown in Table 2, anthocyanogens pass into the wort with different intensities; they are labile and their maximum amount is observed when wort hopping with Magnum for 15 minutes and with Tetnanger for 30 minutes. A long boiling time does not provide their presence in the wort and they are more active to the malt wort protein substances responsible for colloidal haze during beer storage. Anthocyanogens amount could be determined in aqueous solutions when using Magnum hops for 15 minutes and anthocyanogens were converted and outside the detection limits in other cases. When Tetnanger hop variety hopping time was 30 minutes the maximum amount of anthocyanogens was observed in aqueous solutions. Dry-hopping conditions did not provide complete anthocyanogens release from hops in wort and aqueous solutions. The polyphenolic substance transition processes were consistent in both research objects as well as during the aqueous solution and wort hopping.

The aroma formation studies were done due to different compounds in hopped wort and aqueous extract. The data is presented in Figure 1 and Figure 2 respectively.

It ought to be remarked that a longer boiling results in deeper organoleptic aromas such as spice, rough, hop, etc. in the case of Magnum bitter hops variety (Figure 3). The herbal flavor is more present in the aroma with minimum heat treatment time while the flower and clove aromas increase when the heat treatment time is longer in the case of Tetnanger aroma hops variety hopping (Figure 4).

It ought to be remarked regarding dry-hopping that noble hop, floral and spicy tones come out first. The wort has a strongly marked hop aroma with a spicy-herbal-floral touch in the case of Tetnanger and resinous-spicy in the case of Magnum.

Researchers note that floral and citrus tones characterize the presence of linalool while floral, citrus and rose-like – geraniol, resinous – myrcene, clove – caryophile which means that they belong to terpene hydrocarbons **(Kishimoto, 2008)**.

Dry-hopping allows enriching the aqueous solution hopped with Magnum by strong hop, resinous, spicy, and mouthpiece shades while with Tetnanger – floral-spicy aromas where the hop is at the sub-tone level. Thus, there is a fundamental difference in the manifestation of different hop varieties aromas specifications: organic compounds of malt wort (starch and non-starch polysaccharides, protein substances, etc. as well as polyphenolic compounds) responsible for secondary subtones. Aqueous solutions obtained after hopping cause more clear secondary specifications for a particular hop variety tone which is explained by a less transfer of bitter hop compounds into aqueous solutions comparing to wort and characterized by the presence of the compounds in the composition such as acids, mineral, and organic compounds, etc. which reduce the external environment polarity coefficient facilitating the transfer of bitter resins into the wort.

## Studies of the pH and different stabilizers impact on the aroma formation and polyphenol dissolution in model aqueous solutions

Since polyphenol′s are labile compounds capable of exhibiting not only antioxidant properties but also oxidation and transformation, the pH impact on the organoleptic parameters state and the polyphenol content in aqueous extracts of aromatic hop varieties were researched; furthermore, due to the studies (Table 2), the aromatic hops precisely contains more polyphenolic compounds. The data contains in Table 3 and Figure 5.

Studies done with malt wort polyphenol′s have indicated that lowering the pH changed the chemical structure of some malt polyphenols imparting them more light absorption at 275 nm and potentially more bitterness **(Jurić et al., 2015)** unlike hop polyphenol′s as Table data 4 shows. The medium pH is of great importance for the polyphenol′s and related substances extraction since the hop's aroma is formed due to the hops ether compounds – terpenes, terpenoids, oxygenated sesquiterpenes, sulfur compounds, and glycosylated species **(Almaguer et al., 2014; Dresel, Dunkel and Hoffman, 2015)** – Figure 5.

pH values up to 5.2 cause an increase in the release of polyphenolic compounds. More alkaline extraction conditions cause a conversion of polyphenol′s and anthocyanogens negatively affecting the extracts aroma. Accordingly, the spatial polyphenols configuration with associated ester compounds should be in a more acidic environment.

What is since polyphenol′s and anthocyanidins color particularly are pH-dependent **(Tsao, 2010**) being chemically stable in acidic solutions which are confirmed by experiments results (Table 3). Besides, it is well known that anthocyanidins are unstable and decompose at high temperatures and neutral pH **(Dangles and Fenger, 2018)**. When the medium reacts alkaline at pH 7 or higher, a reduction reaction occurs in the medium. Besides, the composition OH groups responsible for the polyphenol′s stability are oxidized and the polyphenol′s lose their stability and due to the detachment of the carbohydrate residue as well.

On the other hand, it is known that flavanols and proanthocyanidins can react to associating with other polyphenol′s and forming coloration up to brown **(Shoji, 2007)**.

Figure 5 indicates that polyphenol′s critically affect aroma at a pH close to neutral when the aroma shades specifications of the ester compounds disappear and only the phenolic-pharmaceutical aroma is visualized inherent in the hop polyphenols group of hop polyphenols.

We have researched the possible use of amino acids and monosaccharides in a model solution to stabilize various polyphenol′s as well as to examine the various compounds′ impact on changes in the polyphenol structure and state.

**Table 1** The studied samples list depending on the hops introduction model.

| Sample No | Technological stage of hop varietes introduction | | | |
|---|---|---|---|---|
| | 15 min after beginning | wort boiling 30 min after beginning | 45 min after beginning | into the current of the wort pumped into the whirlpool |
| | | Magnum | | |
| 1w/1wt* | + | - | - | - |
| 2w/2wt | - | + | - | - |
| 3w/3wt | - | - | + | - |
| 4w/4wt | - | - | - | + |
| | | Tettnanger | | |
| 5w/5wt | + | - | - | - |
| 6w/6wt | - | + | - | - |
| 7w/7wt | - | - | + | - |
| 8w/8wt | - | - | - | + |

Note: 1w/1wt* – to compare the indicators characterizing the extraction rate of various groups of polyphenols, hops were introduced into water and a similar procedure was carried out as with wort – these are samples 1wt.

**Table 2** Polyphenolic compound content in MW and AS at various hop addition modes.

| Indicators content | Value, mg.L$^{-1}$, at the introduction stage (reliability limit $p$ <0.05) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | wort boiling | | boiling time during hopping, min | | | | | | dry-hopping | |
| | befor | after | 45 | | 30 | | 15 | | | |
| | | | AS | MW | AS | MW | AS | MW | AS | MW |
| | | | | | Magnum hop variety | | | | | |
| POL | 196.8 ±17.7$^{a}$ | 147.6 ±13.2$^{a}$ | 8.2 ±0.74$^{a}$ | 131.2 ±11.8$^{a}$ | 10.3 ±0.93$^{a}$ | 139.4 ±12.6$^{a}$ | 82.0 ±7.38$^{a}$ | 123.0 ±11.0$^{a}$ | 65.6 ±5.90$^{a}$ | 196.8 ±17.7$^{a}$ |
| ANTH | 13.48 ±0.94$^{a}$ | 8.55 ±0.6$^{a}$ | nf* | 6.4 ±0.45$^{a}$ | nf | 16.3 ±1.14$^{a}$ | 3.62 ±0.25$^{a}$ | 8.50 ±0.60$^{a}$ | 1.97 ±0.14$^{a}$ | 12.9 ±0.90$^{a}$ |
| | | | | | Tettnanger hop variety | | | | | |
| POL | 196.8 ±17.7$^{a}$ | 147.6 ±13.2$^{a}$ | 53.3 ±4.78$^{a}$ | 141.0 ±12.7$^{a}$ | 84.0 ±7.56$^{a}$ | 182.0 ±16.4$^{a}$ | 41.0 ±3.69$^{a}$ | 262.4 ±23.6$^{a}$ | 131.2 ±11.8$^{a}$ | 205.0 ±18.4$^{a}$ |
| ANTH | 13.48 ±0.94$^{a}$ | 8.55 ±0.6$^{a}$ | 3.62 ±0.25$^{a}$ | 8.55 ±0.60$^{a}$ | 4.60 ±0.32$^{a}$ | 16.22 ±1.14$^{a}$ | 1.42 ±0.10$^{a}$ | 21.15 ±1.48$^{a}$ | 8.55 ±0.60$^{a}$ | 14.0 ±0.98$^{a}$ |

Note: nf* – not finding. [a] Each value represents the mean of three independent experiments (±SD).

**Table 3** Hoped aqueous solution values at different pH medium.

| Indicators | Characteristics at pH value (reliability limit $p$ <0.05) | | |
|---|---|---|---|
| | 4.4 | 5.2 | 7.0 |
| Aroma | jasmine, floral, hop | grass, hop | phenolic, pharmacy, oxidized hop |
| Colore | lemon-mint | lemon-mint | red grapefruit |
| POL, mg.L$^{-1}$ | 42.0 ±3.8$^{a}$ | 41.0 ±3.7$^{a}$ | 27.5 ±2.5$^{a}$ |
| ANTH, mg.L$^{-1}$ | 1.97 ±0.14$^{a}$ | 1.42 ±0.10$^{a}$ | Nf |

Note: nf – not found. [a] each value represents the mean of three independent experiments (±SD).

**Table 4** The amino acids influence on polyphenol content in hoped aqua solution at pH = 7.0.

| Indicators | Characteristices of indicators with applaing amino acid (reliability limit $p$ <0.05) | | | | | |
|---|---|---|---|---|---|---|
| | Gly | | Arg | | Asp | |
| | 5 | 150 | 10 | 150 | 20 | 150 |
| Aroma | pharmacy-phenolic | oxidized hop, hay | oxidized hop | oxidized hop, hay | oxidized hop | hop, citrus, spicy |
| Color | deep crimson | | | | | lemon-yellow |
| pH | 7.0 | | | | | 5.2 |
| POL, mg.L$^{-1}$ | 82.0 ±0.74$^{a}$ | 147.6 ±13.2$^{a}$ | 82.0 ±0.74$^{a}$ | 164.0 ±14.8$^{a}$ | 82.0 ±0.74$^{a}$ | 139.4 ±12.5$^{a}$ |
| ANTH, mg.L$^{-1}$ | 0.33 ±0.02$^{a}$ | 1.42 ±0.10$^{a}$ | 0.88 ±0.06$^{a}$ | 3.62 ±0.25$^{a}$ | 1.97 ±0.14$^{a}$ | 4.71 ±0.33$^{a}$ |

Note: [a] each value represents the mean of three independent experiments (±SD).

**Table 5** The different factors influence on polyphenol content in hoped aqua solution (AS) and dry hop solution (DHS).

| Indicators, mg.L$^{-1}$ | AS characteristics with different treatment during boiling with the substances in concentration (reliability limit $p$ <0.05) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH | | | Amino acid | | | Monosugar | | DHS |
| | 4.4 | 5.2 | 7.0 | Gly | Arg | Asp | Gl | Fr | |
| Concentration, mg.100 mL$^{-1}$ | - | | | 150 | | | 4000 | | - |
| pH | 4.4 | 5.2 | 7.0 | 7.0 | | 5.2 | 7.0 | | 7.0 |
| POL | 82.0 ±7.4 | | | 147.6 ±13.2 | 164.0 ±14.8 | 139.4 ±12.5 | 82.0 ±7.4 | | 131.2 ±11.8 |
| ANTH | 0.33 ±0.02 | 1.97 ±0.14 | nf | 1.42 ±0.10 | 3.62 ±0.25 | 4.71 ±0.33 | nf | 0.33 ±0.02 | 8.55 ±0.60 |
| Isoxantogumol | 2.16 ±0.21 | 1.90 ±0.19 | 4.44 ±0.44 | 3.94 ±0.39 | 3.78 ±0.38 | 1.99 ±0.20 | 3.73 ±0.37 | 3.55 ±0.36 | 0.96 ±0.1 |
| Rutin | 7.77 ±0.77 | 8.16 ±0.82 | 4.32 ±0.43 | 3.36 ±0.33 | 7.86 ±0.79 | 7.54 ±0.75 | 6.43 ±0.64 | 3.50 ±0.35 | 2.29 ±0.23 |
| Quercetin | 0.04 ±0.004 | | | | | | | | |
| vanillic acid* | 0.19 ±0.02 | 0.17 ±0.02 | 0.20 ±0.02 | 0.24 ±0.02 | 0.19 ±0.02 | 0.11 ±0.01 | 0.18 ±0.02 | 0.15 ±0.02 | 0.16 ±0.02 |
| syringic acid* | 0.07 ±0.01 | 0.14 ±0.01 | 0.14 ±0.01 | 0.10 ±0.01 | 0.15 ±0.01 | 0.11 ±0.01 | 0.18 ±0.02 | 0.18 ±0.02 | 0.14 ±0.01 |
| vanillin* | 0.13 ±0.01 | 0.16 ±0.02 | 0.16 ±0.02 | 0.12 ±0.01 | 0.13 ±0.01 | 0.10 ±0.01 | 0.18 ±0.02 | 0.17 ±0.02 | 0.14 ±0.01 |
| syringic aldehyde* | 0.08 ±0.01 | 0.06 ±0.01 | 0.18 ±0.02 | 0.10 ±0.01 | 0.11 ±0.01 | 0.07 ±0.01 | 0.16 ±0.02 | 0.15 ±0.02 | 0.08 ±0.01 |
| synapic acid* | 0.12 ±0.01 | 0.11 ±0.01 | 0.19 ±0.02 | 0.18 ±0.02 | 0.16 ±0.02 | 0.11 ±0.01 | 0.17 ±0.02 | 0.16 ±0.02 | 0.12 ±0.01 |
| coniferyl aldehyde* | 0.34 ±0.03 | 0.31 ±0.03 | 0.45 ±0.04 | 0.04 ±0.004 | 0.08 ±0.01 | nf | 0.22 ±0.02 | 0.21 ±0.02 | 0.11 ±0.01 |
| cynapic aldehyde* | 0.64 ±0.06 | 0.71 ±0.07 | 0.23 ±0.02 | 0.09 ±0.01 | 0.18 ±0.02 | 0.62 ±0.01 | 0.37 ±0.04 | 0.25 ±0.03 | 0.36 ±0.04 |
| Summ of low molecular weight phenolic acids | 1.57 | 1.66 | 1.55 | 0.87 | 1.00 | 1.12 | 1.36 | 1.27 | 1.11 |

Note: nf – not found. Each value represents the mean of three independent experiments (±SD), *Low molecular weight phenolic acid.

It′s clear, that polyphenol′s are synergistic with several organic substances such as organic acids, amino acids, carbohydrates, etc. **(Baxter et al., 1997; Codorniu-Hernández et al., 2005; Gauche, da Silva Malagoli and Bordignon Luiz, 2010; Guerra and Yaylyan, 2014; Renard, Watrelot and Le Bourvellec, 2017)**.

For example, experiments with glycine indicated that (+) – catechin forms various adducts with this amino acid through a dehydration reaction in the ring as well as with the Schiff bases formation at the attachment point to the oxidized b-ring **(Guerra and Yaylyan, 2014)**.

Malt wort contains several amino acids in minor concentrations **(Otter and Taylor, 1976)**, as well as reducing substances represented by low-molecular dextrins, non-staining with iodine, maltose, glucose, fructose **(Floridi et al., 2001)**.

It was of interest to study the polyphenol system stabilization at pH = 7; for this purpose, the amino acids present in malt wort were used – glycine (Gly), leucine (Leu), alanine (Ala), argenine (Arg), aspartic acid (Asp.), and monosugar (glucose (Gl) and fructose (Fr)). The data are presented in Table 4 and Table 5.

According to data in Table 4 and Table 5, glycine, leucine, arginine, aspartic acid and fructose monosaccharide significantly affect polyphenol stability. Alanine and glucose use led to anthocyanogen degradation. Therefore, compounds were selected – amino acids stabilizing polyphenol′s better than others such as arginine, glycine, and aspartic acid. This fact is explained by the hydrophilic nature and ability to interact with polyphenols easier than other amino acids in the case of arginine and aspartic acid **(Higgs et al., 2008)**. There is a carboxyl group binding to the polyphenolic compounds′ active sites on the surface in the main chains of these amino acids.

An interesting effect was observed in the case of adding a monosaccharide, fructose, to the medium (data in Table 5). Considering the amount increase of anthocyanogens (proanthocyanidins, prodelphinidins, etc.) comparing with the control it prevented their oxidation binding to them in other words. This is confirmed by the literature proving the

same **(Andrade et al., 2017)**. Researchers attribute interactions with carbohydrates due to van der Waals interactions providing a degree of polymerization, molecular flexibility, number of external hydroxyl groups, or number of terminal galloyl groups **(Bordenave, Hamaker and Ferruzzi, 2014)**.

In our opinion, it is interesting to note that the anthocyanogens presence affects the aroma and color change of the aqueous extract desired while the sugar adding caused no impact on the total polyphenols content.

However, a significant effect, judging by Table 4 and Table 5, we did not observe. Therefore, it was decided to examine hydrophilic amino acids at a concentration equal to the total sum of amino acids contained in malt wort – 150 mg.100mL$^{-1}$ **(Otter and Taylor, 1976)**, data are shown in Table 5.

According to Table 5, a distinct medium acidification effect was achieved by the aspartic acid adding since the medium pH became 5.2 which is bound to increased polyphenols and anthocyanogens content. The pH changes effect on the anthocyanogens color change has been examined indicating that the acidic to neutral medium acidity changes caused complete but reversible discoloration of the anthocyanidin molecule due to the colorless isoforms formation **(Basílio and Pina, 2016)**.

The results obtained using aspartic acid exceed the control sample obtained by boiling hops in water with a pH of 5.2, provided by the citric acid adding (Table 5). In this case, the anthocyanogens amount tripled indicating their great resistance to critical conditions of extraction from hops at the temperature of 100 °C. Terpene hydrocarbons and alcohols are the main aromatics in hops. Hydrocarbons are isomerized by the ionic mechanism in case of acidic pH values with the amino acids adding **(Bejblová, Žilková and Čejka, 2008)**. Isomers are reversibly formed, enhancing the characteristic aroma. The isomerization transformations of alcohols (linalool, etc.) are more diverse than the transformations of hydrocarbons, due to the presence of a hydroxyl group **(Basílio and Pina, 2016)**. Unsaturated terpenoids undergo irreversible polymerization when a pH close to neutral and alkaline causing an odor loss **(Bejblová, Žilková and Čejka, 2008)**, thus, when a pH close to neutral, oxidized hop tones are present; pH at 5.2 shows unoxidized hop tones and the anthocyanogens and other polyphenol′s color are lighter indicating a creep of the structure of the unoxidized compound **(Basílio and Pina, 2016)**. After all the anthocyanins color change is bound to an intramolecular binding of p-coumaric residues to the anthocyanin chromophore **(Mori, Kondo and Yoshida, 2009)**.

Table 5 data indicates the change in the polyphenol accumulation in different aqueous extracts groups under different experimental conditions. According to Table 5 data the isoxanthohumol extraction is consistent with the pH medium: the closer to a neutral and alkaline medium the higher the prenylflavanoid extractability which is consistent with the literature data **(Moens et al., 2020)**. Most of all isoxanthohumol is extracted during boiling, rather than with vigorous stirring. The polar amino acid addition affects the isoxanthohumol extraction intensity since the addition of the amino acids changes the water solution polarity and increases its dissolution **(Radzicka and Wolfenden, 1988)**. When comparing the boiling method with the cold hopping vigorous stirring at pH 7.0, the isoxanthohumol goes least of all into solution with stirring without exposure to high temperatures. This can be caused by its isomerization **(Magalhães et al., 2008)**.

The quercetin extraction did not depend on external conditions (temperature, pH, stirring), and a stabilizer was added remaining at the same level. The rutin extraction was maximum under the acidic pH value conditions provided by organic or amino acid. However, the temperature factor (t = 99 °C) allowed extracting double more rutin comparing with the extraction at t = 25 °C along with stirring during the same time.

We can say that pH values have a more significant effect comparing with other factors (thermal, the presence of sugar, stirring) regarding the recoverable low molecular weight phenolic compounds (LMPS) amount. The LMPS are most intensively extracted in the citric acid 10% solution state at pH 5.2 during boiling. The LMPS amount is somewhat lower at the same pH value achieved by adding aspartic acid.

It is also necessary to pay attention to the variability of the hopped water aroma depending on the amino acid used (Table 5). Which could hypothetically contribute to the aroma and flavor development in hopped wort made from a variety of grains. There is a wide variety of amino acids containing in the wort qualitatively and quantitatively in this case **(Zhuang et al., 2017)**. Thus, one can testify that the beer taste and aroma formation begins at the hopping stage, when amino acids and mono sugars extracted from various grain raw materials in different percentages react with the hop anthocyanogens but not at the fermentation stage as was stated before so far **(He et al., 2014)**.

## CONCLUSION

The research indicated that many factors affect the hop polyphenol′s dissolution in brewing technology such as the original hop variety, hopping method, the hop contact time with a hopped liquid (wort or water), polarity and pH of the hopped liquid. According to the research, a perceptible impact on the polyphenolic substances aroma begins under unfavorable conditions when the polyphenolic molecule configuration is unstable and undergoes irreversible changes at pH values of 7 or more. Under favorable conditions, the polyphenol′s aroma is a sub-tone and cannot be clearly distinguished by sensor detection. The hop aroma is revealed most fully in the hop aroma-forming substances total amount accompanied with other compounds the wort taste fullness specifications.

Thus, the aroma formation due to the hop polyphenol′s mainly depends upon the wort pH and amino acid composition extracted during mashing from grain raw materials which is mainly hydrophilic. While transformed during fermentation, they affect the beer aroma.

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# BEE STIMULATION TO FORM PROTEIN FOOD RESERVES

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## ABSTRACT
We have investigated different ways of bees' stimulation to lay protein food while using artificial honeycombs. It has been proved that the use of artificial combs to get bee-bread upon the condition of the post-treatment processing of its elements by wax and honey syrup does not stimulate bees to lay and process protein food in their cells. It has been identified that upon the condition of the direct involvement of the working bees into the formation of bee-bread supplies the protein food has been mostly consumed. This proves that the working bees use the freshly-gathered pollen pellet for their own needs in the period of its active gathering. It has been determined that the most effective way of bee stimulation to reprocess pollen pellet into bee-bread is it's single densifying in artificial honeycombs with the follow-up processing of the upper layer of the feed by honey. This way encourages bees to form stocks of bee bread and decreases their activity of consuming protein food from cells of artificial honeycombs. The processing of thickened pollen pellet by honey probably oppresses the bees' need to consume protein food from the packed cells redirecting them to other honeycombs of the bee family's nest which has areas with bee-bread reserves.

**Keywords:** ethnology of bees; bee-bread; artificial honeycombs; bee colonies; stimulation

## INTRODUCTION
Pollen is the only source of protein that bees collect from nature. This is very important for the development of the offspring, and thus for the development of bee colonies **(Anđelković et al., 2012)**. In one day, the bee colony can collect from 50 to 250 g of pollen **(Komosińska-Vassev et al., 2015)**. Bee family can collect from 15 to 40 kg of pollen per year **(Zuluaga, Serrato and Quicazan, 2015)**. The average weight of pollen carried by the bees is about 7.5 mg.

The next stage of pollen transport is the pollen being collected from the foraging bees to the hive and packed into honeycomb cells **(Fuenmayor et al., 2014)**. Non-flying bees fill the mixture into honeycomb cells for ¾ of the cell volume **(Barajas, Cortes-Rodriguez, Rodríguez-Sandoval, 2012)**. When being packed into the cells, the pollen is further enriched by the bees with honey, as well as digestive enzymes and organic acids that are contained in the secretions of the salivary glands of bees **(Barajas, Cortes-Rodriguez, Rodríguez-Sandoval, 2012; Deveza et al., 2015; Zuluaga, Serrato and Quicazan, 2015)**. The process of lactic fermentation of pollen caused by bacilli of *Lactobacillus* bacteria spontaneously occurs in the honeycombs, under anaerobic conditions. This type of lactic acid fermentation is similar to that in yogurts (and other fermented milk products) and renders the final product more digestible and enriched with new nutrients **(Krell, 1996)**. The concentration of lactic acid in bee bread is six-fold higher than in pollen **(Nagai et al., 2004)**. The processes of pollen transformation to bee bread are accompanied by biochemical reactions. It is postulated that this is the result of microorganisms' activity, particularly lactic acid bacteria **(Anđelković et al., 2012)**. The presence of lactic acid preserves the bee bread, which, in turn, affects the ability of longer storage of the resulting product.

Pollen sheaths are dissolved just in the process of pollen transformation into the bee bread and in this form, it becomes more readily absorbed. Fermentation to the form of bee bread not only protects the pollen against the loss of properties but also gives rise to new components as a result of enzymatic transformations. The pollen proteins are degraded to peptides and amino acids during the fermentation process **(Barajas, Cortes-Rodriguez and Rodríguez-Sandoval, 2012)**.

Proper hive management promotes the bee-bread collection, aimed at marketing it for human consumption since it can be considered as a food supplement due to its content of a wide range of nutrients. One of the contributions to their high nutritional value is the presence of significant amounts of proteins, flavonoids, vitamins, and phenolic compounds as natural antioxidants **(Horčinová Sedláčková et al., 2020b; Hudz et al., 2017b; Vergun et al., 2019)** The potential application of "bee bread" as a food and as a nutraceutical supplement depends in large part on its chemical composition which varies directly with the flora of the region and the time of collection by the bees **(Čeksterytė et al., 2008)**. Bee bread differs from pollen by lower pH (3.8 – 4.3), it contains fewer proteins and fats, but more carbohydrates and lactic acid. Bee bread has a better

bioavailability because the walls of pollen, which cannot be destructed by gastrointestinal liquids, have been partly destructed by fermentation and the functionally and energetically rich content of pollen can be assimilated and used easier **(Fatrcová-Šramková, Nôžková and Ostrovský, 2010; Mizrahi and Lensky, 1997)**. Pollen from entomophilous plants is characterized by higher nutritional value compared to that from anemophilous ones **(Anđelković et al., 2012)**. The study conducted by **DeGrandi-Hoffman, Chen and Simonds (2013)** showed that protein concentration in the pollen is higher than that in bee bread, while amino acid concentrations were lower in most cases. An elevated level of free amino acids may be the result of an activity of specific proteolytic enzymes that cause the breakdown of peptide bonds within the polypeptide chain. The concentration of certain amino acids (tryptophan) may be lower in bee bread compared to pollen. This phenomenon may be related to the reduction process, which is caused by microbial activity. Some microorganisms can use amino acids as a source of carbon and energy for their growth. Furthermore, free amino acids can also be incorporated into the structure of proteins, which, in turn, reduces their concentration. The content of these compounds in bee bread may be determined not only by the source of pollen but also by the genotype of bees performing the conversion to the bee bread **(DeGrandi-Hoffman, Chen and Simonds, 2013; Lokutova, 2015)**. Bee bread has antimicrobial **(Didaras et al., 2020; Urcan et al., 2018)**, antibacterial **(Abouda et al., 2011; Baltrušaityté, Venskutonis and Čeksteryte, 2007; Nikolaieva et al., 2019)**, antioxidant **(Bleha et al., 2019a, Bleha et al., 2019b; Hudz et al., 2017a)**, hepatoprotective **(Yildiz et al., 2013)**, immuno-modulating and antiradiation activity **(Breed, Mizrahi and Lensky, 1998)**, adaptogenic properties **(Haščík et al., 2020)**. It stimulates protective forces of the human body, normalizes metabolism, has a positive influence on the liver, nervous and endocrine system functions **(Capcarová et al., 2020),** and enhances regeneration of tissues, physical and mental persistence of the human body **(Bogdanov, 2015)**.

Results of other studies **(Brindza et al., 2015; Ivanišová et al., 2015)** demonstrate that bee bread is a very good source of bioactive compounds not only with antioxidant but also antimicrobial effect. The best results were observed in most of the parameters in the sample from Poltava oblast. Bee bread can be used more in the future not only in medicine, pharmacy but also in the food industry. For confirmation of biological effect is necessary more and intensive study, *in vivo* test for evaluating bioactive components and digestibility properties; very important is not only determining some negative compounds which can decrease the quality of bee bread (heavy metal, radionuclide, and microbes) but also evaluation of pollen sizes **(Grygorieva et al., 2018; Horčinová Sedláčková, Grygorieva and Brindza, 2020a ; Motyleva, Gruner and Semenova, 2018a; Motyleva et al., 2018b)** and mineral composition of pollen **(Motyleva et al., 2018b)**. Results are an important tool for recognizing bee bread as being a beneficial source of natural nutrients **(Hudz et al., 2017b; Ivanišová et al., 2015)**.

### Scientific hypothesis

The bees use bee pollen pellets to store bee bread as a source of nutrition for the bee colony in the honeycomb cells formed. In experiments, we assumed that bees will also use artificial honeycombs for this process, to which a pollen pellet will also be supplied. Proof of the acceptance of artificial honeycombs by the bee colony will be the preparation of bee bread for its nutrition.

## MATERIAL AND METHODOLOGY

### Description of the Experiment

Taking into account the peculiarity of bees' reaction to non-natural materials for them, we have to solve two problems, namely, encourage bees to domesticate the artificial honeycomb and make them concentrate protein feed in its cells (Figure 1). To do the research, we have used several ways of attracting bees to domesticate cells. The first one dealt with the preparation of the containers themselves. Thus, to control the process we have used artificial honeycombs without any previous preparation. Having joined the details (containers, middle wall, frieze rail) we have put them into the nest of bee colonies between the feeding and brood parts. During the whole period of the investigation, we have daily examined and identified the number of cells in the containers that are filled with bee bread.

In other cases, the cells with honeycombs have been processed. In research group 1 the honeycombs have been processed by wax. To do this, the containers have been put into the melted wax for a few seconds, and then honeycombs have been formed out of them and have been put into the nests of bee colonies into the same areas of nests as in the test group. In the research group, 2 artificial honeycombs, except the ones that have been processed by wax, have been irrigated by honey syrup. Having installed honeycombs into the nests of bee colonies, the records have been done in the same way as in the test group **(Brovarskyi et al., 2016)**.

The next type of reaction of bees on laying protein feed was encouraging them to concentrate on making bee bread pellets using attracting workers by putting pollen pellet directly into the cells of containers of the artificial honeycomb (Figure 2). We have used 4 methods in these investigations. They had one thing in common. Artificial honeycombs couldn't be processed by wax. We have manually put 3 – 5 pollen pellets into their cells. The thing that made these methods different was the fact that the dry pollen pellet has been put into the cells of honeycombs (group 3), having filled up 3 – 4 dried pollen pellets, the cells have been irrigated by honey syrup and their content has been trampled down using the thickener we have designed (research group 4). In group 5 freshly harvested pollen pellet has been put into the cells, in group 6 it has been additionally thickened, but it hasn't been processed by honey syrup as in research group 4 (Figure 3).

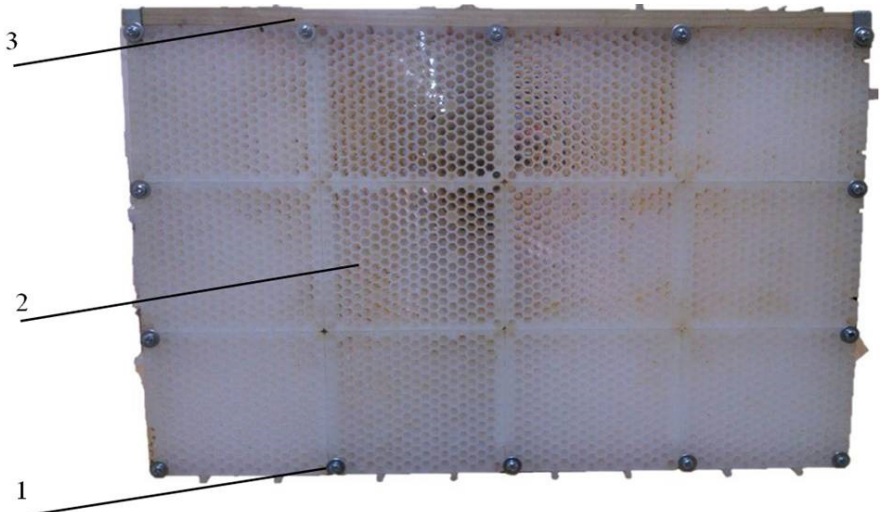


**Figure 1** General view of artificial combs to get bee bread. Note: 1. Clamping 2. Artificial combs 3. Wooden block.

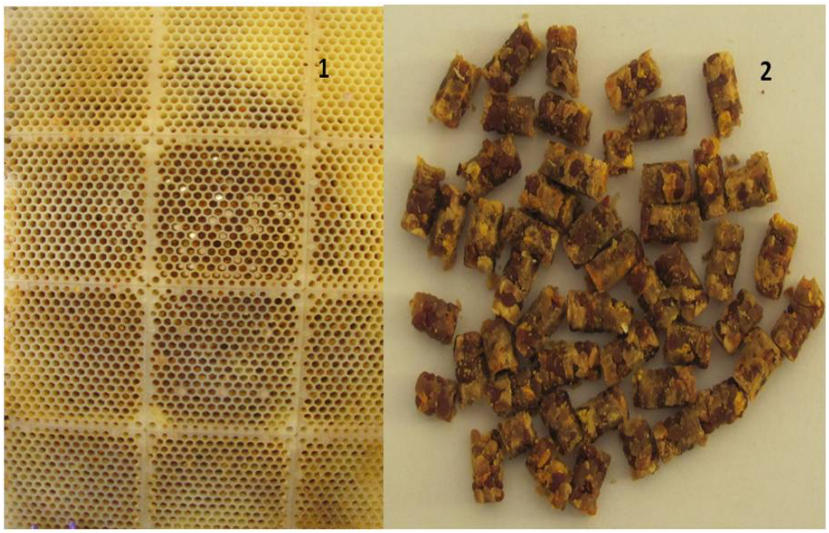


**Figure 2** General view of artificial combs to get bee bread (1), bee bread pellets derived from new technology (2).




**Figure 3** General view of bee bread pellets obtained in a primitive way (A) and newly developed (B).



**Figure 4** Placement of an artificial honeycomb with densely obstructed nets in the family.





**Figure 5** The process of filling the cell freshly pollen basket.

In research groups 3 – 6 during a daily inspection of artificial honeycombs and the record of cells filled with protein food they appeared to be empty (Figure 4, Figure 5). The bees were shaken out of the honeycombs and 3 – 5 pollen pellets were put into empty cells according to the investigation methods. All in all, this procedure has been done 4 times. Five honey bee colonies have been involved in these groups. These colonies have been chosen according to prototypes **(Brovarskyi et al., 2007)**. The results of the records have been analyzed after the mathematical processing.

### Statistical Analysis

For the characteristics of the files, we used the basic descriptors of variability: average, minimum measured value, maximum measured value, the coefficient of variation (%). Data were analyzed with ANOVA test and differences between means compared through the Tukey-Kramer test ($\alpha = 0.05$). The degree of variability was determined by the coefficient of variation values. The given parameter is independent of the unit of the evaluated character.

## RESULTS AND DISCUSSION

To stimulate honey bee colonies to store up protein food, lots of work has been done to form their nests. In every group, we have chosen honeycombs from every bee family. These honeycombs contain lots of cells filled with bee bread. Taking into account the fact that bees mostly consume bee bread while feeding slugs, we have chosen one honeycomb with sealed brood out of the nests. Instead of them, we have put the ones with unsealed brood. The honeycombs with sealed brood have been moved into the

nests of bee colonies of the user group. The frames with slugs for experimental bee colonies have been chosen from them as well. According to our plan, the protein food deficit and a great number of the unsealed brood had to stimulate the bees to activate the process of collecting pollen.

According to the results of undertaken studies, the following things have been determined and brought to light (Table 1). In the methods that used only the preparation of artificial honeycombs (test group and research group 1, 2) the bees passively domesticated the cells of the containers. The bees in the test group started to lay protein food into single cells only 4 days after the nests of the artificial honeycombs have been installed. During the 4th record, the bees of the test group have involved only 0.004% of containers of the artificial honeycombs out of 5496 available for bee bread. Besides that, in the cells of the artificial honeycombs, the bees didn't grow brood and didn't create the reserves of carbohydrates.

The results that we got, having tested the artificial honeycomb in the test group, indicate that bees passively domesticate cells in the sections and it's impossible to use the artificial honeycomb effectively without involving the additional stimulation.

The results of testing artificial honeycombs in bee colonies of research groups 1 and 2 haven't been encouraging. During the first record in research group 1, in which honeycombs have been processed by wax, as well as in group 2, in which the cells have been processed by wax and honey syrup, the bees don´t have to involve either of the cells to store food. In a day (record 2) we have seen the presence of protein food in separate cells of artificial honeycombs of research bee colonies 1 and 2. During the next two records, the intensity of forming protein reserves in these groups was almost of the same level. The bees of these colonies have involved the additional space for laying pollen pellet, but it referred only to at least 10 cells which constituted 0.004 and 0.003% from their overall amount of the honeycomb. It was noticed that in the process of forming bee bread reserves the bees not only filled the cells of the honeycomb with pollen pellet but also took food out of them.

Thus, using the artificial combs for making bee bread, even with extra processing of its elements with wax and honey syrup, does not encourage bees to lay and process protein food in cells.

The research of the etiology of bees with the help of different methods of putting dry and freshly harvested bee pollen pellets has been very encouraging. We have used dried pollen pellet in researches 3 and 4.

As it appeared to be, the bees in the bee family in the 3rd research group passively reacted to fill the cells with pollen pellet. The percentage of the cells filled with bee bread, regarding their overall amount on the artificial honeycombs, was from 0.007 to 0.4%, on the condition that we refilled empty honeycombs with pollen pellet daily. Even when we processed pollen pellet with honey syrup (research 4) and thickened them, the bees insignificantly activated the process of laying the bee bread into the cells of artificial honeycombs. Using this method, we have identified a slight increase in the number of cells with bee bread located on artificial honeycombs – 0.2 – 1%. It is important to underline that the bees enlarged the space for bee bread in these honeycombs with every following fill-up of the empty cells with pollen pellet. If to compare these results with the test group, the activity of bee families of this group in laying protein food was much higher.

Thus, the use of dry pollen pellet on the condition that it's put into the box of artificial honeycombs in small amounts doesn't stimulate bees to lay the bee bread intensively, even if the thickening and the processing of the food by honey syrup are done 4 times.

A completely different reaction of bees on laying the bee bread reserves took place when freshly harvested pollen pellet was used. In research group 5 the bees enlarged the space for forming protein food reserves even though 3 – 5 pollen pellets were put into the cells of artificial honeycombs without being thickened. At the same time, if to look at the total amount of the cells of honeycombs in terms of quantity, these results would be still minor, but a sequence higher than in research 4 and other previous groups of bee colonies. For example, after the first fill-in of the fresh pollen pellet, the bees have put bee bread into the cells on the area that exceeded the research group 4 2.2 times. During the second record, the differences between these groups were 5.9 times, during the 3rd record – 3.6, the 4th – 4.6 times. If to compare it with the test group, the difference in data was highly probable ($p$ <0.01; $p$ <0.001). The freshly harvested pollen pellet may be more preferably for bees to thicken it in the cells because it contains plenty of was in it. Taking into account the results of these investigations, it can be inferred that the first thing that attracts bees to lay protein food reserves is, at first, the quality of pollen pellet. And the processing of the constitutive elements of the artificial honeycombs doesn't reinforce the bees' instinct of reprocessing the pollen into bee bread in cells. Besides that, as the bees use only fresh pollen pellet to stock bee bread, it is the most attractive thing for them to process protein food, unlike the dried pellets. It is not improbable that the dry pollen can be more difficult to yield to fermentation after-ripening of bee bread. We think that these processes are necessary to thoroughly research with the help of up-to-date biochemical, microbiological, physical, and other methods **(Dranca, Ursachi and Oroian, 2020; Kubík et al., 2017; Kubík, Kažimírová and Brindza, 2020; Oroian, Ursachi and Dranca, 2020)**.

The most encouraging results were received in the 6th research group. In this group after 3 – 5 pollen pellets have been put into the cells and thickened, the artificial honeycombs were moved to the nests of bee colonies so that they could form protein food reserves. The research showed that during the first record the bees started to prepare the reserves of bee bread in 11.2% of cells of their total amount in the honeycombs. Compared to research 5, the bees of this group have enlarged the space for reprocessing pollen pellet into bee bread in honeycombs by 25.7 times.

After the next filling of the empty cells, the bees of this group slowed down the pace of forming protein food reserves. The number of cells with bee bread after the second record has increased by 3.2%. During the 3rd record, the number of cells filled with bee bread in the artificial combs exceeded the area of section 4. After the cells have been filled with pollen pellet for the 4th time, the percentage of laying bee bread by workers was 81.7%.

**Table 1** The effectiveness of putting the protein food by working bees under various conditions of preparation of artificial combs, n = 5.

| The way of comb preparation | Domesticated cells among 5496 in the comb<br>The number of iterations | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | | II | | III | | IV | |
| | M ±SD | % | M ±SD | % | M ±SD | % | M ±SD | % |
| **Without processing the cell (control)** | – | – | – | – | – | – | 0.2 ±0.2 | 0.004 |
| **The cells processed with wax (Exp 1)** | – | – | 0.2 ±0.20 | 0.004 | 0.8 ±0.58 | 0.015 | 0.2 ±0.20 | 0.004 |
| **The cells processed with wax and honey syrup (Exp 2)** | – | – | 1.2 ±0,58 | 0.022 | 1.4 ±0.60 * | 0.025 | 1.8 ±0.37 ** | 0.033 |
| **In every iteration, the empty cells were filled with pollen pellet** | | | | | | | | |
| **3 – 4 dried pollen pellet put into cells (Exp 3)** | 0.4 ±0.24 | 0.007 | 2.2 ±0.58 ** | 0.400 | 4.4 ±0.93 *** | 0.080 | 10.8 ±2.15 ** | 0.197 |
| **3 – 4 dried pollen pellet put into cells processed with honey syrup and thickened (Exp 4)** | 11.0 ±2.00 *** | 0.200 | 17.0 ±2.43 *** | 0.309 | 54.2 ±7.66 *** | 0.986 | 52.2 ±8.02 *** | 0.950 |
| **3 – 4 newly collected pollen pellet put into cells (Exp 5)** | 24.0 ±3.61 *** | 0.437 | 100.0 ±4.64 **** | 1.820 | 194.6 ±25.76 *** | 3.540 | 239.2 ±24.36 **** | 4.352 |
| **3 – 4 freshly collected pollen pellet put into cells and thickened (Exp 6)** | 617.0 ±37.87 **** | 11.226 | 636.6 ±36.74 **** | 11.583 | 976.6 ±58.67 **** | 17.769 | 4492.6 ±362.02 **** | 81.743 |

Notes: I – 1st sampling; II – 2nd sampling; III – 3rd sampling; IV – 4th sampling; mean – arithmetic mean; SD – standard error of the mean; *$p$ <0.1; ** $p$ <0.02; *** $p$ <0.01; **** $p$ <0.001 in compare with control group; Exp 1–6 – experiment 1 – 6.

**Table 2** The effectiveness of different methods of getting bee bread with the help of an artificial honeycomb, n = 5.

| Index | The ways of getting bee bread | | | | | |
|---|---|---|---|---|---|---|
| | **Test (stimulation of bees to laying bee bread by thickening small portion of pollen pellet)** | | **Research 1 (single thickening of freshly harvested pollen pellet of 2/3 of the cells)** | | **Research 2 (single thickening of freshly harvested pollen pellet of 2/3 of the cells and processing their surface with honey)** | |
| | the number of cells filled with bee bread, item | the amount of bee bread received from one honeycomb (g) | the number of cells filled with bee bread, item. | the amount of bee bread received from one honeycomb (g) | the number of cells filled with bee bread, item | the amount of bee bread received from one honeycomb (g) |
| ***M* ±SD** | 4400.2 ±371.06 | 706.6 ±163.56 | 4750.2 ±227.44 | 1135.2 ±159.28 | 4886.4 ±219.30 | 1368.7 ±108.70* |
| ***min-max*** | 3162 – 5139 | 278.19 – 1205.06 | 4181 – 5255 | 711.82 – 1618.54 | 4274 – 5357 | 1073.54 – 1687.46 |
| **V%** | 18.86 | 51.76 | 10.71 | 31.38 | 10.04 | 17.76 |

Note: mean – arithmetic mean; SD – standard error of the mean; min, max – minimal and maximal measured values; V – coefficient of variation %; * $p$ <0.05 compared to the test group.

Bees mainly laid the bee bread reserves starting from the bottom sections of the artificial honeycomb located closer to the back wall of the beehive. Besides that, the bees domesticated the cells and laid the bee bread better on those sides of the artificial honeycombs which were oriented to the central part of the nests of honey bee colonies, namely to the hive entrance block. The cells, which the bees laid bee bread into, could be identified according to several features. Examining the honeycombs in the cells, we found freshly harvested pollen pellet. The bees often dipped into them and the shiny spew appeared on the surface of protein food where the process of forming the pellets of the bee bread was finished.

In the process of forming protein food reserves, the bees managed not only to store bee bread but also consumed it even from those cells in which the upper layer of the pollen the bees processed by honey. However, the bees mostly consumed the protein food for the needs of their colonies from those cells which haven't been filled up with pollen pellet completely. As a rule, the bees did not consume the bee bread from the central sections and they ate bee bread in small amounts from the peripheral zones. **Anderson et al. (2014)** found that the bees significantly prefer to consume fresh pollen stored for <72 h. The tendency to eat fresh vs. older pollen stores may involve the strength or type

of chemical signal emanating from stored pollen of variable age.

So, the workers on the artificial honeycombs to store protein food actively start to domesticate those cells which contain the first portions of the thickened freshly harvested pollen pellet. Processing of the cells by wax or honey syrup doesn't stimulate the bees to domesticate artificial honeycombs.

The sections of artificial honeycombs that were located closer to the back wall of the beehive were mostly the area for storing protein food. The bees gradually domesticate all the cells of the artificial honeycombs to process pollen pellet into bee bread. To make the bees lay and thicken pollen pellet it is necessary to fill the empty cells with freshly harvested pellets and thicken them not less than 4 times a day. It is not appropriate to use dried pollen pellet to stimulate the bees to lay bee bread into the cells of artificial honeycombs. The bees mostly take dried pellet out of the cells and unwillingly lay protein food reserves into them.

Taking into account that the most effective way of stimulating bees to store bee bread on the artificial honeycomb is the method of thickening of 3 – 5 pellets of freshly chosen pollen pellet in the cells, it was used in further researches. Besides that, we were interested in researching the question of bees' reaction to protein food if 2/3$^{rd}$ of the cells were filled with freshly harvested pollen pellets.

While researching the ethnology of bees that laid protein food it was found that they did not break pellets into pieces in the process of thickening pollen pellet. Thus, they do not add ferments into them. That is why we have decided to do additional researches with the help of artificial honeycombs. We have used two more methods in our research, except the method of stimulating bees, to store protein food by trampling down a small quantity of pollen pellet (test group). The first method was a single thickening of pollen pellet up to 2/3$^{rd}$ of the height of the cells without processing it (research 1). The second method included the processing of the upper layer by honey (research 2). These ways of getting bee bread by using artificial honeycombs checked the method of stimulating the bees to store protein food by thickening a small amount of freshly harvested pollen pellet in the cells and the process has been repeated 4 times after that. To do this research we have used 3 bee colonies each of which had 5 bees. The honeycombs in the nest were placed in the same area as in the previous research. During this research, we have recorded the number of cells with bee bread and identified the total weight of the pollen pellet we have got after the honeycombs have been in the nests of bee colonies for 2 weeks. The data received during these researches can be found in Table 2.

According to the intensity of laying protein food reserves into artificial honeycombs using different ways of stimulating bees to process pollen pellet into bee bread, it has been identified that the bee colonies of the test and research groups 1 and 2 had a different level of activity while doing this jobs. The common thing in the behavior of the bees in all the bee family groups was the thing that while laying bee bread and in the process of finishing its forming, the bees consumed protein food in small amounts. You could see that when the bees have finished processing upper levels of protein food by honey and started eating unripe bee bread the next day. The bees mostly consumed food from the cells of those sections which were located at the bottom of the sides of artificial honeycombs. The bees did not consume bee bread on the central sections of the honeycombs.

After 2 weeks, lots of sections that were located on the peripheral sides of artificial honeycombs had cells with a small amount of bee bread in them, and there were also the ones that appeared to be empty. The intensity of consuming protein food by bee colonies that belong to the same group was different. The bees of some colonies consumed less bee bread and some of them consumed more. The reason for such behavior of bees could not be identified. It is necessary to investigate these processes more thoroughly to solve this problem. It can be inferred that the intensity of consuming protein food depends on different factors. At first, it refers to the age of bee workers, their physiological state, the type and the amount of work that would be done, the quantity of unsealed brood in the nests of bee colonies, etc.

It has been proved that the bees of test and research groups used a different number of cells in artificial honeycombs to process pollen pellet into bee bread depending on the ways of stimulating bees to store protein food. Thus, at the end of the experiment, i.e. 14 days after the protein food reserves have been stored, we have found that 80.1% of the cells on artificial honeycombs were completely or partially filled by bee bread. Co-boundary data (Lim) of this group were between 3162 and 5139 cells. The input of protein food that varied according to the intensity of the process influenced the variation index, which was 18.9% in this group that shows the instability of this index because of the influence of different factors. In general, taking into account the fact that the bees have partially consumed protein food in the cells, single extraction of bee bread in terms of one artificial honeycomb in this group was 706.6 g, with a minimum of 278.2 g and a maximum one of 1205.1 g (Lim), whereas the variation index was 51.76%. It's necessary to mention that in this group, in which the bee workers did not take part in storing bee bread, protein food was consumed to the biggest extend. This indicates that bee workers use freshly harvested pollen pellet for their own needs at the time when it is actively produced. To decide, which the most attractive food for the bee family is pollen pellet or bee bread, it is necessary to thoroughly investigate the behavior and nutrition of bees.

In the first research group, where a single thickening of the artificial honeycombs section cells with freshly harvested pollen pellet was used, alongside the processing of bee bread, the bees consumed protein food in small amounts. This process was less active than in the test group, though. During the two weeks that were spent on keeping artificial honeycombs of bee colonies 86.4% of section cells were filled with bee bread. If to compare this method with the test group, the bees involved 7.9% cells more for storing bee bread. Because the bees were less active in consuming protein food from section cells, the amount of bee bread we got from one artificial honeycomb was 1135.2 g on average, which is 60.6% more than in the test group. However, if to calculate the number of cells involved and the amount of bee bread received, the difference between the test and the first research group was improbable ($p$ >0.1).

While thickening pollen pellet in the cells of artificial honeycombs with processing food by honey (research 2) the bees worked on storing bee bread more actively. In this group, the percentage of the cells filled with bee bread was almost 90%. Unlike the test group, the bees of the colonies of research group 2 used 11% more cells for storing protein food whereas in research group 1 the percentage was 2.9%. However, alongside this, the consumption of protein food in this group was minimum, unlike in the methods previously used. This influenced the average amount of bee bread received in terms of one artificial honeycomb. Thus, according to the results of the records we got out of each artificial honeycomb, we got 1368.7g in research group 2, which is 93.7% more than in the test group ($p$ <0.05) and was 20.6% higher than the index of research group 1 (the difference is improbable, $p$ >0.1). In research group 2 the co-boundary data were less (according to the number of the cells filled with bee bread as well as with the total amount of the pollen pellet received (Lim) if to compare other methods of preparing protein food to the processes of conservation.

By industrially maintaining bee-keeping, bee-keepers get not only honey from the bee colonies but other goods as well. It widens the range of apicultural products in the market and promotes enterprises' rise in profitability. At the same time, despite the increasing needs for separate kinds of apicultural goods, bee-bread in particular, their overall production level is very slight because of the lack of modern and effective technologies and equipment. So, to get bee-bread, bee-keepers use methods that are based on the destruction of wax combs **(Lebedev and Kubrak, 1997; Nekrashevych, Kashyryn and Vynokurov, 2002; Psheslavskyi, 2010)**. Nowadays to get bee-bread, honeycombs made of artificial materials have been designed **(Brovarskyi, Velychko and Kolesnik, 2013; Brovarskyi, Brindza and Velychko, 2015; Velychko, Brovarskyi and Brindza, 2011)**; the overall study of the morphology of the pellets **(Velychko, Brovarskyi and Brindza, 2013)**, biochemical composition, and microbiology of this variety of the product have been also conducted **(Brovarskyi et al., 2013; Brovarskyi and Velychko, 2014; Hryniewicka et al., 2016; Ivanišová et al., 2015; Kubík et al., 2017; Shevtsova et al., 2016)**. However, bees unwillingly domesticate the cells of such honeycombs, and the ways of regulating the processes of forming protein food reserves in them have not been designed at all. That is why the investigation of the behavior of bees and working out the ways of stimulating bee colonies to lay protein food are of vital importance from a theoretical as well as practical point of view.

To decrease the pressure of involving the bees in wax building activity, it needs to avoid problems of damaging frames (transporting bee colonies, pumping out honey, etc.), cut costs on buying empty honeycombs, effectively use the biological potential of bee colonies while getting a different kind of goods and improve their quality, prevent affection and spread of diseases, artificial materials for producing honeycombs are widely implemented nowadays. Among them are nest honeycombs, plastic empty honeycombs, plastic honey cells, honeycombs for getting even-aged brood in the artificial raising of queen bees, balls of queen-bee cells made of polymer materials, etc. **(Brovarskyi et al., 2014; Brovarskyi et al., 2017)**. To make bees domesticate artificial honeycombs or cells (balls of queen-bee cells, Dombrovskyii honeycombs), different ways of activating this process are used (reducing the number of nests, processing the elements or cells of honeycombs by wax or carbohydrates, etc.) **(Brovarskyi et al., 2013; Brovarskyi and Velychko, 2014; Brovarskyi, Velychko and Brindza, 2015; Brovarskyi et al., 2015)**. Nowadays, because of the lack of knowledge of honeybees' biology, there is a need for doing research on stimulating bee families to domesticate artificial honeycombs or their separate elements and design manufacturing technology of apicultural goods on their basis, bee-bread in particular. We have emphasized this particular problem in our investigations.

The aim and the task of the research were to investigate the ethnology of bees by different ways of stimulating them to form protein food reserves in artificial honeycombs. According to the set aim, the following things had to be done defining the effectiveness of laying bee bread by bees using different ways of preparing containers of artificial honeycombs; research the intensity of forming protein food reserves using different ways of stimulating bees.

## CONCLUSION

The most effective way of stimulating bees to process pollen pellet into bee bread is its single thickening in artificial honeycombs with the following processing of the upper layer of food with honey. Such a method encourages the bees to form bee bread reserves and lessens their activity of consuming protein food from the cells of artificial honeycombs. It is likely that the processing of the thickened pollen pellet with honey oppresses the bees' necessity to consume protein food from the filled cells and redirects them to other honeycombs of the bee family nest which has areas filled with bee bread reserves.

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Experiments with hives were carried out on hives owned by the author of the publication, Mr Serhii Velychko, who is an officially registered beekeeper in the National Organization of Beekeepers of Ukraine.

**Ethical Statement:**
Proposed experiment on hives was discussed by the Ethics Committee of the Ministry of Agriculture of Ukraine without comment, because he did not use in the experiments any techniques or synthetic chemicals that caused health problems or death of bees.

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# EVALUATION OF NUTRITIONAL COMPOSITION AND SENSORY PROPERTIES OF CHEESE, CHEESE SPREADS AND TRADITIONAL BUTTER FROM SLOVAK PRODUCTION

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## ABSTRACT

Cheese production is associated with the analysis of its nutritional composition as well as with the sensory evaluation of the acceptance of the products. The paper is aimed at the determination of basic chemical parameters such as the content of proteins, fat, dry matter, salt and, pH and also on the evaluation of sensory properties of hard cheeses by the sensory panel. The GC-MS analysis of cheeses, cheese spreads, and traditional butter was performed to evaluate the aroma profile of the dairy products analyzed. The dry matter in the analyzed cheeses varied between 56.75 and 71.83%, the fat content varied from 18.73 to 30.83%, and the salt from 1.21 to 2.61%. The presence of proteins was found between 27.76 and 32.61% and the pH of the cheeses ranged from 5.21 to 6.01. The results of the sensory analysis were processed using a PCA map. The results showed that sample no. 5 was rated within all attributes as the best. Sample 3, 2, and 1 followed. Sample 4 received the lowest score. The volatile aromatic compounds that contribute to a taste perception were analyzed by GC-MS in dairy products. The aroma profile was built by the volatile compounds that belonged to chemical families of alcohols, aldehydes, ketones, esters, amides, amines, imines, and terpenes. In particular, cheeses contained mostly alcohols (3-methyl-1-butanol), aldehydes (3-methylbutanal and benzaldehyde), and ketones (acetoin, 2,3,3-trimethylcyclobutanone, 2-heptanone, 2,3-butanedione, acetone, 2-butanone, 2-nonanone, 2- pentanone). The spreads most often contained alcohols (represented by 2-methyl-1-butanol, 3-methyl-1-butanol and cyclopropane-1,2,3-d3-methanol), aldehydes (2-methylbutanal, 3-methylbutanal, benzaldehyde) and ketones (acetoin, 2-heptanone, 2-pentanone, 2-butanone and 2,3-butanedione).

**Keywords:** cheese; cheese spread; butter; chemical composition; sensory analysis; aroma profile

## INTRODUCTION

Milk and dairy products such as cream, butter, yogurt, kefir, and cheese have been consumed around the world for millennia. Milk and other dairy products are indispensable for daily life, and their aroma characters are unique and provide great value in many processed foods, candies, cookies, and others **(Tamura et al., 2021)**. Due to the diverse options in ingredients, recipes, and processing conditions, manufacturers have many possibilities for producing cheeses and spreads with different physicochemical properties, leading to different flavours, functional properties, and uses according to consumer preferences **(Hamad and Ismail, 2013; Visioli and Strata, 2014)**. The nutritional composition of the cheese varies depending on the type of cheese, but in general, cheeses are high in fat, saturated fat, and cholesterol. The protein content of cheese ranges from 3 to 40% **(O'Callaghan, O'Connor and O'Brien, 2017)**. An important component of cheese is also salt, which has three main functions in the cheese: it acts as a preservative, contributes directly to the aroma, and is a source of sodium in the diet. Together with the required pH, water activity, and redox potential, the salt helps to preserve the cheese by minimizing degradation and preventing the growth of pathogens **(Guinee, Carić and Kalab, 2004)**. The raw flavour is obtained from a wide variety of compounds that results from the hydrolysis or metabolism of carbohydrates, proteins, and fats, along with compounds added during processing, ripening, or directly from milk **(Kilcawley, 2017; Le Quéré, 2011)**. The important role in the development of dairy product aroma play especially the degradation of proteins (caseins) that leads to the formation of the most important flavour components which contribute to the sensory perception of dairy products. Caseins are degraded into peptides and amino acids and the latter are major precursors for volatile aroma compounds **(Smit et al., 2005)**. The volatiles responsible for the typical aroma of cheese is produced mainly by lipolytic and proteolytic pathways and by the

metabolism of lactose, lactate, and citrate **(Bertruzzi et al., 2018)**. Perception of cheese flavour is due to the combined detection of aroma and taste compounds present in cheese by their respective sensory receptors. The flavour of the cheese is related to milk quality, processing operation, microbial activity, and added flavouring agents such as spices and herbs **(El-Sayed and Youssef, 2019; Smit et al., 2005; Bertruzzi et al., 2018; Drake, 2007)**.

The basic nutritional composition of the cheese can be determined by traditional chemical reference methods for measuring fat (e.g. ether extraction), protein content (Kjeldahl), moisture (gravimetric determination by oven drying), and salt (titration with silver nitrate according to Volhard), which are accurate but also too slow and impractical for rapid analyzing of large numbers of cheese samples in laboratories. At present, fast process and more cost-effective methods are used, such as near-infrared spectrometry (NIR), mid-infrared spectroscopy (MIR), or near-infrared transmittance spectroscopy (NIT) for the simultaneous determination of multiple chemical parameters (fat, protein, salt, and moisture) **(Margolies and Barbano, 2018; Alinovi et al., 2019; Visconti et al., 2020; Madalozzo et al., 2015; Manuelian et al., 2017)**.

Aroma profile and volatile compounds analysis in food is possible by various olfactometry methods such as Charm analysis, AEDA (aroma extract dilution analysis), odour unit, and other methods **(Tamura et al., 2021)**. In the last decades, volatile compounds responsible for the aroma were identified with the use of gas chromatography-based techniques. The volatile profile of cheese and dairy products is determined especially by an electronic nose (e-nose), gas chromatography coupled with mass spectrometry (GC-MS). E-nose is an odour detection device using an array of sensors **(Delgado-Rodríguez et al., 2012; Schlossareck and Ross, 2019)**. Electronic nose with sensors is used in many industries and for various applications such as quality control, process monitoring, shelf-life evaluation, origin or authenticity assessment **(Buratti et al., 2018; Li et al., 2017)**. Gas Chromatography-Mass Spectrometry (GC-MS) is the reference method in the analysis of volatile organic compounds (VOCs) in the field of environmental, food, flavour and fragrance, medical and forensic sciences. The technique allows determining volatile compounds present at trace levels that have a low perception threshold but significantly contribute to the flavour profile of food **(Bertruzzi et al., 2018)**.

Dairy products and their aroma profile and flavour have been intensively studied. **Niimi et al. (2014)** reported that the same aromatic active compounds can be present in several types of cheese however they usually differ by varying the amount of a particular ingredient in such a product.

The paper aims to evaluate selected chemical and physical parameters and sensory properties of cheeses and characterization of the aroma profile of cheeses, cheese spreads, and traditional butter of Slovak origin.

### Scientific Hypothesis

Cheeses were studied to evaluate the consistency of chemical composition, acceptability of cheeses by a sensory panel. An exploration of cheeses, cheese spreads, and traditional butter by GC-MS was performed to confirm the differences in the aroma profile.

## MATERIAL AND METHODOLOGY

### Samples

Cheeses and cheese spreads and butter were obtained from cheese producer Dubník, the Slovak Republic in three different batches. Samples of five types of hard cheese differ in flavouring spices and length of ripening. Hard cheeses were analyzed for basic physical and chemical composition by NIR and sensory panel. 11 samples of dairy products (including above mentioned 5 types of hard cheese, 5 types of cheese spreads, and one type of traditional butter) differed in the technological process of their production ). All 11 samples were analyzed by GC-MS for aroma profile determination. The hard cheese samples were analyzed by NIR and sensory panel.

All analyzed samples are characterized as follows: no. 1 – hard cheese with chili flakes „Ľahké pierko s čili“; no. 2 – 6 months rippening hard cheese „Kryštálová Kamenica“; no. 3 – 3 months rippening hard cheese „Sýta Rubaň“; no. 4 – hard cheese with chives „Jemný Dubník pažítka“; no. 5 – hard cheese with 4 spices „Ľahké pierko 4 korenia“; no. 6 – cheese spread with smoked cheese „Syrová nátierka s údeným syrom“; no. 7 – cheese spread with onion „Syrová nátierka sladká cibuľka“; no. 8 – cheese spread with hot paprika „Syrová nátierka pálivá paprika“; no. 9 – cheese spread with sweet paprika „Syrová nátierka dolniacka paprika“; no. 10 – cheese spread with emmental cheese „Syrová nátierka Ementálček“; no. 11 – traditional butter „Tradičné maslo“.

### Chemicals

The analyses did not require special chemicals. We used deionized water and ethanol (96%, p.a., nondenatured, Centralchem Ltd., Slovakia) for washing the equipment.

### Animal and Biological Material

No animal and biological material was used.

### Instruments

NIR spectrometer, type MPA (Bruker Optik GmbH, USA) was applied for the determination of the basic chemical composition of cheeses. Selected chemical parameters (dry matter, fat, salt, pH, proteins) were measured at the AgroBioTech SPU research center. The cheese samples were homogenized into a fine powder, then transferred to a Petri´s dish (type Duraplan) and placed in the apparatus. The NIR instrument was calibrated by O.K. SERVIS BioPro, Ltd. for hard cheeses in the range as follows: fat (5 – 43.6%), NaCl (0.04 – 2.85%), proteins (13.6 – 37.10%), dry matter (24.2 – 72.6%) and pH (4.93 – 6.35).

The aroma profile was analyzed by gas chromatography coupled to mass spectrometry (GC-MS). Agilent 7890B gas chromatograph coupled with an Agilent 5977A mass spectrometer (Agilent Technologies Inc., Palo Alto, CA, USA) was used. Separation was performed by a DB-WAXms column (30 m × 0.32 mm × 0.25 μm; Agilent Technologies) working with the temperature program and MS conditions according to **Sádecká et al. (2014)**. Identification of the compounds was performed by matching the measured peaks with Kovats´ retention indices with NIST library (The National Institute of Standards and Technology Library) by software Alpha Soft V14 (Alpha M.O.S.) **(Štefániková et al., 2019)**.

## Description of the Experiment

### Sample preparation:

The samples were used after cutting/homogenization into appropriate size and by adjusting the temperature (for NIR and sensory panel analysis).

NIR analysis required homogenization of the cheese samples and then the samples were put into glass Petri´s dishes (type Duraplan) in amount 20 g.

For GC-MS analysis, the cheese samples were cut into small pieces. 4 g of each sample (cheese, spread, or butter) was weighed into clean 20 mL headspace vials covered by a magnetic cap with a septum. The samples prepared were stirred at 50 °C or 15 minutes on a shaker included as a part of the GC headspace autosampler (Combi Pal, Alpha M.O.S.).

Sensory analysis was performed in the sensory laboratory. The equipment of the sensory laboratory and the course of the evaluation were performed based on the **ISO 8589 (2007)** standard. The cheese analysis involved 6 experienced experts. The cheese temperature was adjusted to 10 °C and the room temperature was 21 °C. The organoleptic properties of the individual samples were evaluated using a 1 – 9 point test (1 – insufficient to 9 – very intense). The sensory panel evaluated the attributes: appearance, overall odour, aroma odour, consistency, overall taste, flavour, overall acceptability.

**Number of samples analyzed:** 3 batches of 11 samples

**Number of repeated analyses:** 3 times repeated measurements

**Number of experiment replication:** 2 parallel replications

## Statistical Analysis

Statistical analysis was performed in the program Excel with XLSTAT (vs. 2020.3.1) statistical software for Excel. Chemical parameters were analyzed with descriptive statistics (means, standard deviations, minimum, maximum values) and results were visualized in boxplots. Data from the sensory panel were tested for normality with the Shapiro-Wilk test on a significance level $p = 0.05$. A graphical evaluation was performed with the use of principal component analysis (PCA) and visualized in a biplot map, which presents individual observations, as well as variables examined in one plot. Data obtained in GC-MS analysis – a type of chemicals and area percentage of aromatic compounds were visualized in XLSTAT as a heat map. The columns of the data matrix were re-ordered according to the hierarchical clustering result, putting similar observations close to each other. The result is a heat map with a double dendrogram in a vertical and horizontal position.

## RESULTS AND DISCUSSION

The results obtained can be divided into three parts – chemical composition, sensory evaluation, and aroma profile.

## The basic chemical composition

The chemical composition results are summarized in Table 1 and Figure 1. The dry matter in the analyzed cheeses ranged from 54 to 68.4%. In general, it can be stated that cheeses containing herbs had a lower salt content compared to ripening cheeses such as “Kryštálová Kamenica” or “Sýta Rubaň”.

The fat content ranged from 17.42 to 30.88% and the salt from 1.2 to 2.1%. Proteins accounted for 27.4 to 32.43%. The acidity of the cheeses, characterized by a pH value, was in the range of 5.04 to 6.03. The highest values in the most of parameters were determined in Sýta Rúbaň cheese, except for the protein content.

The lowest values of these parameters were found in cheese with 4 types of spices, again except for protein content. A visual expression of the consistency of the individual cheese chemical composition data is shown in Figure 1. In our case, the results indicate the consistency of the chemical composition of the samples over time. Since the data were collected from 3 series of measurements during the 3 months (the cheese producer provided samples at monthly intervals), the results reflect the stability of the chemical composition of the products over time. As can be seen in the case of dry matter content, the closest data were measured for the “Kryštálová Kamenica” and “Ľahké pierko s čili” cheeses. “Kryštálová Kamenica” is a cheese that has been ripening for 6 months, therefore the composition of this cheese becomes stable. Cheeses such as “Sýta Rubaň” and “Ľahké pierko 4 korenia” had a higher dry matter content, which may be related to seasonal changes in milk parameters. In the case of fat content, the closest data were observed in the “Sýta Rúbaň” cheese and the largest variance was recorded in the “Ľahké pierko 4 korenia”. The salt as a parameter showed a relatively constant proportion in cheeses such as “Jemný Dubník pažítka“, “Ľahké pierko s čili” and “Ľahké pierko 4 korenia”. We found the highest variability of the salt content values in the “Sýta Rubaň” cheese. The pH values were most balanced in the “Kryštálová Kamenica” and “Jemný Dubník pažítka”. On the other hand, “Ľahké pierko 4 korenia” had the highest variance in pH values. In the protein content, we recorded the most consistent data for all analyzed cheeses.

Different types of cheese were analyzed in literature to evaluate basic chemical composition. Ricotta cheeses were analyzed by **Madalozzo et al. (2015)** NIR method for protein and fat content. The average fat content was 10.63%, protein 12.37%, and moisture 70.23%. **Margolies and Barbano (2018)** analyzed Cheddar cheeses using the MIR technique. The average fat content was 34.0%, the protein content 24.0%, the moisture content 36.82% and the salt 1.8%. **Manuelian et al. (2017)** analyzed commercial cheeses using the NIT technique. The moisture, fat, and protein contents averaged 43.24 ±0.97%, 27.24 ±0.47%, and 24.87 ±0.54%. **Jo et al. (2018)** analyzed the sensory and chemical properties of Gouda cheeses. All cheeses had a water content of less than 45%, a fat content of more than 46%, and a pH value in the range of 4.9 to 5.6. The salt content of the cheese differs significantly from the type of cheese, ranging from about 0.5 – 0.7% in acidic cottage cheese and Emmental cheeses to about 4-6% in pickled cheeses such as Domiati and feta **(Guinee, 2004)**.

Kuflu cheese, a Turkish mould-ripened variety was studied by **Hayaloglu et al (2008)**. This type of cheese was characterized by moisture content 49.97%, 12.18% fat content, 37.84% total protein contents, and pH 6.29. Our results corresponded most to the chemical parameters in ripening Cheddar cheeses.

**Table 1** Chemical composition of cheese samples.

| | Sample | Dry matter (%) | | Fat (%) | | NaCl (%) | | pH | | Proteins (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | x̄ | *SD* | x̄ | *SD* | x̄ | *SD* | x̄ | *SD* | x̄ | *SD* |
| 1 | Ľahké pierko s čili | 60.65 | 0.42 | 20.56 | 0.21 | 1.72 | 0.07 | 5.61 | 0.02 | 31.75 | 0.01 |
| 2 | Kryštálová Kamenica | 65.98 | 0.05 | 25.06 | 0.67 | 1.90 | 0.11 | 5.86 | 0.02 | 32.61 | 0.18 |
| 3 | Sýta Rúbaň | 71.83 | 3.43 | 30.83 | 0.05 | 2.61 | 0.51 | 6.01 | 0.02 | 31.22 | 0.06 |
| 4 | Jemný Dubník pažítka | 62.66 | 0.80 | 29.02 | 0.40 | 1.45 | 0.04 | 5.50 | 0.06 | 27.76 | 0.36 |
| 5 | Ľahké pierko 4 korenia | 56.75 | 2.75 | 18.73 | 1.31 | 1.21 | 0.01 | 5.21 | 0.17 | 30.15 | 0.09 |

Note: x̄ – mean, *SD* – standard deviation.

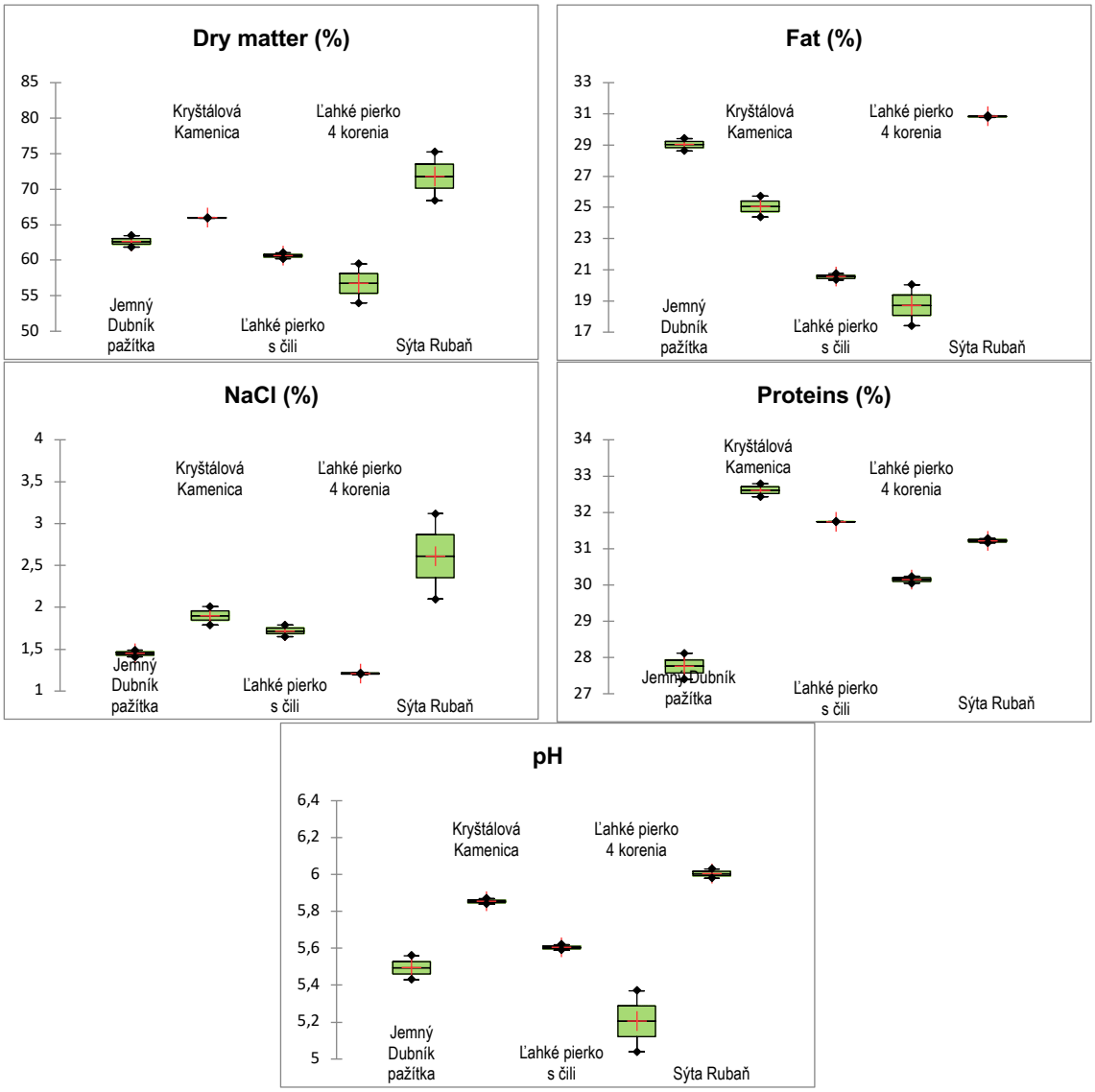


**Figure 1** Boxplots of chemical composition variability. Note: + Mean ♦Minimum/Maximum.

## Sensory analysis

Sensory analysis was performed in the sensory laboratory. Trained sensory panels are important tools for assessing the quality of food and non-food products **(Tomic et al., 2010)**. Sensorial features of cheese are more or less a consequence of the combined actions of all steps involved in its processing **(Kongo and Malcata, 2015)**. The organoleptic properties of the individual samples were evaluated using a 1 – 9 point test (1 – insufficient to 9 – very intense). The sensory panel evaluated the attributes: appearance, overall aroma, aroma odour, consistency, overall taste, flavour, overall acceptability. The data obtained from the sensory evaluation were processed and statistically tested by the Shapiro-Wilk test for normality at a significance level of P=0.05. A principal component analysis (PCA) was used to determine element variability and visualization of sensory analysis data. The result is PCA biplot (Figure 2). For a better interpretation of the results obtained using the statistical method of analysis of the main components, we used visualization using the so-called biplot. This PCA map represents a biplot that shows simultaneously our observations as well as the variables examined in a single graph. The coordinate axes (x and y) of the biplot explain 79.52% of the total variation of observation.

This type of graph allows a better interpretation of the share of individual characteristics in the main components. A PCA biplot shows both PC scores of samples (dots) and loadings of variables (vectors). The further away these vectors are from a PC origin, the more influence they have on that PC.

In our biplot, the most important variables in the sensory evaluation were overall acceptability and appearance, almost no influence had consistency and aroma odour.

The results of the sensory analysis (Figure 2) showed that sample no. 5 "Ľahké pierko 4 korenia" was rated within all attributes as the best. Sample 3, 2, and 1 followed. Sample 4 "Ľahké pierko s pažítkou" received the lowest score. **Toro, Valencia, and Molina (2016)** evaluated nutritional parameters and sensory features of processed cheese. For the development of this type of food product, it is necessary to consider nutrition value for different groups of people and its versatility and ease of consumption.

## Aroma profile

The GC-MS method is often used to characterize the aromatic profile of dairy products. We have found the presence of the following aromatic compounds. The volatile compounds that were identified belong to several chemical groups: alcohols, aldehydes, ketones, carboxylic acids, esters, amides, amines, imines, and terpenes (Table 2a and 2b). Data obtained by GC-MS analysis and area percentages were visualized by heat map (Figure 3). The vertical dendrogram represents volatile compounds in the samples and the horizontal dendrogram the reordered samples. The

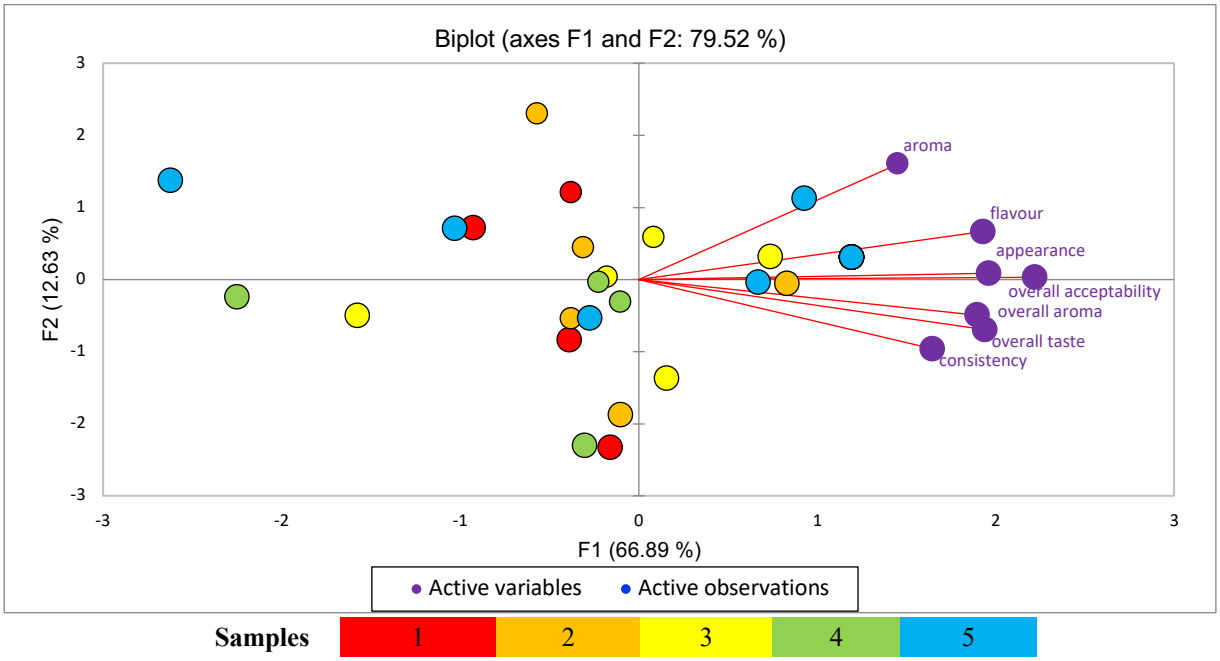


**Figure 2** Sensory analysis by the sensory panel. Note: no. 1 – hard cheese with chili flakes „Ľahké pierko s čili"; no. 2 – 6 months rippening hard cheese „Kryštálová Kamenica"; no. 3 – 3 months rippening hard cheese „Sýta Rubaň"; no. 4 – hard cheese with chives „Jemný Dubník pažítka"; no. 5 – hard cheese with 4 spices „Ľahké pierko 4 korenia".

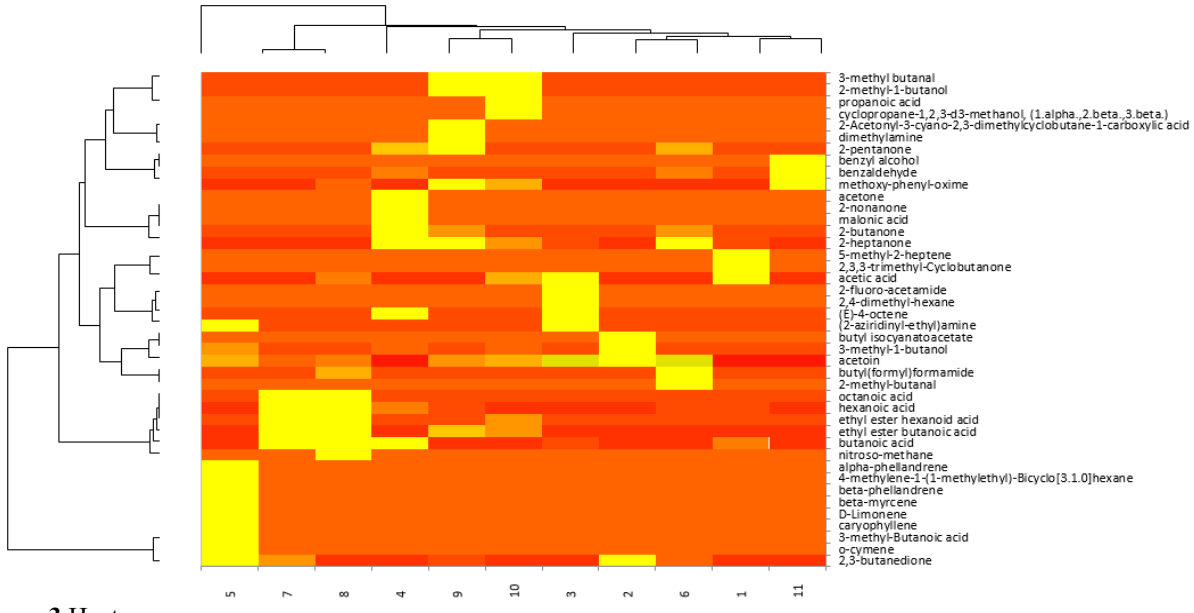


**Figure 3** Heat map.

heat map reflects the range of area percentages as well as the proportion of specific volatile aromatic compounds in the individual samples. The clustering showed that the compounds are divided into four groups (left dendrogram) according to the observed similarities. The pattern inside the map shows the groups of compounds described above as follows: the first cluster is represented by the compounds 5-methyl-2-heptene to 2-methyl-butanal, the second cluster is represented by compounds from acetone to propanoic acid. The third group can be clearly distinguished by the presence of 2-pentanone to butanoic acid. This means agglomeration appears to have moderate amounts of most of the compounds. Compounds from D-limonene to 2,3-butanedione represent group four. Horizontal clustering revealed the fact that almost no similarities were observed between the samples, based on the composition of the aroma profile and the amount of volatiles.

***Cheese aroma profile***

Aldehydes (specifically 3-methylbutanal and benzaldehyde) were an important group of volatile compounds in cheeses with a presence in several samples. Due to their low odour detection thresholds, these compounds contribute significantly to the aroma features of the cheeses. Among the carbonyl compounds, ketones were also represented, namely acetoin, 2,3,3-trimethyl-cyclobutanone, 2-heptanone, 2,3-butanedione, acetone, 2-butanone, 2-nonanone, 2-pentanone. Alcohols also have a low odour detection threshold, which gives dairy products herbal, woody and fatty tones. We identified alcohols represented by 3-methyl-1-butanol in our cheese samples. Terpenes (specifically β-phellandrene, α-phellandrene, β-myrcene, o-cymene, D-limonene, and caryophyllene) were also identified in sample 5 (cheese "Ľahké pierko 4 korenia") to confirm the spicy aroma of the sample. Carboxylic acids such as acetic acid, butanoic acid, caproic acid (hexanoic acid), heptanoic acid, malonic acid, and 3-methyl-butanoic acid were present in the cheese samples.

Sample 1 with chive flavour had the largest proportion of acetic and butyric acid. In sample 2 with chili flavour, acetoin was present in the highest concentrations, followed by 3-methyl-1-butanol, 2,3-butanedione. Sample 3 was characterized by the highest proportion of acetoin and acetic acid, followed by (2-aziridinylethyl)amine. In sample 4 (Kamenica matured Parmesan-style cheese) the most abundant was butanoic acid, followed by acetone.

In the last sample were dominant 2,3-butanedione, (2-aziridinylethyl) amine, acetoin, D-limonene, followed by terpenes such as α-phellandrene, caryophyllene, o-cymene, β-phellandrene, which originated from the addition of spices.

Cheddar cheeses were analyzed on aroma profile by GC-MS by the authors **Chen et al. (2020)**. They found forty-nine flavour compounds in the samples, including 11 acids, 5 aldehydes, 8 alcohols, 8 ketones, 9 esters, 3 aromatic hydrocarbons and heterocyclic compounds, and 4 other compounds. The active ingredients were mainly aldehydes (2-methylbutanal, 3-methylbutanal, benzaldehyde), ketones (2-heptanone, 2-octanone), carboxylic acids (butyric, isovaleric acid, octanoic acid, n-decanoic acid, hexanoic acid), and esters (ethyl hexanoate, ethyl acetate, ethyl butyrate, and butyl acetate). The authors identified, according to the value of the aroma intensity (AI values), the compounds with the highest aromatic activity in the cheese. Several compounds have relatively high aroma intensity values such as butyric acid (cheesy/rancid), ethyl acetate (fruity/sweet), 2-heptanone (cheesy/spicy/fruity), ethyl butyrate (fruity/sweet), benzaldehyde (almond), 3-methylbutanal (nutty/malty), 2-methylbutanal (nutty/chocolate), and 3-methylbutanoic acid (cheesy/sweaty/rancid). Of these, the odours of 3-methylbutanal and 2-methylbutanal were rated by the sensory evaluation panel as pleasantly nutty, and the almond odour imparted by benzaldehyde was also considered to be a good nutty flavour. **Štefániková et al. (2019)** analyzed by GC-MS traditional slovak „Parenica" cheese. A Parmesan and parmesan-style cheese aroma were studied by **Barbieri et al. (1994)**, **Moio and Addeo (1998)**. The backbone of Grana Padano cheese aroma seemed to consist of carboxylic acids and 14 potent neutral odourants imparting fruity, green, nutty, and coconut notes. The authors reported that the concentration of volatile components responsible for the fruity and green notes was inversely proportional to the length of ripening, whereas the concentration of volatile agents with spicy, nutty, and earthy notes tended to increase during maturation. Bryndza cheese aroma profile was studied by **Sádecká et al. (2014)**. The overall aroma of Bryndza cheese was found to be formed by the following volatile components: esters, alcohols, aldehydes, ketones, fatty acids, and hydrocarbons. Nineteen of twenty-seven aroma-active compounds were identified. Mascarpone cheese aroma was analyzed by **Capozzi et al. (2020)** and the volatile compounds contributing to aroma were classified into 9 chemical classes (9 ketones, 5 alcohols, 4 organic acids, 3 hydrocarbons, 2 furans, 1 ester, 1 lactone, 1 aldehyde, and 1 oxime).

The most abundant compounds were ketones (especially 2-heptanone and 2-pentanone), then 2-propanone, 2-nonanone, 2-butanone, 1-pentanol, 2-ethyl-1-hexanol, furfural, and 2-furan methanol. **Thomsen et al. (2014)** studied different French commercial cheeses belonging to the same type: non-cooked semi-hard cheeses, including famous types like French Port Salut or Gouda. The volatiles found in the samples belonged to sulfur compounds (e.g. dimethyl disulfide, described as cauliflower, garlic flavour), alcohols (3-methyl butane-1-ol was found in the greatest amount and this alcohol has been described in cheeses with a fruity aroma **(Pérès, Viallon and Berdagué, 2001)**, aldehydes (e.g. 3-methylbutanal, responsible for cheesy aroma), ketones (the most abundant ketone was 3-hydroxybutan-2-one or acetoine, perceived alone with a dairy aroma), carboxylic acids (the most abundant carboxylic acid was 3-methylbutanoic acid, described as a potent odourant in goat cheese flavour **(Poveda et al., 2008)**, esthers (e.g. ethyl acetate and ethyl propanoate), lactones and furans.

Our results corresponded more closely with analyzes of the Cheddar cheeses, which belong to ripening cheeses, and therefore we also found these compounds in our samples of ripening cheeses. The similarities in dominant compounds were also found with non-cooked semi-hard cheeses.

**Table 2a** Cheese aroma profile.

| No. | Compound | Percentage area | | Aroma |
|---|---|---|---|---|
| | | $\bar{x}$ | *SD* | |
| 1. | acetic acid | 20.82 | 1.86 | sour fruity |
| | butanoic acid | 5.41 | 0.74 | unpleasant |
| | 2,3,3-trimethyl-cyclobutanone | 1.99 | 1.27 | unknown |
| | 5-methyl-2-heptene | 1.64 | 0.46 | nutty |
| | 2-heptanone | 0.76 | 0.20 | cheesy |
| | hexanoic acid | 2.85 | 0.40 | fatty |
| 2 | 2,3-butanedione | 12.68 | 3.16 | buttery |
| | 3-methyl-butanal | 3.94 | 1.67 | aldehydic |
| | acetoin | 26.3 | 3.48 | buttery |
| | 3-methyl-1-butanol | 9.79 | 2.13 | fermented |
| | butyl-isocyanatoacetate | 3.76 | 0.26 | sulfuric |
| 3 | (2-aziridinylethyl)amine | 16.46 | 6.58 | unknown |
| | acetic acid | 22.28 | 2.09 | sour fruity |
| | 2-fluoro-acetamide | 2.35 | 1.17 | unknown |
| | acetoin | 20.52 | 17.50 | buttery |
| | butanoic acid | 1.30 | 0.20 | cheesy |
| | 2,4-dimethyl-hexane | 0.58 | 0.12 | unknown |
| | (E)-4-octene | 1.28 | 0.21 | unknown |
| | 2-heptanone | 0.65 | 0.14 | cheesy |
| 4 | acetone | 11.15 | 1.84 | fruity |
| | 2-butanone | 13.53 | 8.02 | ethereal |
| | malonic acid | 0.76 | 0.04 | odourless |
| | malonic acid | 1.11 | 0.65 | odourless |
| | 2-pentanone | 4.44 | 0.14 | fruity |
| | 3-methyl-1-butanol | 0.45 | 0.21 | fermented |
| | butanoic acid | 17.38 | 1.84 | cheesy |
| | (E)-4-octene | 0.84 | 0.19 | unknown |
| | 2-heptanone | 8.78 | 2.19 | cheesy |
| | benzaldehyde | 0.66 | 0.25 | almond |
| | hexanoic acid | 8.45 | 0.38 | fatty |
| | 2-nonanone | 3.42 | 0.84 | fruity |
| 5 | (2-aziridinylethyl)amine | 14.36 | 1.94 | unknown |
| | 2,3-butanedione | 18.73 | 6.34 | buttery |
| | acetoin | 15.28 | 2.9 | buttery |
| | 3-methyl-1-butanol | 1.59 | 0.24 | fermented |
| | butanoic acid | 0.26 | 0.02 | cheesy |
| | 3-methyl-butanoic acid | 1.23 | 0.21 | sour fruity |
| | β-phellandrene | 1.86 | 0.29 | minty |
| | 4-methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane | 1.37 | 0.28 | woody |
| | β-myrcene | 0.61 | 0.05 | spicy |
| | α-phellandrene | 6.16 | 0.62 | terpenic |
| | o-cymene | 2.01 | 0.27 | unknown |
| | D-limonene | 13.59 | 0.88 | citrusy |
| | caryophyllene | 4.15 | 0.23 | spicy |

Note: no. 1 – hard cheese with chili flakes „Ľahké pierko s čili"; no. 2 – 6 months rippening hard cheese „Kryštálová Kamenica"; no. 3 – 3 months rippening hard cheese „Sýta Rubaň"; no. 4 – hard cheese with chives „Jemný Dubník pažítka"; no. 5 – hard cheese with 4 spices „Ľahké pierko 4 korenia".

***Cheese spreads aroma profile***

Processed cheese is an attractive product that enjoys great popularity. The basic raw material for processed cheese and spreads are natural cheeses and other dairy materials and non-dairy raw materials, as well as emulsifying salts **(Černíková et al., 2017)**.

These components contribute to the aroma of the products. The aroma profile of the cheese spreads in our study was characterized by the following aromatic compounds.

Oxygen derivatives of hydrocarbons such as alcohols (represented by 2-methyl-1-butanol, 3-methyl-1-butanol, and cyclopropane-1,2,3-d3-methanol), aldehydes (2-methylbutanal, 3-methylbutanal, benzaldehyde) occurred ), ketones (acetoin, 2-heptanone, 2-pentanone, 2-butanone, and 2,3-butanedione), carboxylic acids (propanoic acid, butanoic acid, hexanoic acid, octanoic acid, acetic acid, 2-acetonyl-3-cyano-2,3-dimethylcyclobutane-1-carboxylic acid) and esters (ethyl butanoate and ethyl hexanoate), the nitrogen derivatives were amines (dimethylamine) and oximes (methoxyphenyloxime) and amides (butyl(formyl) formamide).

Acetoin was dominant in sample 6 with the addition of smoked cheese, followed by 2-heptanone, butyl (formyl) formamide, 2-pentanone. Sample 7 with onion flavour had the largest proportion of hexanoic acid, butanoic acid, and acetoin.

**Table 2b** Cheese spreads and butter aroma profile.

| No. | Compound | Percentage area | | Aroma |
|---|---|---|---|---|
| | | x̄ | *SD* | |
| 6 | butyl(formyl)formamide | 3.33 | 2.21 | fruity |
| | 2,3-butanedione | 1.53 | 0.6 | buttery |
| | 2-butanone | 2.57 | 1.75 | ethereal |
| | 3-methyl-butanal | 1.27 | 0.44 | aldehydic |
| | 2-methyl-butanal | 0.68 | 0.37 | chocolate |
| | 2-pentanone | 3.15 | 1.42 | fruity |
| | acetoin | 20.4 | 6.25 | buttery |
| | 2-heptanone | 6.98 | 2.06 | cheesy |
| | benzaldehyde | 0.81 | 0.23 | almond |
| | hexanoic acid | 1.72 | 0.96 | fatty |
| 7 | 2,3-butanedione | 5.56 | 1.55 | buttery |
| | 3-methyl-butanal | 1.02 | 0.49 | aldehydic |
| | acetoin | 10.35 | 0.2 | buttery |
| | butanoic acid | 19.75 | 1.47 | cheesy |
| | ethyl butanoate | 2.01 | 0.24 | fruity |
| | hexanoic acid | 33.65 | 5.57 | fatty |
| | ethyl hexanoate | 3.07 | 0.21 | fruity |
| | octanoic acid | 3.93 | 0.79 | fatty |
| 8 | butyl(formyl)formamide | 0.85 | 0.83 | fruity |
| | nitroso-methane | 4.64 | 2.3 | unknown |
| | acetic acid | 4.28 | 0.87 | sour fruity |
| | acetoin | 12.46 | 1.84 | buttery |
| | butanoic acid | 17.77 | 1.96 | cheesy |
| | ethyl butanoate | 2.06 | 0.12 | fruity |
| | methoxy-phenyl-oxime | 0.85 | 0.13 | unknown |
| | hexanoic acid | 34.65 | 2.24 | fatty |
| | ethyl hexanoate | 2.93 | 0.58 | fruity |
| | octanoic acid | 4.75 | 0.32 | fatty |
| 9 | 2-acetonyl-3-cyano-2.3-dimethyl cyclobutane-1-carboxylic acid | 2.2 | 2.07 | unknown |
| | dimethylamine | 3.09 | 0.42 | unknown |
| | 2,3-butanedione | 0.83 | 0.45 | buttery |
| | 2-butanone | 2.25 | 1.69 | ethereal |
| | 3-methyl-butanal | 1.03 | 0.14 | aldehydic |
| | 2-methyl-butanal | 1.03 | 0.66 | chocolate |
| | 2-pentanone | 11.53 | 1.89 | fruity |
| | acetoin | 13.28 | 1.56 | buttery |
| | 2-methyl-1-butanol | 1.30 | 0.99 | roasted |
| | ethyl butanoate | 1.08 | 0.32 | fruity |
| | 2-heptanone | 7.59 | 1.69 | cheesy |
| | methoxy-phenyl-oxime | 3.50 | 0.92 | unknown |
| | hexanoic acid | 1.99 | 0.92 | fatty |
| 10 | cyclopropane-1,2,3-d3-methanol | 8.96 | 2.29 | unknown |
| | acetic acid | 8.86 | 0.63 | sour fruity |
| | 3-methyl-butanal | 1.39 | 0.31 | aldehydic |
| | 2-methyl-butanal | 1.76 | 0.2 | chocolate |
| | propanoic acid | 21.57 | 1.87 | soury |
| | acetoin | 15.60 | 0.39 | buttery |
| | 3-methyl-1-butanol | 0.65 | 0.12 | fermented |
| | 2-methyl-1 butanol | 1.79 | 0.5 | roasted |
| | ethyl butanoate | 0.67 | 0.2 | fruity |
| | 2-heptanone | 3.63 | 0.74 | cheeesy |
| | methoxy-phenyl-oxime | 1.92 | 0.68 | unknown |
| | ethyl hexanoate | 0.9 | 0.2 | fruity |
| 11 | methoxy-phenyl-oxime | 5.72 | 2.86 | unknown |
| | benzaldehyde | 5.96 | 3.78 | almond |
| | benzyl alcohol | 7.92 | 9.07 | flowery |

Note: no. 6 – cheese spread with smoked cheese „Syrová nátierka s údeným syrom“; no. 7 – cheese spread with onion „Syrová nátierka sladká cibuľka“; no. 8 – cheese spread with hot paprika „Syrová nátierka pálivá paprika“; no. 9 – cheese spread with sweet paprika „Syrová nátierka dolniacka paprika“; no. 10 – cheese spread with emmental cheese „Syrová nátierka Ementálček“; no. 11 – traditional butter „Tradičné maslo“.

Sample 8 was spicy with the addition of hot peppers, and hexanoic acid, butanoic acid, and acetoin were the most abundant. Acetoin and 2-pentanone had the largest proportion in sample 9. And in the last Emmental-flavoured sample 10, propanoic acid was the most abundant, followed by acetoin, acetic acid, cyclopropane-1,2,3-d3-methanol.

**Ningtyas et al. (2019)** determined flavour profile in reduced-fat cream cheese. The volatile profiles of the cream cheese contained β-glucan, esterified phytosterols, and emulsified phytosterols. Twenty-nine volatile compounds at varying levels were identified: 9 fatty acids, 9 ketones, 9 aldehydes, 2 alcohols. Additionally, high levels of acetic acid (vinegar), hexanoic acid (pungent/sour), octanoic acid, and decanoic acid (rancid/fatty) were also identified in the samples. **El-Wahed and Hassanien (2019)** analyzed sensory characteristics of cheese spreads and cheese spreads analogues. In control, processed cheese spread the highest concentration of major short-chain volatile fatty acids namely butyric, caproic, caprylic, and capric acids were found. **Hickey et al. (2018)** reported volatile compounds from the comparative study of processed Cheddar cheese. The treatment of the experimental variant lied also of buttermilk. The differences were found in the abundance of the compounds as follows: hexanol, 2-methyl-propanal, 2-methyl-butanal, benzeneacetaldehyde, nonanal, butanoic acid, hexanoic acid, octanoic acid, methional, σ-decalactone.

***Butter aroma profile***

Butter has appreciated sensory attributes such as flavour, aroma, and melting near the body temperature, which results in a pleasant sensation in the mouth **(Galindo-Cuspinera, Valença de Sousa and Knoop, 2017)**. The volatile compounds methoxyphenyloxime, benzaldehyde, and benzyl alcohol were found in our butter samples. **Tamura et al. (2021)** analyzed butter for volatile compounds and their contribution to fresh sweet creamy butter flavour. Out of the sixty-four identified compounds, 23 were selected, which, according to the authors, had the most significant effect on the aroma. They belonged here e.g. compounds such as δ-dodecalactone, δ-decalactone, γ-decalactone, acetaldehyde, and decanoic acid (capric), which were the most prominent aromatic compounds responsible for the butter flavour. **Yoshinaga et al. (2019)** also confirmed the presence of lactones in butter and fermented butter. The main lactones were δ-decalactone, δ-dodecalactone, δ-tetradecalactone, δ-hexadecalactone, and γ-dodecalactone. In margarine, dominant lactones were δ-decalactone and δ-dodecalactone. Our results, represented by benzaldehyde with a fruity aroma, benzyl alcohol with floral tones, and methoxyphenyloxime, the aroma of which is not described, do not correspond to the results of the above study.

We assume that an insufficient number of standards have influenced the identification of GC-MS compounds, so a more detailed study of the aroma profile in butter would need to take this into account, and investment in standards for compounds that have the highest probability to occur in butter should be considered.

## CONCLUSION

In this paper, we evaluated selected chemical parameters (protein content, fat, dry matter, salt, and pH) and sensory properties of hard cheeses, and aroma profiles of cheeses, cheese spreads, and butter of Slovak production. In general, the dry matter content was higher for ripening cheeses compared to cheeses with herbs and spices. The same trend was also confirmed for the salt content of the cheeses, where the ripening cheeses had the highest salt contents probably due to the increasing concentration of salt during the ripening process. The fat content did not follow this trend. We observed a lower pH in samples with herbs and ripening cheeses were characterized by a higher pH. The protein content was relatively balanced in all samples except for the samples with the addition of chives. The consistency of the parameters measured was confirmed within the 3-month monitoring. Within the sensory panel, the sensory properties of cheese samples and cheese spreads were evaluated and the sensory acceptability of the samples was evaluated. The highest score obtained cheese with 4 spices, however, all samples were evaluated as acceptable for the consumers.

The application of sophisticated analytical methods in the study of aroma profiles of food is currently very widespread. The implementation of GC-MS and e-nose in the analysis of dairy products in the food industry consists of e.g. in the classification of products in terms of sensory properties, type, geographical origin, ripening stage (in the case of cheeses), or prediction of their durability. GC-MS and e-nos thus become powerful tools for monitoring indicators of food quality and origin. We focused on the use of the GC-MS method to evaluate the presence of volatile substances responsible for the aromatic properties of selected dairy products - cheeses, spreads, and butter of Slovak production. In general, it can be stated that the samples did not show many similarities in terms of the presence and abundance of the analyzed volatile compounds. The identified volatile compounds belonged to several chemical groups, namely oxygen derivatives of hydrocarbons such as alcohols, aldehydes, ketones, carboxylic acids, esters, amides, nitrogen derivatives such as amines, imines. Compounds from the group of terpenes, the origin of which can be attributed to plant ingredients, were also present in the samples of herbs and spices flavoured.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# USE OF AROMATIC ROOT VEGETABLES IN THE TECHNOLOGY OF FRESHWATER FISH PRESERVES

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## ABSTRACT
The expediency of using freshwater fish and aromatic root vegetables in the technology of preserves has been substantiated. Based on the organoleptic analysis, the compatibility of freshwater fish and aromatic vegetables as part of preserves has been determined. The conditions for pretreatment of salted semi-finished products to ensure their maturation as part of preserves have been theoretically substantiated and experimentally determined. It has been found that pretreatment of freshwater fish flesh with 1.0% and 1.5% malic acid for 60 minutes provides soft, tender and juicy consistency, which corresponds to an organoleptic rating of 5 points. Changes in the fatty acid composition of preserves are mainly associated with an increase in the amount of polyunsaturated fatty acids that have been introduced with linseed oil, which is a positive factor. It has been found that, in comparison with the control sample, the level of all mineral elements in preserves with aromatic root vegetables is significantly increased, with fiber present, which indicates the expediency of introducing aromatic root vegetables into this product to enrich it with essential mineral and carbohydrate elements to obtain a high-value and healthy food product. Enriching the formulation of preserves made of freshwater fish with a variety of herbal additives increases their nutritional value and allows to get a product of high value enriched with such vital nutrients as carbohydrates, calcium, potassium, phosphorus, sulfur, sodium, magnesium, manganese, and iron. Aromatic root vegetables such as horseradish, parsley, and ginger have been found to exhibit antiseptic properties and delay the activity of microbial enzymes as they content phenol. Therefore, the use of aromatic root vegetables helps to inhibit the oxidation and hydrolysis of fats, which may be due to the presence of phenols in their composition.

**Keywords:** preserves; linseed oil; aromatic root vegetables; hydrobionts; polyunsaturated fatty acid

## INTRODUCTION
Providing the population with high-quality food products of increased nutritional and biological value is an urgent issue of today, due to a violation of the nutritional status, and a lack of several essential nutritional factors. In this regard, the concept of creating new food products, chemically balanced, enriched with functional components, and ensuring their immutability, health safety after technological processing, as well as during storage, is becoming increasingly important **(Sukhenko et al., 2017)**.

The current state of nutrition of the Ukrainian population requires the development and implementation of production technologies based on natural raw supplies and expected composition, for now, it is represented by products that do not meet the human needs for the key nutrition factors. In Ukraine, there is an increase in the volume of cultivation and fishing in freshwater facilities, the product range of which is limited to live and chilled fish. Organoleptic parameters of freshwater fish and their nutritional value require streamlining of technologies for its processing by combining it with vegetable raw products **(Golembovskaya and Lebskaya, 2015)**.

Carp are the fish that are mostly grown in the water farms of Ukraine. This type of fish contains a lot of protein with all essential amino acids, biologically effective fatty acids, but the flesh of this fish has a low percent, or no dietary fiber, and lacks several trace elements essential in nutrition following the requirements of modern nutritionology concepts. Besides, freshwater fish have a low taste property, which indicates the feasibility of their improvement **(Mushtruk et al., 2020a)**. There are aromatic root vegetables, grown in Ukraine, which are only partially used in the technology of fish products. Previous studies have found that the use of vegetable raw products in the technologies of freshwater hydrobionts, including aromatic root vegetables, facilitates the harmonization of organoleptic indicators, and obtaining food products with functional ingredients.

However, systemic studies in the activation of the maturation processes of freshwater fish in the composition

of preserves have not been conducted. Thus, the development of the technology of preserves made of freshwater fish and aromatic root vegetables is an urgent issue, the solution of which will facilitate the production of high-quality and healthy biologically valuable fish products from the Ukrainian raw products and expand the range of products including freshwater fish.

The analysis of current trends in fish production technologies allows to identify the following key directions that are related to the technologies of multicomponent food products, improvement of technologies of preserves made of low maturity hydrobionts, the enrichment of the recipe composition, pretreatment of raw products in various ways, the application of physico-chemical and biotechnological techniques for the production of conventional foods made of fish such as canned food, preserves, pâté, salted, dried, culinary products and washed minced products for *surimi* production **(Shanina et al., 2020)**.

The record shows that the use of enzymatic agents during fish salting makes it possible to obtain good quality products from immature fish species. It is possible to use expensive enzymatic agents in the production of fish preserves, made of plant and microbial raw supplies, for example, culture liquid *H. Mediteranei* to soften pollock flesh or the proteolytic enzyme preparation *Sal Intersor EC* for the production of fish fillet preserves.

These scientists' works highlight the fact that some marine hydrobionts contain highly active complexes of proteolytic enzymes that can be widely used. The muscle tissue of freshwater fish has a low level of activity of its proteinases, which does not allow achieving the desired degree of maturation without additional application of proteolytic enzyme preparations that intensify this process **(Shanina et al., 2019)**.

S. L. Chernyavskaya has developed the technology of a proteolytic enzyme preparation from zebra mussel, proving the effectiveness of using Protofermol proteolytic enzyme preparation from a zebra mussel, bivalve mollusk, without preliminary separation of the flaps to intensify the maturation process of preserves made of Baltic herring pieces **(Chernyavskaya et al., 2019)**.

Bùi Xuân Đông has developed a new range of preserves made of fillets of pond fish species, whose enzyme system of muscle tissue was activated by injecting an enzyme preparation derived from their internal organs **(Bùi, 2011)**.

Similar studies were conducted by T. J. Park and co-authors **(Park et al., 2020)**, which highlighted the production of proteolytic enzyme preparations from the entrails of pond fish such as carp, silver carp, grass carp, and pike. O. P. Dvorianynova, in collaboration with other scientists, obtained an enzyme complex from the muscle tissue of pond carp, and determined the proteolytic activity of the obtained enzymes **(Dvoryaninova et al., 2010)**. Other authors theoretically substantiated and experimentally confirmed the expediency and effectiveness of using new methods for intensifying and regulating the maturation of preserves made of poorly maturing fish species by introducing white cabbage as a source of plant proteases, and the enzyme preparation *Sal Intensor EC* of proteolytic action into the formulation.

T. A. Davletshyna substantiated the mechanism of the antimicrobial drug action on microorganisms, their proteolytic and lipolytic enzymes, which reduce bacterial contamination of semi-finished products and finished fish products **(Davletshina et al., 2019)**.

Technologies for the production of non-fish preserves, namely squid and whelk ones, which are low-maturing raw products for the production of preserves, were of considerable interest, too **(Saltanova and Malinovskaya, 2012)**. The technology of making preserves using these shellfish involves the pretreatment of raw products by blanching, or the use of gamma radiation to soften the flesh.

The effectiveness of processing fish preserves with orange juice, which helps them mature, has been highlighted. The use of orange juice as a dietary supplement provides the product with functional properties, as it enriches the preserves with biologically active substances, C, PP, B vitamins, beta-carotene, as well as potassium, calcium, iron, and selenium.

The considered fish raw products' technologies speak for the prospects for improving the preserves technologies, as products that preserve the natural properties of raw products to a greater extent due to the absence of heat processing.

Thus, the change in the structure of fishing, the general trend of reducing the catch of conventional marine fish species, an increase in the number of freshwater fish as aquaculture objects makes it necessary to improve complex technologies for processing raw products and expand the range of freshwater fish food products. One of the promising ways to address these issues is to develop the technology of freshwater fish preserves enriched with vegetable ingredients, based on the principles of food combinatorics. The use of aromatic root vegetables is one of the priority areas of research in the future.

## Scientific hypothesis

The concept of creating new food products that are balanced in chemical composition and enriched with functional components and ensuring their health safety after technological processing, as well as during storage, is becoming increasingly important. Based on the generalization of the theoretical content, the expediency of aromatic root vegetables in the technology of freshwater fish preserves has been substantiated, which facilitates the maturation of salty semi-finished products and allows to obtain a food product with high organoleptic properties and increased biological value.

## MATERIAL AND METHODOLOGY

### Samples

Study of preserves of control and experimental samples (preserves with ginger, horseradish, and parsley) was carried out using the following raw products: live fish of spring and autumn catch (carp) according to **DSTU 2284 (2010)**, grown in reservoirs of Cherkasyrybhosp PJSC; table salt according to **DSTU 3583 (2015)**; ground allspice according to **GOST ISO 973 (2016)**; ground black pepper according to **DSTU ISO 959-1 (2008)**; cloves according to **DSTU ISO 2254 (2008)**; sunflower oil according **to DSTU 4492 (2017)**; bay leaves according to **GOST 17594 (1981)**; parsley root (*Petroselinum crispum*) according to **DSTU 6010 (2008)**; ginger root (*Zingiber officinale*) according to **DSTU ISO 1003 (2018)**; horseradish root

(*Armoracia rusticana*) according to **DSTU 294 (1991)** from farmlands of Cherkasy and Kyiv regions; table vinegar according to **DSTU 2450 (2006)**; linseed oil according to **TU U 15.4-14235416-001 (2010)**.

**Chemicals**

Petroleum ether (excise, AR grade, Khimlaborreaktyv LLC, Ukraine).

Nitric acid (A brand, CP, Khimlaborreaktyv LLC, Ukraine).

Potassium dichromate (AR grade, Khimlaborreaktyv LLC, Ukraine).

Hydrochloric acid (A brand, AR grade, Khimlaborreaktyv LLC, Ukraine).

Sodium hydroxide (A brand, AR grade, Khimlaborreaktyv LLC, Ukraine).

Sodium tripolyphosphate (technical, p 85%, Khimlaborreaktyv LLC, Ukraine).

Sulfuric acid (A brand, CP, Khimlaborreaktyv LLC, Ukraine).

**Instruments**

Drying cabinet **(**SNOL, Khimlaborreaktyv LLC, Ukraine).

Muffle furnace (SNOL, Khimlaborreaktyv LLC, Ukraine).

Fat analyzer (SOX 406, Khimlaborreaktyv LLC, China).

Mineralizer (Velp Scientifica, Khimlaborreaktyv LLC, Italy).

Distiller for steam distillation (Velp Scientifica UDK 129, Khimlaborreaktyv LLC, Italy).

Fiber extractor (FIWE Raw Fiber Extractor, Khimlaborreaktyv LLC, Italy).

Atomic absorption spectrophotometer (Shimadzu AA-6200, SPEKTROLAB LLC, Ukraine).

Gas chromatograph (Kupol_55, Shimadzu Corporation, Japan).

Amino acid analyzer (LC-2000, Biotronik, Khimlaborreaktyv LLC, Ukraine).

**Laboratory Methods**

Characterizing of the chemical composition of fish preserves has been carried out according to the following methods: the mass fraction of moisture by drying the product sample down to a fixed weight at a temperature of 100 – 105 °C according to **DSTU 8029 (2015)**; the mass fraction of ash by weight method, after mineralization of the product's sample weight in a muffle furnace at a temperature of 500 – 600 °C according to **DSTU 8718 (2017)**; the mass fraction of lipids by Soxhlet method, which consists in the fact that fat is weighed after its extraction with a solvent from the dry sample weight in the Soxhlet apparatus, based on determining the change in the sample's weight after fat extraction with a solvent by **DSTU 8718 (2017)**; the mass fraction of protein by determining the total nitrogen by the Kjeldahl method. Cinefaction of samples was performed on the Velp Scientifica DK6 series (Italy) with a vacuum pump (JP). Distillation was made on a steam distillation device Velp Scientifica UDK 129 (Italy), **DSTU 8030 (2015)**.

Determination of the fiber's mass fraction was carried out by removing acid-alkaline-soluble substances from the product and determining the residue weight, conventionally fiber following **DSTU 8844 (2019)**.

Determination of the fatty acid content was carried out by chromatographic method on the Kupol 55 chromatograph (Russia) to **GOST ISO 17764-1 (2015)**.

The mineral composition (the content of potassium, calcium, magnesium, phosphorus, manganese, and so on) was determined by atomic emission spectrometry with inductive plasma, and the content of heavy metals (lead, cadmium, arsenic, mercury, copper, and zinc) was determined by atomic absorption spectrometry according to **DSTU EN ISO 11885 (2019).**

**Description of the Experiment**

**Sample preparation:** After deboning, the carp fillet was salted in brine at room temperature for 24 hours until it was 5% salty. Then the fillet was portioned into pieces and placed in plastic jars with vegetable raw products added (parsley, ginger, horseradish). Recipes for preserves of test samples consisted of carp meat – 75% and pickle solution – 25%. Preserve samples were stored in plastic containers with a capacity of 200 мL at a temperature ranging from 0 to +4°C (Figure 1). The control sample, ginger preserves, horseradish preserves, and parsley preserves were sent for analysis. Then, an average sample was taken from each unit, which was then characterized after grinding by the total chemical composition, energy value, mineral content, amino acid composition of proteins, and fatty acid




**Figure 1** Preserves with carp with the addition of spicy-aromatic roots.

composition of lipids.

**Number of samples analyzed:** In the study of the samples, four types of preserves were used: a control without the addition of spicy-aromatic root crops and three samples with the addition of ginger, horseradish and parsley.

**Number of repeated analyses:** Each study was carried out five times with the number of samples being four, which amounted to twenty repeated analyses.

**Number of experiment replication:** The study was repeated 5 times, with the experimental data processed using mathematical statistics methods.

### Statistical Analysis

The statistical analysis data were performed by Microsoft excel and Statistica 15. The accuracy of the obtained experimental data was determined using the Student's t-test with a confidence coefficient ≤ of 0.05 with several parallel definitions of at least 5. Linear programming problems were solved using the MS Excel table processor's 'Search for a solution' setting (Excel Solver).

## RESULTS AND DISCUSSION

Previous research has shown that the use of vegetable raw materials **(Rodak and Fil, 2016; Pobedash, 2020; Paska et al., 2020; Fedorova and Kuzmenko, 2015; Shubina et al., 2019; Fedorova and Dasheev, 2017; Shubina et al., 2017)** and, including spicy-aromatic roots, in the technology of freshwater aquatic organisms contribute to the harmonization of organoleptic characteristics.

The results of studies of the chemical composition of carp preserves and aromatic root vegetables are shown in Table 1. It should be noted that the mineral composition of the test samples differs from that of the control sample, which is due to the content of minerals in vegetable additives (ginger, horseradish, parsley), which in combination with fish raw products enrich the preserves. If we compare with the studies **(Shulgina et al., 2017; Alekseeva, 2019)** of other authors who do not add vegetable raw materials, we get a much lower mineral content of an average of 1.4%.

The fatty acid composition was determined in preserves with sunflower oil and a mixture of sunflower oil and linseed oil in a ratio of 1:1. Similar studies **(da Silva et al., 2019; Lansing et al., 2018; Fernandez et al., 2019; Eveleva and Cherpalova, 2019; Brazhnaya, 2016)** describe similar studies, but the authors used different types of oils (sunflower and olive, soybean and sunflower, corn and others) and the ratio of oils (1:2, 1:3, 1:5 and 1:10). The fatty acid composition of preserves with sunflower oil is shown in Table 2.

According to experimental data, the content of all FA groups in the preserves studied is exceeded in all groups, but the control sample, in which the sum of saturated acids

**Table 1** Characteristics of the chemical composition of fish preserves.

| Sample of preserves | Mass fraction, % | | | | |
|---|---|---|---|---|---|
| | moisture content | protein | fat | mineral substances | fiber |
| Control sample | 70.49 ±1.88 | 11.85 ±0.73 | 9.36 ±0.65 | 8.29 ±0.61 | - |
| Preserves with ginger | 66.90 ±1.47 | 11.93 ±0.74 | 10.61 ±0.98 | 9.55 ±0.23 | 0.03 ±0.03 |
| Preserves with horseradish | 65.61 ±1.78 | 11.88 ±0.81 | 11.99 ±1.05 | 9.93 ±0.41 | 0.05 ±0.03 |
| Preserves with parsley | 67.65 ±1.69 | 11.68 ±0.31 | 10.01 ±0.75 | 10.15 ±0.66 | 0.14 ±0.03 |

Note: ($n = 5$, $p \leq 0.05$)

**Table 2** Fatty acid composition of lipids of preserves made of carp and aromatic raw products with sunflower oil.

| Fatty acids | FA code | Content of fatty acids, % of their total amount | | | | Recommended quantity, g/day (Onishchenko, 2004) |
|---|---|---|---|---|---|---|
| | | Control sample | Preserves with ginger | Preserves with horseradish | Preserves with parsley | |
| **Saturated fatty acids, incl.** | | **24.80** | **29.92** | **30.78** | **29.93** | **25** |
| myristic acid | 14:0 | 0.09 | 0.12 | 1.07 | 0.08 | |
| palmitic acid | 16:0 | 20.52 | 25.63 | 25.44 | 25.59 | |
| stearic acid | 18:0 | 3.64 | 3.59 | 3.62 | 3.57 | |
| arachic acid | 20:0 | 0.14 | 0.14 | 0.18 | 0.15 | |
| nonadecanoic acid | 19:0 | 0.41 | 0.45 | 0.47 | 0.54 | |
| **Monounsaturated fatty acids, incl.** | | **33.14** | **38.05** | **38.13** | **38.17** | **30** |
| palmitooleic acid | 16:1 | 5.10 | 5.09 | 5.11 | 5.12 | |
| $\omega_9$ oleic acid | 18:1 | 15.85 | 20.79 | 20.85 | 20,88 | |
| $\omega_9$ elaidic acid | 18:1 | 12.14 | 12.15 | 12.15 | 12.17 | |
| gadoleic acid | 20:1 | 0.06 | 0.02 | 0.02 | 0.02 | |
| **Polyunsaturated fatty acids, incl.** | | **12.83** | **14.06** | **13.16** | **14.12** | **11** |
| linolenoic acid $\omega_3$ | 18:2 | 6.09 | 7.15 | 6.15 | 7.17 | |
| linoleic acid $\omega_6$ | 18:3 | 6.09 | 6.26 | 6.30 | 6.30 | |
| eicosadienoic acid | 20:2 | 0.64 | 0.65 | 0.71 | 0.65 | |
| **Unidentified** | | **29.23** | **17.98** | **17.93** | **17.79** | |

**Table 3** Fatty acid composition of lipids of preserves made of carp and aromatic raw products with sunflower and linseed oil.

| Fatty acids | FA code | Content of fatty acids, % of their total amount | | | | Recommended quantity, g/day (Onishchenko, 2004) |
|---|---|---|---|---|---|---|
| | | control sample | preserves with ginger | preserves with horseradish | preserves with parsley | |
| **Saturated fatty acids, incl.** | | **24.80** | **10.05** | **10.09** | **10.82** | **25** |
| tridecanoic acid | 13: 0 | - | 0.17 | 0.21 | 0.33 | |
| palmitic acid | 16: 0 | 20.52 | 5.70 | 5.71 | 6.43 | |
| stearic acid | 18: 0 | 3.64 | 3.88 | 3.88 | 3.85 | |
| arachic acid | 20: 0 | 0.14 | 0.30 | 0.28 | 0.21 | |
| nonadecanoic acid | 19: 0 | 0.41 | - | - | - | |
| myristic acid | 14: 0 | 0.09 | - | - | - | |
| **Monounsaturated fatty acids, incl.** | | **33.14** | **22.37** | **22.13** | **22.18** | **30** |
| palmitooleic acid | 16: 1 | 5.10 | - | - | - | |
| $\omega_9$ oleic acid | 18: 1 | 15.85 | 22.37 | 22.13 | 22.18 | |
| $\omega_9$ elaidic acid | 18: 1 | 12.14 | - | - | - | |
| gadoleic acid | 20: 1 | 0.06 | - | - | - | |
| **Polyunsaturated fatty acids, incl.** | | **12.83** | **67.33** | **67.51** | **66.76** | **11** |
| linolenoic acid $\omega_3$ | 18: 2 | 6.09 | 59.97 | 60.09 | 59.58 | |
| linoleic acid $\omega_6$ | 18: 3 | 6.09 | 6.46 | 6.51 | 6.38 | |
| linolenoic acid $\omega_6$ | 18: 3 | - | 0.29 | 0.26 | 0.22 | |
| eicosatetraenoic acid | 20: 4 | - | 0.61 | 0.65 | 0.58 | |
| eicosadienoic acid | 20: 2 | 0.64 | - | - | - | |
| **Unidentified** | | **29.23** | **0.25** | **0.27** | **0.24** | |

**Table 4** Indicators of biological effectiveness of lipids of preserves made of carp and aromatic raw products with sunflower and linseed oil

| Lipids | Ratio of FA types | | | |
|---|---|---|---|---|
| | SFA:MFA:PUFA | PUFA:SFA | C18:2:C18:1 | $\omega_6$:$\omega_3$ |
| **Ideal lipid (Tsypriian, Manasar, Slobodkin, 2007)** | 1:1:1 | 0.2:0.4 | > 0.25 | 10: 1 |
| **with sunflower oil** | | | | |
| **Control sample** | 1: 1.34: 0.52 | 0.52: 1 | 1: 2.60 | 1: 1.00 |
| **Preserves with ginger** | 1: 1.27: 0.47 | 0.47: 1 | 1: 0.37 | 1: 1.14 |
| **Preserves with horseradish** | 1: 1.24: 0.43 | 0.43: 1 | 1: 0.36 | 1: 0.98 |
| **Preserves with parsley** | 1: 1.28: 0.47 | 0.47: 1 | 1: 0.37 | 1: 1.14 |
| **with sunflower and linseed oil** | | | | |
| **Control sample** | 1: 1.34: 0.52 | 0.52: 1 | 1: 2.60 | 1: 1.00 |
| **Preserves with ginger** | 1: 2.23: 6.69 | 6.69: 1 | 1: 0.37 | 1: 9.28 |
| **Preserves with horseradish** | 1: 2.19: 6.69 | 6.69: 1 | 1: 0.36 | 1: 9.23 |
| **Preserves with parsley** | 1: 2.05: 6.17 | 6.17: 1 | 1: 0.37 | 1: 9.35 |

**Table 5** Effect of added aromatic root vegetables on the mineral composition of freshwater carp preserves, mg/100 g

| Mineral element | Preserves under study: | | | | 10% of the adequate daily consumption requirement (Onishchenko, 2004) |
|---|---|---|---|---|---|
| | control sample | with ginger | with horseradish | with parsley | |
| **Potassium** | 83.51 ±2.11 | 93.89 ±2.02 | 108.87 ±3.09 | 97.21 ±2.43 | 250 |
| **Calcium** | 15.43 ±0.69 | 21.69 ±0.61 | 21.81 ±1.06 | 28.99 ±1.02 | 125 |
| **Sulfur** | 19.11 ±0.80 | 22.73 ±0.30 | 26.77 ±1.15 | 20.23 ±1.13 | 1.0 |
| **Iron** | 0.15 ±0.02 | 0.59 ±0.03 | 0.22 ±0.03 | 0.26 ±0.03 | 1.5 |
| **Manganese** | — | 0.17 ±0.02 | 0.01 ±0.005 | 0.03 ±0.006 | 0.2 |

Note: (n = 5, $p \leq 0.05$).

approaches the recommended amount. Among saturated acids, palmitic acid is dominant, the content of which in experimental samples with various aromatic root vegetables exceeds the control sample by 5%.

The fatty acid composition of preserves with sunflower and linseed oil is shown in Table 3.

Analyzing the data presented in Table 3, it was found that the content of saturated and monounsaturated FA

compared to the recommended amount in the experimental samples is lower, but the enrichment of preserves takes place due to the high content of polyunsaturated FA, namely linoleic acid the linseed oil is rich in.

The results of indicators of biological effectiveness of lipids of preserves made of carp and aromatic raw products with sunflower oil and sunflower-and-linseed oil are shown in Table 4.

It should be particularly noted that the high content of fatty acids of the $\omega_3$ family in the lipids of preserves, which we have found, speaks for their high biological efficiency **(Mushtruk et al., 2020a; Barboza et al., 2020; Golembovskaya, 2020)**.

The characteristics of the effect of aromatic root vegetables on the mineral composition of preserves made of freshwater carp fish are shown in Table 5.

Based on these results, the level of all mineral elements is significantly increased in preserves with aromatic root vegetables, compared to the control sample and studies by other authors **(Dementeva and Bogdanov, 2017; Petrik, 2016; Abramova and Goferber, 2017)**, which speaks for the expediency of adding aromatic root vegetables to this product to enrich it with essential mineral elements and obtain a healthy food product of high value.

Taking into account the different needs of a human body for minerals, we have calculated the level of macro- and microelements to meet daily needs provided that 200 grams of preserves are consumed (Figure 2).

The control sample was characterized by a lower degree of meeting the daily requirement for most trace elements, which can be explained by a less balanced mineral composition of fish raw products.

These data speak for the fact that the degree of manganese supply provided that the preserve with ginger is consumed is the highest and makes up 85% of its daily requirement, respectively. Preserves with horseradish and parsley meet the need for this element by 5% and 15%, while in the control sample it is absent.

Preserves with ginger are also rich in ferrum and potassium – 39% and 38%, respectively. Comparing them with other samples, we can conclude that they are most enriched with minerals due to the addition of ginger.

Determination of the content of toxic elements in the composition of preserves is one of the essential characteristics of their health safety (Table 6).

According to the results of Table 6, it was found that the addition of aromatic root vegetables to the composition of preserves is not accompanied by an increase in the level of toxic elements, which guarantees their health safety and suitability for consumption.

The obtained results for determining the chemical composition of preserves made of carp and aromatic root vegetables (Table 1) speak for the fact that the protein content in test samples of preserves ranges from 11.68% to 11.93 %. The lowest protein content was observed in the control sample.

The fat content in the finished preserves ranged from 9.36% to 11.99%, which has a positive effect on the product's taste properties.

Determination of the moisture content in the finished preserves has shown that there is a certain fluctuation in this indicator in the range from 65.61 (preserves with

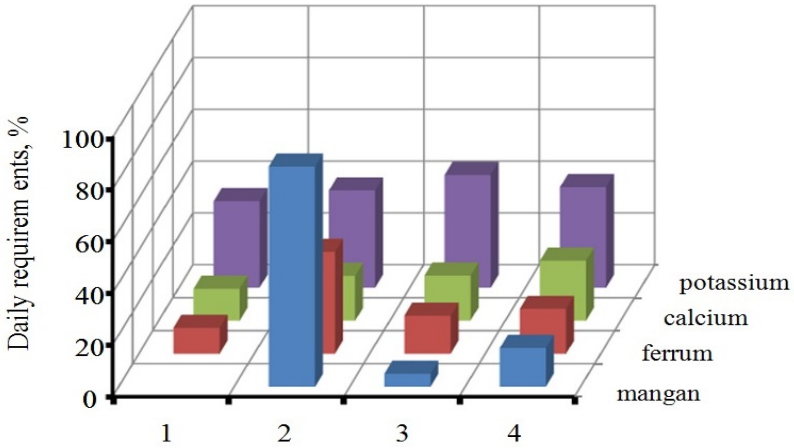


**Figure 2** The degree of meeting a person's daily need for minerals provided that 200 g of fish preserves are consumed, %. Note: 1 – control sample; 2 – preserves with ginger; 3 – preserves with horseradish; 4 – preserves with parsley.

**Table 6** Mass fraction of toxic elements in preserves with aromatic root vegetables, mg.100g$^{-1}$.

| Indicator | Preserves under study: | | | | Permissible levels, max (Tsypriian, Manasar, Slobodkin, 2007) |
|---|---|---|---|---|---|
| | control sample | with ginger | with horseradish | with parsley | |
| **Copper** | 0.03 ±0.005 | 0.05 ±0.007 | 0.03 ±0.001 | 0.06 ±0.010 | 1.0 |
| **Zinc** | 0.20 ±0.010 | 0.59 ±0.020 | 0.19 ±0.020 | 0.25 ±0.020 | 4.0 |
| **Rubidium** | 0.09 ±0.005 | 0.25 ±0.009 | 0.01 ±0.003 | 0.01 ±0.006 | undefined |
| **Strontium** | 0.39 ±0.010 | 0.39 ±0.010 | 0.64 ±0.020 | 0.82 ±0.020 | undefined |
| **Nickel** | 0.02 ±0.004 | 0.009 ±0.0002 | 0.02 ±0.001 | 0.01 ±0.005 | undefined |

Note: (n = 5, $p \leq 0.05$).

horseradish) to 70.49 (control sample preserves).

In terms of mineral content, the test samples differ in comparison with the control sample. In our opinion, this is because vegetable supplements contain a wide range of minerals, which in combination with minerals contained in fish raw products enrich ready-made preserves **(Lebskaya, Pavluchenko and Moisiienko, 2017; Slobodyanyuk et al., 2019)**.

The sum of individual groups of carp lipid fatty acids corresponds to or exceeds the recommended amount of daily need **(Tsypriian, Manasar, Slobodkin, 2007)**.

Fatty acids play an essential part in human metabolic processes.

Therefore, the composition of fatty acids in food products is vital for ensuring normal metabolism and its correction in various disorders. Accordingly, the composition of fatty acids of the final form of a food product hinges on the characteristics of the ingredients that are used in its production. Among the results of the fatty acid composition of preserve lipids with the use of sunflower oil, MUFAs dominate (33.14 – 38.17%), with the use of sunflower and linseed oil in combination, PUFAs dominate (12.83 – 67.51%).

Changes in the fatty acid composition of preserves (Tables 2, 3) are mainly associated with an increase in the amount of polyunsaturated fatty acids that were introduced with the addition of linseed oil, which is a positive factor.

Among PUFAs, linolenic acid makes up the largest amount (59.58 – 60.09%). The record shows that this acid is vital for humans, it serves mainly as an energy supplier but plays an essential part in the treatment and prevention of cardiovascular diseases, such as coronary heart disease, hypertension, hyperlipoproteinemia, thanks to its lipid-lowering, antiplatelet and hypotensive effects **(Loizzo et al., 2016)**.

The degree of lipid fatty acids absorption hinges on the ratio of individual fatty acids and characterizes their biological effectiveness. It does not diverge from practical data. The ratio of individual classes of lipids does not correspond to the recommendations proposed by nutrition specialists. However, data on the ratio of fatty acids $\omega_6$:$\omega_3$ in all types of preserves speak for their high biological efficiency. The record shows that the recommended ratio of PUFAs of $\omega_6$ family/PUFAs of the $\omega_3$family in the diet of a healthy person is 10:1, and in cases of lipid metabolism pathology, it is 5:1 and even 3:1 **(Shulgina, et al., 2017; Zheplinska et al., 2019)**. However, the greatest deficiency in the diet is noted for fatty acids of the $\omega_3$ family. Therefore, the high content of $\omega_3$ family acids in preserve lipids that we have found speaks for their high biological efficiency.

The mineral composition of preserves is characterized by a fairly high content of potassium (93.89 – 108.87 mg.100g$^{-1}$), calcium (21.69 – 28.99 mg.100g$^{-1}$), sulfur (20.23 – 26.77 mg.100g$^{-1}$) compared to the control sample. Based on the study of the mineral composition of fish raw products, the need to optimize it due to the insignificant amount or absence of some essential elements, in particular potassium, manganese, iodine, bromine, selenium, has been proven.

Conducting corresponding research would be very appropriate, as it would make it possible to enrich the recipes of preserves based on freshwater fish with a wide range of vegetable additives, increasing their nutritional value and allowing to get a product rich in such vital nutrients as carbohydrates, calcium, potassium, phosphorus, sulfur, sodium, magnesium, manganese and iron.

## CONCLUSION

Studies on the chemical composition of preserves gave positive results and proved that the addition of aromatic root vegetables increases the mineral content compared to the control sample.

The biological effectiveness of the fatty acid composition of carp preserves is due to an increase in the amount of polyunsaturated fatty acids, namely linoleic acid $\omega_6$, which was introduced with the addition of linseed oil to fish flesh, and compared with the daily requirement exceeds the recommended content.

In comparison with the control sample, in the preserves under study, the level of all mineral elements is significantly increased, which speaks for the expediency of adding aromatic root vegetables to this product to enrich it with these essential elements to obtain high-value and healthy food product. As for the content of toxic elements, preserves with aromatic root vegetables are good for health.

The chemical composition of the experimental formulations differed from the control sample with a higher protein content: 15% vs. 11%, fat: 10 – 12% vs. 9%, and minerals: 9 – 10% vs. 8%, but the energy value of the experimental preserves was higher 144 – 172 in compared with control: 131.

Studies of changes in the quality and safety of preserves during storage have determined the allowable shelf life of preserves no more than 28 days at temperatures from 0 to +4 °C.

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# STUDIES ON THE CHEMICAL COMPOSITION OF FRUITS AND SEEDS OF *PSEUDOCYDONIA SINENSIS* (THOUIN) C.K. SCHNEID.

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## ABSTRACT

*Pseudocydonia sinensis* (Thouin) C. K. Schneid. less known plant species in the Ukraine conditions, but the fruits were widely used in traditional Chinese medicine for the treatment of asthma, colds, sore throat, mastitis, rheumatoid arthritis, and tuberculosis. The content of protein, ash, and lipids in the seeds was found to be greater than in the pulp and peel. Monosaccharide analysis of neutral carbohydrate part showed the presence of two main sugars fructose and sucrose in the seeds, pulp, and peel. There is a higher beta-carotene content in the rind of the fruit than in the seeds and pulp. The total amount of fatty acids varied from sample to sample and contained mainly oleic acid, palmitic acid, linolenic acid, and linoleic acid. Linoleic acid in the seeds was 48.02% of total fatty acids, slightly less in the rind 42.70%. Palmitic acid, oleic acid, and linoleic acid in the pulp samples were 45.38, 21.32, and 14.93%, respectively. The total amount of amino acids found in the seeds was 105.0 $g.kg^{-1}$ DM, including total essential amino acids (32.70 $g.kg^{-1}$ DM). Glutamic acid was found in seeds to be the dominant free amino acid followed by aspartic acid and arginine in the seed. In our study, the antioxidant activity carried out by the DPPH method and measured by molybdenum reducing antioxidant power of peel, pulp and seeds were 9.41, 7.08, 6.21, and 158.81, 92.83, 78.58 mg $TEAC.g^{-1}$ DM, respectively. Micro and macronutrients and amino acids predominated in the seeds, total fatty acids predominated in the pulp. The highest content of bioactive compounds (total polyphenols, flavonoid, and phenolic acid) and antioxidant activity was found in the peel. *P. sinensis* can be considered as a nourishing fruit with a copious potential with health-promoting roles and medicinal properties.

**Keywords:** Chinese quince; fruit; seed; chemical composition; nutrients

## INTRODUCTION

Increasing interest in less know, non-traditional, neglected, and underutilized plant species, which could serve as a valuable source of natural bioactive compounds, has been emerged worldwide and they play important role in procuring food security to improve health and nutrition, ecological sustainability, and livelihoods. These species of rich in valuable biologically active compounds include *Aronia mitschurinii* A. K. Skvortsov & Maitul., *Cornus mas* L., *Chaenomeles* spp., *Diospyros virginiana* L., *Lycium* spp., *Lonicera* spp., *Morus nigra* L., *Ziziphus jujuba* Mill., *Vaccinium* spp., *Sambucus nigra* L. **(Monka et al., 2014; Ivanišová et al., 2017; Klymenko, Grygorieva and Brindza, 2017; Grygorieva et al., 2018; Grygorieva et al., 2020; Klymenko et al., 2019; Horčinová Sedláčková et al., 2018; Horčinová Sedláčková et al., 2019; Szot, Zhurba and Klymenko, 2020; Vinogradova et al., 2020)**. The direction adopted by the European Community towards sustainable crop production intensification involves "growing a wider range of plant species and varieties using combinations, sequences and rotations" **(Save and Grow, 2011)**. It is important to cultivate little-known edible plants more widely, as they are a potential source of new biologically active substances needed for the functioning of the human body.

*Pseudocydonia sinensis* (Thouin) C. K. Schneid. (Chinese quince) is a less known species of the family Rosaceae Juss. and the only species in the genus *Pseudocydonia* C. K. Schneid., native to eastern Asia in China. This species is closely related to the East Asian genus, *Chaenomeles* Lindl., and to the European genus, *Cydonia* Mill. **(Suzuki, 1994)**. Sometimes it is called *Chaenomeles sinensis*. They are distinguished by the absence of thorns and single, not clustered flowers. *Cydonia oblonga* differs from the *Pseudocydonia sinensis* plant in the presence of toothed leaves and the absence of pubescence on the fruits **(Klymenko, Grygorieva and Brindza, 2017)**.

In Europe, *Pseudocydonia sinensis* is only grown in botanical gardens and arboretums but has already proved to be an annually fruiting crop that is resistant to the climatic conditions of the continent. A more detailed study of the biology of this species will help to introduce it into widespread cultivation more quickly.

Since the fruits of *Pseudocydonia sinensis* are very acidic and tart, they are used only in their raw form; they are used to make marmalades, jams, fruit jellies, candied pulp, syrups and juices, wines, liqueurs, and in the preparation of flour products **(Hamauzu et al., 2006; Monka et al., 2014; Klymenko, Grygorieva and Brindza, 2017)**.

According to literature data, fruits of the studied species contain organic acids, flavonoids (rutin and quercetin), procyanidins, and volatile compounds **(Hamauzu et al., 2005; Hamauzu and Nakamura, 2014)**. The skin of *Pseudocydonia sinensis*' fruits contains the following volatile compounds: (E,E)-α- -farnesene, isobutyl octanoate, ethyl octanoate, isobutyl 7-octanoate, and hexyl hexanoate **(Mihara et al., 1987)**. Aromoactive compounds such as ethyl 2-methylpropanoate, ethyl (E)-2-butenoate, ethyl 2-methyl butanoate, methionyl, (Z)-3-hexenyl acetate, β-ionone, ethylnonanoate, and γ-decalactone were also found in the skin **(Choi et al., 2018)**.

The fruits of the *Pseudocydonia sinensis* were widely used in traditional Chinese medicine for the treatment of asthma, colds, sore throat, mastitis, rheumatoid arthritis, and tuberculosis **(Mihara et al., 1987; Chung, Cho and Song, 1988; Hamauzu and Nakamura, 2014)** as confirmed by their antibacterial, antihemolytic, anti-inflammatory, antipruritic, antioxidant, antiviral, anti-ulcerative, gastroprotective, antitumor, and antimicrobial properties **(Osawa et al., 1997; Osawa et al., 1999; Oku, Ueda and Ishiguro, 2003; Hamauzu et al., 2006; Hamauzu et al., 2007; Hamauzu, Kishida and Yamazaki, 2018; Sawai et al., 2008; Chun et al., 2012; Sawai-Kuroda et al., 2013; Monka et al., 2014; Kabir et al., 2015; Essuman, Nagajyothi and Tettey, 2017; Grygorieva et al., 2020)**.

This work was carried out to determine the chemical composition of fruits and seeds of less known species *Pseudocydonia sinensis* to assess the possibility of using this species in the future.

## Scientific Hypothesis

As *Pseudocydonia sinensis* is widely used in Chinese traditional medicine, its fruits and seeds contain bioactive components. When introduced in Ukraine and Slovakia, the amount of beneficial substances in fruits and seeds is not reduced, which makes it possible to recommend this species for wide cultivation.

## MATERIAL AND METHODOLOGY

### Samples

*Pseudocydonia sinensis* seeds (Figure 1) and fruits (pulp and peel) (Figure 2) were collected in November 2019 from trees growing in an M.M. Gryshko National Botanical Garden (Kyiv, Ukraine; 197 m a.s.l.).



**Figure 1** *Pseudocydonia sinensis* (Thouin) C. K. Schneid.




**Figure 2** Fruits (A) and seeds (B) *Pseudocydonia sinensis* (Thouin) C. K. Schneid.

**Chemicals**

Ethanol (Centralchem s.r.o., Bratislava, Slovakia, p.a.), acetonitrile (Fisher Chemical, Loughborough, UK, HPLC grade), petroleum ether (Sigma-Aldrich,Merck KGaA, Darmstadt, Germany,Sigma Grade, ≥99%), ninhydrin (Ingos, Czech Republic), nitric acid (Analytika Praha Ltd, Czech Republic), hydrochloric acid (Analytika Praha Ltd, Czech Republic), methyl cellosolve (Ingos, Czech Republic), filter with 0.45 μm pore size (Labicom, Czech Republic), tin chloride ($SnCl_2$) (Centralchem s.r.o., Bratislava, Slovakia, p.a.), Folin-Ciocalteu reagent (Sigma-Aldrich,Merck KGaA, Darmstadt, Germany), sodium carbonate (Centralchem s.r.o., Bratislava, Slovakia, p.a.), sodium hydroxide (Centralchem s.r.o., Bratislava, Slovakia, p.a.), gallic acid (Fisher Chemical, Loughborough, UK, HPLC grade), aluminum chloride (Centralchem s.r.o., Bratislava, Slovakia, p.a.), potassium acetate (Centralchem s.r.o., Bratislava, Slovakia, p.a.), quercetin (Fisher Chemical, Loughborough, UK, HPLC grade), Arnova reagent (10% $NaNO_2$+10% $Na_2MoO_4$) (Sigma-Aldrich,Merck KGaA, Darmstadt, Germany), caffeic acid (Fisher Chemical, Loughborough, UK, HPLC grade).

**Instruments**

HPLC system with an ELSD detector (Agilent Technologies 1260 Infinity, Santa Clara, CA, USA).

Vacuum degasser (Agilent Technologies, Santa Clara, CA, USA).

Quarterly pump (Agilent Technologies, Santa Clara, CA, USA).

Autosampler (Agilent Technologies, Santa Clara, CA, USA).

HPLC system with ninhydrin and a VIS detector (Model AAA-400 amino acid analyzer, Ingos, Czech Republic).

UV-VIS spectrophotometer (UV Jenway Model 6405, UV/VIS, England).

ICP-OES system (Ultima 2, Horiba Scientific, France).

ES column (Zorbax SB-C18, 4.6x25.0 mm, 5 μm particle size, Agilent, Santa Clara, CA, USA).

Centrifuge (EBA 21, Hettich, Germany).

Magnetic stirrer (Arex-6 Connect Pro, Velp Scientifica, Italy).

Microwave oven (Milestone 1200, Milestone, Italy).

Vertical shake table (GFL, Germany).

**Laboratory Methods**

***Determination of dry matter, ash, and protein content***

Total dry matter, ash, and protein content were determined according to the EN method **(CSN EN 12145, 1997)**. Total lipid content was determined according to methods specified in the ISO method **(ISO 659, 1998)**.

***Determination of saccharides***

For the determination of saccharides, 1 g of sample was extracted with 10 mL of extraction solution (ultrapure water and ethanol mixed in ration 4:1) in a 50 mL centrifugation tube placed on a vertical shake table (GFL, Germany). After 1 h of extraction, samples were centrifuged for 4 min at 6000 rpm in a centrifuge (EBA 21, Hettich, Germany); the supernatant was filtered using a filter with 0.45 μm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with ultrapure water. An Agilent Infinity 1260 liquid chromatography (Agilent Technologies, USA) equipped with an ELSD detector was used for the determination of saccharides. A Prevail Carbohydrates ES column (250/4.6 mm) was used as a stationary phase and acetonitrile (VWR) mixed with water in a 75:25 volume ratio was used as the mobile phase.

***Determination of carotenoid***

Total carotenoid content expressed as beta-carotene was analyzed at a wavelength of 445 nm spectrophotometrically (VIS spectrophotometer UV Jenway Model 6405 UV/VIS). Sample (1 g) was disrupted with sea sand and extracted with acetone until complete discoloration. Petroleum-ether was added and then water, in purpose to the separation of phases. After the separation, the petroleum ether-carotenoid phase was obtained and the absorbance was measured **(ČSN 560053, 1986)**.

***Determination of mineral contents***

Sample for elemental analysis was prepared using the wet ashing method in a microwave oven (Milestone 1200, Milestone, Italy). A total of 0.25 g sample matrix was decomposed in a mixture of nitric acid (6 mL) (Analytika Praha Ltd, Czech Republic) and hydrochloric acid (2 mL) (Analytika Praha Ltd, Czech Republic). After the decomposition sample was filtered using a filter with 0.45 μm pore size and filled up to 25 mL in a volumetric flask with ultrapure water. Elemental analysis was performed using ICP-OES (Ultima 2, Horiba Scientific, France) according to the procedure described by **Divis et al. (2015)**.

***Determination of amino acids***

Amino acids were determined by ion-exchange liquid chromatography (Model AAA-400 amino acid analyzer, Ingos, Czech Republic) using post-column derivatization with ninhydrin and a VIS detector. A glass column (inner diameter 3.7 mm, length 350 mm) was filled manually with a strong cation exchanger in the LG ANB sodium cycle (Laboratory of Spolchemie) with average particles size 12 μM and 8% porosity. The column was tempered within the range of 35 to 95 °C. The elution of the studied amino acids took place at a column temperature set to 74 °C. A double-channel VIS detector with the inner cell volume of 5 μL was set to two wavelengths: 440 and 570 nm. A solution of ninhydrin (Ingos, Czech Republic) was prepared in 75% v/v methyl cellosolve (Ingos, Czech Republic) and in 2% v/v 4 M acetic buffer (pH 5.5). Tin chloride ($SnC^{l2}$) was used as a reducing agent. The prepared solution of ninhydrin was stored in an inert atmosphere ($N^2$) in darkness at 4 °C. The flow rate was 0.25 ($mL.min^{-1}$) and the reactor temperature was 120 °C.

***Determination of 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.

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 0.1 mL 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 $mg.L^{-1}$; $R^2 = 0.9977$) 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 $mg.L^{-1}$, $R^2 = 0.999$) was used as a standard and the results were expressed in $mg.g^{-1}$ DM caffeic acid equivalents.

**Number of samples analyzed:** 15.
**Number of repeated analyses:** 3.
**Number of experiment replication:** 1.

## 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 ($p$ <0.05). The variability of all these parameters was evaluated using descriptive statistics.

## RESULTS AND DISCUSSION

Determining the chemical composition of *Pseudocydonia sinensis* is of great importance in studies into its nutritional aspects and use as raw material for industry. Proteins are macromolecules, structural units of which are called amino acids and play numerous functions that allow an organism to function and reproduce **(Day, 1996)**. The protein content in seeds, pulp, and peel was 13.20, 1.26, and 2.32%, respectively (Table 1). Protein content in *Cydonia oblonga* varied from 0.49 to 0.70 $g.100g^{-1}$ **(Leonel et al., 2016; Rasheed et al., 2018)**.

After the combustion process of the plant sample at high temperatures, the plant raw transforms into a mineral residue that consists of macro- and microelements. The results obtained show the differences between the parts of the plant and are 4.33, 2.42, and 2.46% in the seeds, pulp, and peel. According to **Leonel et al. (2016)** the fruits of different *Cydonia oblonga* cultivars contain between 0.5 and 0.8 $g.100g^{-1}$ ash, and for the results of **Rasheed et al. (2018)**, the ash content in fruits is 0.62 $g.100g^{-1}$.

Lipids are important structural components of membranes, concentrated in different plant parts and responsible for the growth and survival of the organism. There are essential components of food products **(Sebei et al., 2013)**. The content of lipids makes up in seeds, pulp, and peel was 22.95, 0.40, and 3.65%, respectively. Lipid content in the *Cydonia oblonga* was from 1.5 to 2.4 $g.100g^{-1}$ **(Leonel et al., 2016)**. **Rodríguez-Guisado et al. (2009)** reported lipid content in *Cydonia oblonga* similar to those observed in this study, varying from 1.31 to 2.33%.

Monosaccharide analysis of neutral carbohydrate part showed the presence of two main sugars – fructose (3.40, 34.46, and 26.00 $g.kg^{-1}$, respectively) and sucrose (9.65, 7.97, and 17.43 $g.kg^{-1}$, respectively) in the seeds, pulp, and peel, while other saccharides, such as maltose and lactose were found in low amounts only (<0.5 $g.kg^{-1}$).

**Rodríguez-Guisado et al. (2009)** analyzing the profile of sugars in *Cydonia oblonga* found levels of 5.31 to 10.89% for fructose, 4.08 to 5.44% for glucose, 1.51 to 2.41 of sucrose, and 0.31 to 0.42% for maltose totaling between 11.67 and 16.08% total sugars. **Leonel et al. (2016)** established the amount of total zinc in the range from 9.5 to 11.1 $g.100g^{-1}$. Behind the results of **Rasheed et al. (2018)**, the amount of reducing sugar was 5.15 $g.100g^{-1}$, and the amount of non-reducing sugar was 4.61 $g.100g^{-1}$.

*Pseudocydonia sinensis* contains beta carotene in seeds, pulp, and peel (0.93, 2.45, and 6.67 $mg.kg^{-1}$, respectively). The major quantitative tocopherol in *Pseudocydonia sinensis* seeds, pulp, and peel was α-tocopherol (67.26, 7.63, and 13.72 $mg.kg^{-1}$ DWP, respectively). The oil contents were 22.95 (seeds), 0.40 (pulp), and 3.65% (peel) dry weight plant material.

Total fatty acid profile demonstrated properties and uses of plant oils. Many plant species are an essential source of valuable fatty acid content **(Burčová et al., 2017; Matemu et al., 2017)**.

**Table 1** The contents of some phytochemical compounds of *Pseudocydonia sinensis* (Thouin) C. K. Schneid.

| Components | Seeds (*mean ±SD*) | Pulp (*mean ±SD*) | Peel (*mean ±SD*) |
|---|---|---|---|
| Total dry matter (%) | 91.67 ±2.65 | 90.23 ±2.16 | 92.67 ±1.38 |
| Total content of protein (%) | 13.20 ±0.22 | 1.26 ±0.06 | 2.32 ±0.11 |
| Total content of ash (%) | 4.33 ±0.18 | 2.42 ±0.09 | 2.46 ±0.07 |
| Total content of lipids (%) | 22.95 ±0.32 | 0.40 ±0.02 | 3.65 ±0.11 |
| Beta carotene ($mg.kg^{-1}$) | 0.93 ±0.07 | 2.45 ±0.10 | 6.67 ±0.15 |
| Saturated fatty acids ($g.100g^{-1}$ oil) | 14.40 ±0.10 | 55.94 ±0.18 | 27.86 ±0.16 |
| Monounsaturated fatty acids ($g.100g^{-1}$ oil) | 28.00 ±0.19 | 20.40 ±0.21 | 16.55 ±0.12 |
| Polyunsaturated fatty acids ($g.100g^{-1}$ g oil) | 40.65 ±1.20 | 14.81 ±0.17 | 37.80 ±0.19 |
| Fructose ($g.kg^{-1}$) | 3.40 ±0.08 | 34.46 ±0.19 | 26.00 ±0.22 |
| Maltose ($g.kg^{-1}$) | <0.5 | <0.5 | <0.5 |
| Sucrose ($g.kg^{-1}$) | 9.65 ±0.13 | 7.97 ±0.09 | 17.43 ±1.10 |
| Lactose ($g.kg^{-1}$) | <0.5 | <0.5 | <0.5 |
| Vitamin A (retinyl acetate) ($mg.kg^{-1}$) | <0.1 | <0.1 | <0.1 |
| Vitamin E (α-tocopherol) ($mg.kg^{-1}$) | 67.26 ±1.33 | 7.63 ±0.13 | 13.72 ±1.14 |

Note: mean – arithmetic mean; *SD* – standard error of the mean.

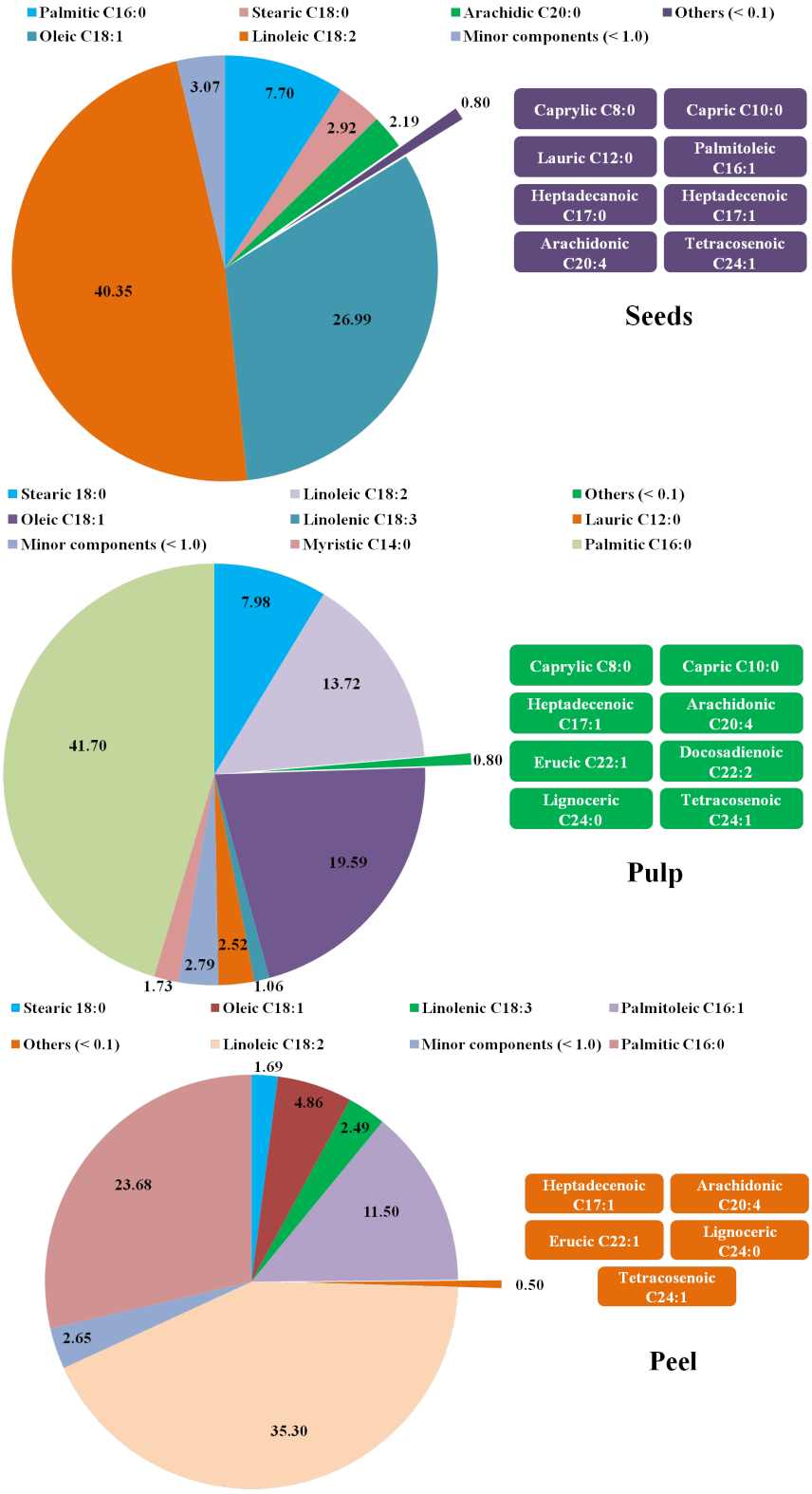


**Figure 3** Fatty acid composition of *Pseudocydonia sinensis* (Thouin) C. K. Schneid. Note: Minor components (<1.0): seeds (Myristic C14:0 (0.45); Linolenic C18:3 (0.28); Eicosenoic C20:1 (0.80); Behenic C22:0 (0.55); Erucic C22:1 (0.22); Docosadienoic C22:2 (0.20); Lignoceric C24:0 (0.57) their total amount is 3.07 g.100g$^{-1}$ oil); pulp (Palmitoleic C16:1 (0.28); Heptadecanoic C17:0 (0.73); Arachidic C20:0 (0.60); Eicosenoic C20:1 (0.54); Behenic C22:0 (0.64) their total amount is 2.79 g.100g$^{-1}$ oil); peel (Caprylic C8:0 (0.14); Capric C10:0 (0.10); Lauric C12:0 (0.42); Myristic C14:0 (0.51); Heptadecanoic C17:0 (0.37); Arachidic C20:0 (0.75); Eicosenoic C20:1 (0.10); Behenic C22:0 (0.10); Docosadienoic C22:2 (0.16) their total amount is 2.65 g.100g$^{-1}$ oil).

In this study, total fatty acids varied in different parts of *Pseudocydonia sinensis* and contained oleic acid, palmitic acid, linolenic acid, and linoleic acid. Linoleic acid in seeds accounted for 48.02% of total fatty acids, followed by oleic acid, accounting for 32.12% of total fatty acids (Figure 3). Palmitic acid was the minor fatty acid in leaves,

accounting for 9.16% of the total fatty acids. Unsaturated fatty acids were the predominant fatty acids in seeds, accounting for 82.41% of the total fatty acids while saturated fatty acids only accounted for 17.59%.

Palmitic acid, oleic acid, and linoleic acid in the pulp samples were 45.38, 21.32, and 14.93%, respectively. Stearic acid was the minor fatty acid in leaves, accounting for 8.68% of the total fatty acids. Saturated fatty acids were the predominant fatty acids in *Pseudocydonia sinensis* pulp, accounting for 61.16% of the total fatty acids, while unsaturated fatty acids accounted for only 38.84%.

In peel, linoleic acid, palmitic acid, and palmitoleic acid accounted for 42.70, 28.64, and 13.91% of total fatty acids, respectively. Oleic acid was the minor fatty acid in the peel, accounting for 5.88% of the total fatty acids. Unsaturated fatty acids were also predominant in the peel, which accounted for 66.30% of total fatty acid while saturated fatty acids accounted for 33.70%.

According to **Zhou et al. (2020)**, *Pseudocydonia sinensis* fruits were rich in oleanolic acid and ursolic acid, and from the twigs isolated five new oxylipins of chaenomic acid **(Kim et al., 2014)**. Amino acids are structural components of proteins and classified into essential and non-essential. Seeds and fruits are the most analyzed parts of plants for amino acid composition **(Kumar et al., 2019)**. Amino acid content has also been reported in various other fruit plants, namely apples **(Gomis et al., 1990)**, medlar **(Glew et al., 2003)**, quince **(Silva et al., 2004)**, plum **(Ogasanović, 2007)**, cherry **(Cubero et al., 2009)**, pawpaw **(Nam, Jang and Ha Rhee, 2018)**, Chinese chestnut **(Yang et al., 2018)**. There are no reports on free amino acid composition in *Pseudocydonia sinensis* fruits.

Amino acid analysis has shown that the studied *Pseudocydonia sinensis* seeds, pulp, and peel contained 18 amino acids (9 essential and 9 non-essential) (Figure 4).

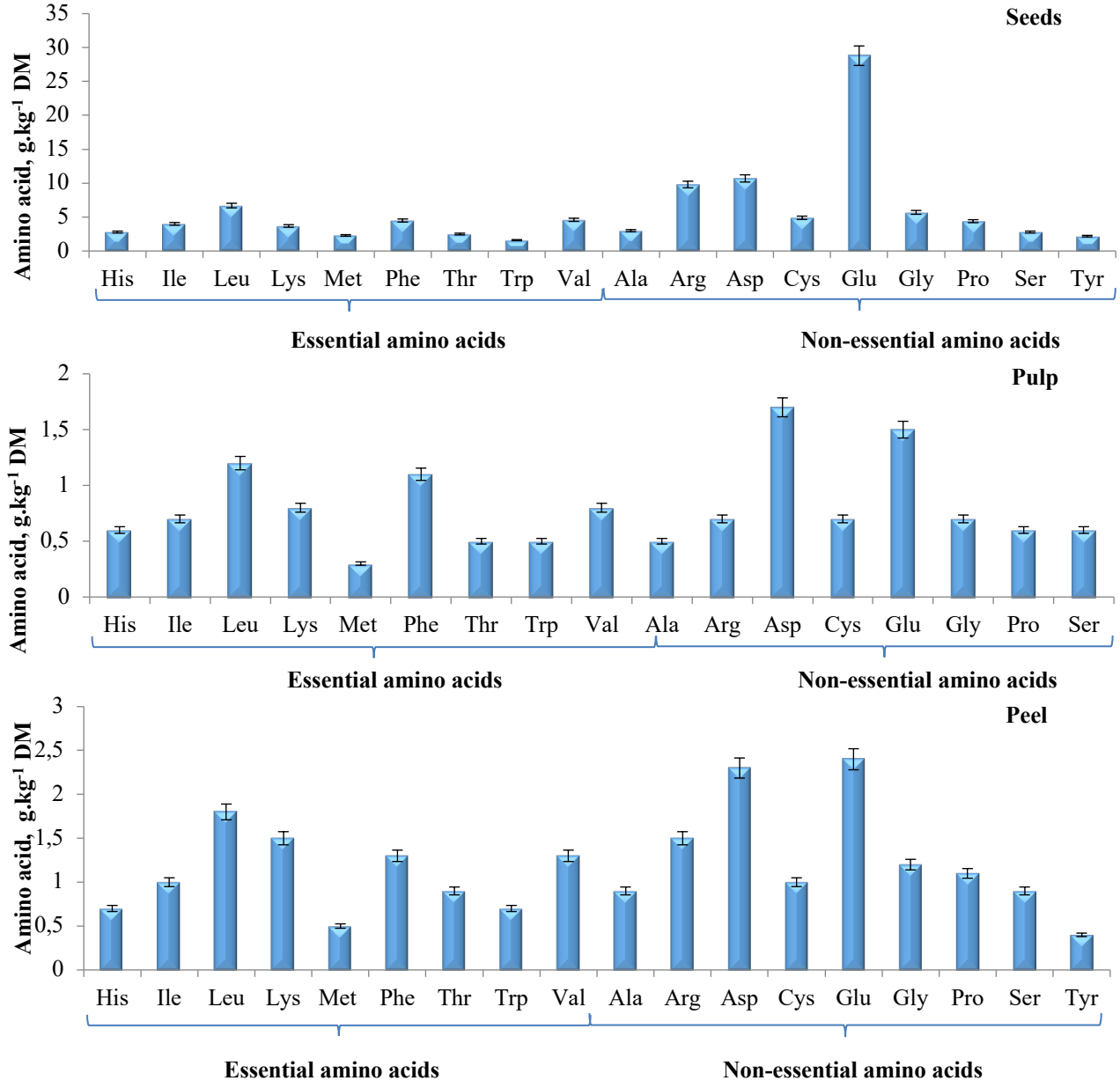


**Figure 4** Amino acid composition of *Pseudocydonia sinensis* (Thouin) C.K. Schneid. seeds, pulp and peel (g.kg$^{-1}$ DM).

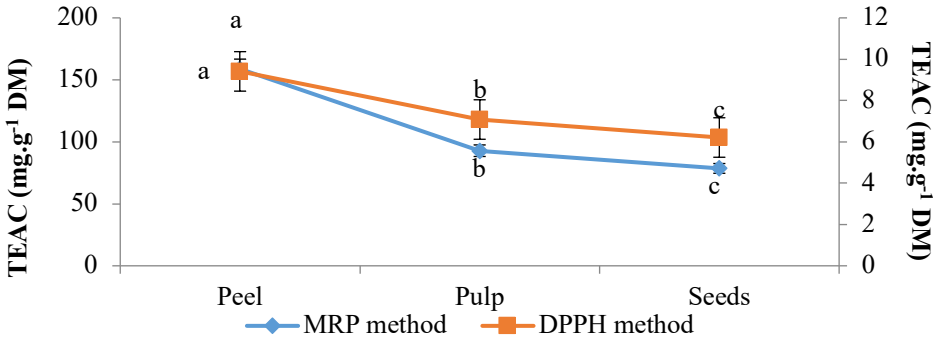


**Figure 5** Antioxidant activity of the peel, pulp and seeds of the fruit of *Pseudocydonia sinensis* (Thouin) C. K. Schneid. Note: evaluated by the DPPH method and molybdenum reducing antioxidant power (different superscripts in each column indicate the significant differences in the mean at $p$ <0.05): TEAC – Trolox equivalent antioxidant capacity.

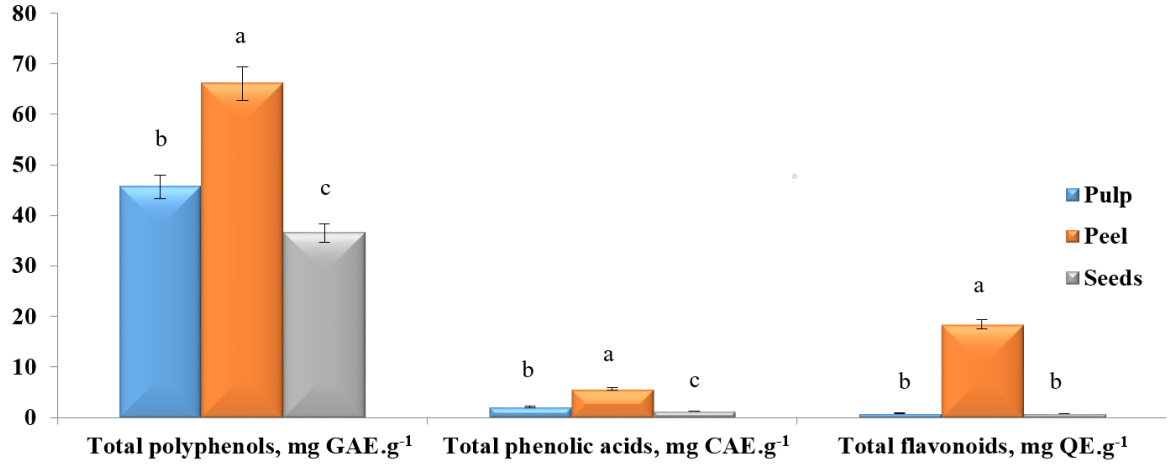


**Figure 6** Total polyphenols, phenolic acids and flavonoids of the peel, pulp and seeds of *Pseudocydonia sinensis* (Thouin) C.K. Schneid. Note: different superscripts in each column indicate the significant differences in the mean at $p$ <0.05.

The total amount of amino acids found in the seeds was 105.0 g.kg$^{-1}$ DM, including total essential amino acids (32.70 g.kg$^{-1}$ DM) and percentage of total essential amino acids (31.14%). Glutamic acid was found to be the dominant free amino acid (28.8 g.kg$^{-1}$) in seeds followed by aspartic acid (10.7 g.kg$^{-1}$) and arginine (9.8 g.kg$^{-1}$). In the peel and pulp, the total amino acid content is found much than in the seeds, amounting to 21.4 and 13.7 g.kg$^{-1}$ DM, respectively. The total non-essential amino acids in peel and pulp amounting to 11.7 and 7.2 g.kg$^{-1}$ DM, respectively, and the percentage of total essential amino acids amounting to 9.7 and 6.5%, respectively).

The *Cydonia oblonga* fruits with 21 free amino acids identified. The sum of the 21 free amino acids ranged from approximately 316 to 1357 mg.kg$^{-1}$ for *Cydonia oblonga* pulps and from 512 to 1820 mg.kg$^{-1}$ for *Cydonia oblonga* peels. In what concerns the quince pulps, generally, the three most abundant free amino acids were aspartic acid, hydroxyproline, and asparagine. As a general rule, the three most abundant compounds present in quince peels were glycine, aspartic acid, and asparagine **(Silva et al., 2004)**.

At present, little is known about the levels of trace elements in *Pseudocydonia sinensis* fruits and their parts such as seeds, peel, or pulp. The average contents of the elements in the different parts of *Pseudocydonia sinensis* are shown in Table 2.

Macroelement and trace element concentrations in the seeds samples revealing the following trend: K > P > Mg > Ca > S > Zn > Fe > Cu > Mn > Na > Al > Ni > As > Cr > Se > Pb > Cd > Hg. These elements were also detected in pulp samples according to the following order: K > Ca > P > Mg > S > Fe > Na > Zn > Cu > Al > Mn > Se > Ni > As > Cr > Pb > Cd > Hg. In the peel samples, the following concentrations were observed: K > Ca > P > Mg > S > Fe > Zn > Na > Cu > Al > Mn > Se > Ni > As > Cr > Pb > Cd > Hg.

Among biological activities inherent in plant raw material can be highlighted an antioxidant activity that had widely studied last time. A plant raw is a valuable source of antioxidants with different nature that has a therapeutic value for human health. The study of antioxidant capacity was carried out by different methods **(Gupta, 2015)**.

In our study, the antioxidant activity carried out by the DPPH method of *Pseudocydonia sinensis* peel, pulp and seeds were 9.41, 7.08, and 6.21 mg TEAC.g$^{-1}$ DM, respectively (Figure 5).

**Table 2** Mineral composition of *Pseudocydonia sinensis* (Thouin) C.K. Schneid. seeds, pulp and peel (mg.kg$^{-1}$).

| Components | Seeds (*mean ±SD*) | Pulp (*mean ±SD*) | Peel (*mean ±SD*) |
|---|---|---|---|
| **P** | 5189 ±218 | 1015 ±121 | 846 ±68 |
| **K** | 10381 ±203 | 12101 ±236 | 7572 ±337 |
| **Ca** | 2863 ±95 | 1285 ±119 | 3045 ±278 |
| **S** | 1670 ±77 | 441 ±58 | 460 ±58 |
| **Fe** | 36.0 ±1.4 | 36.0 ±1.8 | 27.0 ±0.4 |
| **Mn** | 13.2 ±0.5 | 1.7 ±0.05 | 2.3 ±0.1 |
| **Mg** | 3589 ±68 | 650 ±97 | 644 ±68 |
| **Na** | 7.0 ±0.09 | 10.0 ±0.9 | 11.0 ±1.1 |
| **Al** | 1.6 ±0.04 | 2.4 ±0.03 | 4.7 ±0.2 |
| **Cr** | <0.2 | <0.2 | <0.2 |
| **Cu** | 22.0 ±1.8 | 5.0 ±0.01 | 6.0 ±0.5 |
| **Zn** | 45.0 ±1.4 | 9.0 ±1.1 | 15.0 ±0.9 |
| **Se** | <0.2 | 1.03 ±0.01 | 1.48 ±0.7 |
| **As** | <0.3 | <0.3 | <0.3 |
| **Cd** | 0.020 ±0.001 | 0.017 ±0.001 | 0.029 ±0.004 |
| **Ni** | 1.35 ±0.01 | 0.51 ±0.002 | 0.53 ±0.010 |
| **Hg** | 0.005 ±0.0001 | 0.005 ±0.0001 | 0.010 ±0.004 |
| **Pb** | <0.1 | <0.1 | <0.1 |

Note: mean – arithmetic mean; SD – standard error of the mean.

The antioxidant activity of peel, pulp, and seeds extracts measured by molybdenum reducing antioxidant power was from 158.81, 92.83, and 78.58 mg TEAC.g$^{-1}$ DM, respectively.

Earlier in our study **(Grygorieva et al., 2020)** the antioxidant activity of peel and pulp of *Pseudocydonia sinensis* of different genotypes growing in the arboretum (Slovakia) also confirmed the higher antioxidant potential of peel extracts compared to a pulp. Our results were similar according to previous studies that confirmed the higher antioxidant activity of the peel of *Pseudocydonia sinensis* than the pulp **(Monka et al., 2014)**. The higher antioxidant activity of peels of other fruits than pulp has been widely reported, namely mango **(Ajila et al., 2007)**, different varieties of peach **(Liu et al., 2018)**, Chinese jujube **(Xue et al., 2009)**. In a study by other authors, the total antiradical activity of aqueous and methanolic extracts of dry peels was 91.87 – 93.25% and that of dry pulp 80.39 – 84.11% **(Monka et al., 2014)**. In a study by **Baroni et al. (2018)**, the antioxidant activity of *Cydonia oblonga* Mill. pulp, peel, seed, and jam extracts evaluated by the DPPH assay identified that methanolic peel extracts demonstrated the strongest activity, followed by pulp and seed extracts. A study by **Silva et al. (2004)** showed that the phenolic fraction of the seed extracts have stronger antioxidant activity than the peel and pulp extracts. Also, the different studies determined that the antioxidant activity by the DPPH method of methanol extracts of *Malus domestica* Borkh. cultivars were higher in peel extracts (71.7 – 84.9%) than in pulp ones (43.9 – 52.8%) **(Manzoor et al., 2012)**.

Polyphenols are a large group of organic compounds with antioxidant and anti-inflammatory properties that may play a vital role in metabolic processes in the human body **(Cory et al., 2018)**. **Osawa et al. (1999)** and **Oku, Ueda and Ishiguro (2003)** report that the polyphenols of *Pseudocydonia sinensis* are considered to be the most important biologically active ingredients because of their various pharmacological actions and high content. The content of total polyphenols (Figure 6) in the peel, pulp, and seeds was from 66.06, 42.02, and 36.05 mg GAE.g$^{-1}$ DM, respectively.

It was previously reported that the content of total polyphenols in the peel and flesh in different genotypes of *Pseudocydonia sinensis* growing under Slovakian conditions, the content of total polyphenols in the peel and flesh was between 55.61 and 82.02 and between 34.73 and 66.99 mg GAE.g$^{-1}$ DM, respectively **(Grygorieva et al., 2020)**. Studies by **Manzoor et al. (2012)** and **Al-Snafi (2016)** confirmed the high content of phenolic compounds in the rinds of *Malus domestica* and *Cydonia oblonga*.

Flavonoids are a group of natural substances that play variable biological activities as well as other polyphenol compounds such as anti-inflammatory, antimutagenic, anticancer, antioxidative, etc. **(Panche, Diwan and Chandra, 2016)**.

The total flavonoid content in the peel, pulp, and seeds was 18.39, 0.80, and 0.75 mg QE.g$^{-1}$ DM, respectively. The total flavonoid content in the peel and pulp of *Pseudocydonia sinensis* fruits of different genotypes growing in Slovakia was 11.00 to 26.72 and 0.59 to 1.07 mg QE.g$^{-1}$ DM, respectively **(Grygorieva et al., 2020)**. It was previously reported **(Amirahmadi, Abdollahi and Ayyari, 2017)** that the total flavonoid content in fruits of closely related *Cydonia oblonga* species was 6.2 mg QE.g$^{-1}$.

Phenolic acids are a large group of phenolic compounds that possess numerous biological activities, among which antioxidant action **(Kumar and Goel, 2019)**.

It was found that the total phenolic acid content varies significantly between samples (Figure 6). The content of phenolic acids in the peel, pulp, and seeds was 5.68, 2.08, and 1.23 mg CAE.g$^{-1}$ DM, respectively.

The content of phenolic compounds in the fruit of *Pseudocydonia sinensis* was in agreement with previous research **(Hamauzu et al., 2006; Grygorieva et al., 2020)**. According to our previous studies **(Grygorieva et al., 2020)** of fruits from Slovakia, the total phenolic content in peel and pulp was 4.20 – 8.39 and 1.12 – 3.97 mg CAE.g$^{-1}$ DM. According to **Hamauzu et al.**

**(2005)**, the total content of phenols in fruits of *Pseudocydonia sinensis* was 1280 mg.100g$^{-1}$ FW. This was four and twenty times higher than in *Cydonia oblonga* and *Malus domestica*, respectively. Differences between present and previously conducted studies in the chemical composition of fruit of *Pseudocydonia sinensis* could be attributed to the geographical plant origin and different methods of extraction. This has been previously reported in other plant species, namely *Diospyros virginiana* **(Grygorieva et al., 2018)**, *Sambucus nigra* **(Horčinová Sedláčková et al., 2018; Horčinová Sedláčková et al., 2019)**, *Ziziphus jujuba* **(Ivanišová et al., 2017)**, *Corylus avellana* **(Nikolaieva et al., 2019)**, *Solidago canadensis* **(Shelepova et al., 2019)**, *Hippophae rhamnoides* **(Ivanišová et al., 2020)**.

## CONCLUSION

The chemical composition, antioxidant activity, total polyphenol, flavonoid, and phenolic acid content of peel, pulp, and seeds extracts from *Pseudocydonia sinensis* were studied. The content of micro and macronutrients was found in the seeds much higher than in the pulp and peel. It should also be noted that all *Pseudocydonia sinensis* samples are a very valuable source of potassium, which is necessary for the water and electrolyte balance. Total fatty acids predominate in the pulp than in the peel and seeds. Total amino acids predominate in the seeds than in the peel and pulp. The results revealed the highest content of bioactive compounds (total polyphenols, flavonoid, and phenolic acid) and antioxidant activity in peel compared with pulp and seeds. This study demonstrates the potential application of *P. sinensis* as a valuable source of natural phenolic antioxidants and can be used as raw material to elaborate diverse food products, providing important functional properties. When introduced in Ukraine and Slovakia, the amount of beneficial substances in fruits and seeds is not reduced, which makes it possible to recommend this species for wide cultivation.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE EFFECT OF DRY DEMINERALIZED WHEY (DDW) AND COCONUT OIL ON THE RHEOLOGICAL CHARACTERISTICS OF THE LEGUME BUTTER

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## ABSTRACT

This article reveals the possibility of using legume butter to obtain a new combined product of increased quality. The technology of model compositions of finishing semi-finished products, in particular, confectionery legume butter with DDW, has been developed. The results of a study on optimizing the recipe composition of finishing semi-finished products made it possible to develop this technology of multifunctional confectionery butter with the desired (specified) organoleptic and structural and mechanical properties. The analysis of the formation of the structure of legume butter of oppositely directed processes in the enzymatic destruction of the residual cellular structure and spontaneous aggregation of protein particles into the spatial structure due to gradual dehydration of the system was carried out. It is noted that sugar plays the role of a plasticizer, reducing the rate of gelatinization of starch while reducing the viscosity peak. The use of dry demineralized whey (DDW) in legume butter technologies contributes to the formation of a stronger structure of the protein component and the development of a spatial structure, which gives it certain structural and mechanical properties. Three main directions of technological use of confectionery butter were established, which are based on structural characteristics of consistency: 1 – confectionery butter used to cover confectionery products and as a layer for them (CP – Confectionery Products), where the main indicator of consistency is extensibility; 2 – confectionery butter used for making flowers, as a decorative element for flour confectionery (SC – Sugar Confectionery)), where the main indicator of consistency is an equivalent ratio of extensibility and forming ability; 3 – confectionery butter used for modeling shaped products, as a decorative element for flour confectionery products, where the main indicator of consistency is the forming ability. The results of studies of the rheological characteristics of legume butter with dry demineralized whey (DDW) and coconut oil are presented. It was ascertained that DDW and coconut oil lead to changes in the state of legume butter, changing the values of rheological characteristics. The rational content of DDW and coconut oil in the composition of legume butter while ensuring high functional and technological properties was proved. The optimal ratios of components of the developed legume butter were mathematically grounded.

**Keywords:** legume butter; dry whey powder; bean; rheological properties; modeling

## INTRODUCTION

Food products significantly affect human health throughout life and therefore pose a significant potential danger. WHO data show **(WHO, 2015)** that more than 80% of all diseases of adults and children are more or less associated with eating disorders (cardiovascular diseases, type 2 diabetes mellitus (DM), osteoporosis, etc.). The nutritional value of confectionery products **(Nazarova and Chuprina, 2018; Dzjuba et al., 2017)** is and remains an existing complex of carbohydrates, proteins, fats, minerals, vitamins, etc. Therefore, when researching and implementing new technologies for confectionery and semi-finished products, it is still necessary to improve the range of children's and dietary food. The solution of problems recently is based on the addition of fillers of the recipe composition to confectionery butter masses, while the product is enriched with biologically active substances. After all, a necessary complex of substances for the human body, butter has a multifunctional use in confectionery production.

For the creation of new types of food products of increased biological value, there is considerable interest in non-traditional and local kinds of raw materials such as plant-based products, which are cheaper and less labor-intensive during production. We are aware **(Diakonova, 2006)** that raw materials of vegetable origin are divided into several groups: fruit and vegetable products, vegetable products, leguminous products, starch products. First of all, such requirements are met by cereals and legumes.

They have the features necessary for improvement: they are harmless to the human body, generally available, cheap, and most importantly, they are a natural source of finished products enriched with proteins, fats, dietary fiber,

vitamins, minerals, polyphenolic compounds, and other biologically active substances. Rational use of vegetable raw materials and the creation of various forms of food protein (protein flour, concentrates, texturates, isolates) on its basis is a promising direction for solving the problem of reducing protein deficiency.

Modern technologies of the confectionery industry enable the creation of products with a variety of taste characteristics, smells, textures, shapes, which attract the attention of consumers and increase demand. Recently, special attention has been paid to the production of legumes, which are of great importance for improving the supply of food to the population. Currently, a large number of samples of the leguminous collection, including beans, have been collected. The study of some biochemical parameters of these samples **(Ryabchenko and Ulyanchenko, 2008; Balia, 2011)** is of great theoretical and practical value.

Vegetable protein is the most important component of food and feed resources, the use of which significantly affects the state of human health, their well-being, duration, and standard of living. This has become especially important nowadays when there is a considerable increase in the population of our planet, which leads to protein starvation in some countries. At the end of the twentieth century, the share of vegetable protein **(Ryabchenko and Ulyanchenko, 2008)** was 70%, and 30% accounted for animal protein in the total balance of this product.

Vegetable protein deficiency remains one of the most important problems of our time. In recent years, it has not lost its topicality. Most scientists tend to believe that this period should be the century of leguminous crops, due to which the task of increasing the production of vegetable protein for the needs of animal husbandry and nutrition of the population will be solved. In the conditions of modern agricultural production, replenishing the protein deficit at the expense of their resources is becoming an urgent need.

At the present stage, one of the causes of protein deficiency is the insufficient use of plant resources, in particular representatives of the Fabaceae family of species of the genus Phaseolus vulgaris, which includes about 200 species. However, the high productivity of bean plants is possible only if they are maximally provided with nutrients.

Bean is a valuable high-protein crop **(Mazaraki, Kravchenko and Demichkovskaya, 2019)**, which is widely used in the food industry, medicine, cosmetics. The grain contains substances that contribute to the elimination of radionuclides out of the human body **(Skurikhin and Volgarev, 1987)**. Its importance in the national economy is determined by its high taste and nutritional qualities. Bean products can not only meet human needs for vegetable protein but also diversify the diet, so they are in great demand among the population. The most important nutritional component of bean seeds is proteins **(Skurikhin and Volgarev, 1987)**, which are involved in the most important functions of the body and cannot be replaced by other food substances.

Bean has a high potential yield, which is realized if agrotechnical requirements and recommendations are met. Its average yield in the world is about 0.07 $t.ha^{-1}$, and under optimal conditions, it reaches 3.0 – 4.5 $t.ha^{-1}$. Especially as a food item **(Mazaraki, Kravchenko and Demichkovskaya, 2019; Lukomets, 2012)**, beans are in high demand. Its grain contains up to 32% protein, which is close in nutritional value to animal proteins. In addition to protein, the grain contains 41.0 – 54.6% carbohydrates, 0.4 – 3.6% fat, 2.2 – 6.6% fiber, vitamins E, B1, B2, B6, C, pathogenic acid, riboflavin, minerals.

A special feature of the bean is its rich mineral composition which are iron (up to 8%), calcium, phosphorus, potassium, sodium, magnesium, iodine, fiber, citric acid, ash substances. As confirmed by these data, the bean is a food product that contains almost all the substances necessary for normal human nutrition, which puts it among dietary products.

In modern society, the industrialization and globalization of food production, as well as advertising in the media, are increasingly affecting the quality of food, so it is important not to weaken the role of scientific developments and findings of specialists. The bean has long been a traditional crop in Ukraine, but it is not widely distributed. Therefore, further development of the scientific foundations of the technology of its use in the confectionery industry is an important scientific problem.

**Analysis of model approximations of medium types**

Given the current market needs, legumes are playing an increasingly important role in the food development process. Some of them, for example, soy **(Avdeyeva, 2003; Lukomets, 2012)** have long been massively used in the manufacturing of products with combined meat and vegetable composition as a structural and protein additive. Similar studies are also conducted for other legumes – bean, pea, chickpea. The initial assessment of the possibility of using pea and bean butter for organoleptic parameters was carried out in the works **(Avdeyeva, 2003)**. Samples with the addition of beans had high taste properties, which is an important component for semi-finished products.

Various botanical varieties of the white and red bean are widely used in confectionery production, especially in Japan, China, and Korea. Quite clearly, their works **(Honda et al., 2020; Li et al., 2017)** revealed a sweet bean butter made by boiling sugar with beans. It is widely used for gluing cakes, pastries, cookies, as a layer for desserts and flour confectionery, for making sweets, bars and as a plastic mass for making flowers and modeling shaped decorative elements. These developed technologies make it possible to obtain confectionery bean butter of high biological and nutritional value, but it has rather low organoleptic characteristics due to the inherent specific taste and aroma of legumes.

At the same time, to solve the relevant technological problems of bakery and confectionery production, certain types of specialized fat products are being developed, which allows optimizing the manufacturing technology of products of increased nutritional value and storage stability. To ensure the appropriate functional orientation, searches are underway to include valuable additional ingredients in the formula, in particular those with a balanced fatty acid composition, a reduced content of saturated fatty acids, the presence of essential substances of a lipid nature, with a minimum content of cholesterol and trans isomers of fatty acids **(Avdeyeva, 2003)**. The

scientific direction of technological processes can regulate the nutritional value to a certain extent.

The research **(Nazarova and Chuprina, 2018)** reveals the choice of process parameters according to the nature of changes in the structure and properties of prescription components in general. We see a direct connection between the importance of nutrition in human life due to the improvement of formula and technological parameters of production. It is known **(Kravchenko and Rybchuk, 2018)** that legume butter is obtained by boiling sugar and beans in a ratio of 1:1. As a result of this interaction, a complex homogeneous system related to coagulation structures is formed. The process of structure formation is determined primarily by the composition and structure of legume cells and the physical condition of polymers.

The author analyzes the comparative characteristics of the chemical composition of individual legumes **(Kholodova, 2013)**. In terms of the amount of essential amino acids per 100 g of protein, bean dominates over other legumes, except chickpea (Figure 1).

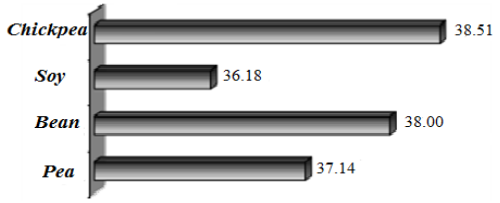



**Figure 1** Content of the total amount of essential amino acids in legumes, g,100g$^{-1}$ of protein.

That is why scientists have recently increased their interest in legumes as an alternative raw material for the production of pastes. At the initial stage of the interaction of the bean with water, swelling occurs, accompanied by a significant increase in volume and mass. During the cooking stage, the bean peeled from the seed shell undergoes complex physical and chemical changes. This causes the resulting mixture to soften. Temperature parameters have an impact on the course of the process. So, an increase in temperature accelerates the movement of moisture inside the cotyledon of beans. Accordingly, the process of swelling of protein substances and carbohydrates of cell walls is more intense, and the gelatinization of starch, which has already begun, accelerates.

Proteins are denatured during cooking, and the moisture absorbed by them is released and absorbed by the gelatinizing starch. In the process of cooking the cell walls of cotyledons of beans are destroyed under the influence of penetrating moisture and temperature. Pectin substances are most destructed **(Avdeyeva, 2003)**, with an average of 40%, to a lesser extent hemicellulose and structural protein of the cell walls of extensions. Under the pressure of gelatinized starch, cell walls break. This leads to the complete decomposition of the integrity of the cotyledons' shape.

The formation of the structure of legume butter **(Kravchenko and Rybchuk, 2019a)** is due to the development of two oppositely directed processes. The first one consists of enzymatic destruction of the residual cellular structure, and the second process is a spontaneous aggregation of protein particles into the spatial structure as a result of gradual dehydration of the system. Sugar in this technology plays the role of a plasticizer. By forming binding zones, sugar reduces the rate of starch gelatinization while reducing the peak viscosity.

The use of dry demineralized whey (DDW) is considered in sufficient detail in our work **(Kozlova, 2014)**. Therefore, due to its high moisture-binding and moisture-retaining ability, it will contribute to the formation of a stronger structure of its protein component in legume butter technologies due to the emergence and development of a spatial structure. As a result of thermal denaturation and coagulation of proteins dissolved in the continuous phase of the dispersion medium, under the appearance of stronger hydrogen bonds, it gives the framework a static shear strength. This allows giving certain structural and mechanical properties to the legume butter.

According to the results of previous studies, it was found **(Kravchenko and Rybchuk, 2019b; Stadnyk et al., 2020)** that DDW at a concentration of 10 – 30% improves the rheological characteristics of legume butter. Increasing the forming capacity indicators gives the butter certain functional and technological properties. Certain concentrations help to "mask" the taste and aroma of legumes. Also, a harmonious taste and aroma composition are created with improved organoleptic characteristics of legume butter. This butter can be used in confectionery products, as finishing semi-finished products.

However, an increase in the concentration of DDW in legume butter technologies contributes to an increase in the adhesive and cohesive strength, which makes it difficult to work with butter. Bean butter, in comparison with sugar one, is characterized by lower strength, so to reduce the adhesive-cohesive strength, it is advisable to consider the fat component, which at a temperature of 21 °C has a solid consistency. This is due to certain crystallization characteristics, which are determined by the composition of fatty acids. As it is known **(Kravchenko, and Rybchuk, 2019a; Stadnyk et al., 2019)**, fats dominated by saturated fatty acid glycerides have a higher hardness. According to the results of physical and chemical properties, the optimal characteristics were determined in coconut oil. By chemical composition, coconut oil is characterized by a high content of saturated fatty acids, in particular lauric and myristic, as well as a small number of volatile acids, namely caprylic and caproic. Coconut oil has high oxidation resistance, this is because 90% of fatty acids are saturated. Therefore, the choice of coconut oil in the formula of legume butter with DDW is explained by acceptable physical and chemical, and organoleptic characteristics, which will help to improve the functional and technological properties of confectionery butter. The consistency and taste of the product depending on the structural and mechanical characteristics of legume butter. Therefore, when manufacturing products, it is necessary to choose the optimal composition of ingredients according to the recipe.

Based on partial research conducted by various authors and our specialists, it was decided to develop a technology for model compositions of finishing semi-finished products, in particular, confectionery legume butter with DDW and additional recipe components. The results of a study on optimizing the formula of finishing semi-finished

products make it possible to develop this technology of multifunctional confectionery butter with the desired (specified) organoleptic and structural-mechanical properties.

We have proposed 3 main directions of technological use of the developed confectionery butter, which are based on the structural characteristics of the consistency. The first category includes confectionery butter used to cover confectionery products and layer them (CPC). The main indicator of consistency is extensibility.

The second category includes confectionery butter used for making flowers as a decorative element for flour confectionery products (CPD). The main indicator of consistency is an equivalent ratio of extensibility and forming ability.

The third one consists of confectionery butter used for figured products modeling, as a decorative element for flour confectionery products (FPM). The main indicator of consistency is the forming ability.

## Scientific hypothesis

The perspective for using white bean of the "Mavka" cultivar with the optimal amount of DDW additive and coconut oil as a possible component of determining the effect of the consistency regulator on rheological properties, to improve the functional and technological properties of butter.

## MATERIAL AND METHODOLOGY.

### Samples

The work uses the components that are required according to the recipe in the production of this system of bean pastes with the relevant current regulations documentation: white food beans variety "Mavka"; Dry demineralized whey; Coconut oil; Granulated sugar.

Dry demineralized whey, made from whey with a 90% level of demineralization following TU U 15.5-00413890-089:2014, white food beans of the Mavka variety. Bean variety belongs to the group of early, yield-resistant to various diseases, has high taste properties, oval-cylindrical shape, smooth. Manufacturer Odessa, TM "Spring". Coconut oil was bought in a store in Kyiv. Manufacturer: Green Volley Oils. India. Packaged by PKF Aroza LLC, Ukraine, Kyiv region. Granulated sugar - GOST 21-94 Rivne, Ukraine, Factory of confectionery jewelry "Decoration" – TU U 10.8-40570177-001:2016.

### Chemicals

For the first stage of cooking, water (chemical formula $H_2O$) was used for soaking, washing, cooking. Water corresponds to the national standard TU U 2.14-0051232-098:2007.

### Instruments

Elastic-elastic and plastic-viscous properties were determined on a plane-parallel elastoplastomer of Tolstoy, made in Russia. Adhesion and cohesion strength was performed on a dynamometer MIG1.3, producer limited liability company ITM KharkivThe temperature was measured with a thermometer TLS Ukrainian manufacturer "Glass Device", which is designed to accurately measure temperature. The obtained results were statistically processed using the standard Microsoft Office software package.

### LaboratoryMethods

Sampling was performed according to **DSTU 4803 (2013)**, Organoleptic evaluation "Descriptive (qualitative) method of profile analysis" **GOST 5897-90 (1992)**.

### *Method of researches of rheological characteristics of model compositions*

The method of rheological characteristics is based on the construction of a graph of the yield curve of the prototype under the action of the applied shear stress (under loading and unloading). It is used to establish the elastic-elastic and plastic-viscous properties of the test samples (Tolstoy's plane-parallel elastoplastometer). During the study of the rheological characteristics of the samples, a fixed load of 65 g was selected at the same temperature (+6 °C) and sample height (7 mm). The separation of the total deformation into inverse and irreversible was carried out by a simplified method of extrapolation of the linear section of deformation to the intersection with the y-axis $\gamma = f(t)$ (proposed by Rebinder). This makes it possible to determine the relative deformation, shear stress, modulus of elasticity, modulus of elasticity, plastic viscosity according to known formulas. Adhesion-cohesive strength - by the method of normal separation.

### *Research of organoleptic characteristics of model compositions*

To determine them, the method of developing organoleptic scales was used: indicators of appearance, taste, smell, consistency, molding ability. It is used when the composition of the sample is known, and the approximate number of them changes the characteristics. This makes it possible to bring the composition of the components as close as possible to the composition of the samples. following the distribution of weights, identified areas of technological use of confectionery pastes. The ability to form and the degree of detection of this indicator determine the manufacturability of confectionery pastes. For PKV the ratio of weights in terms of consistency is 0.1:0.1:0.7:0.1, for VCC – 0.2:0.2:0.3:0.3, for IRF – 0.3:0.2:0.2:0.3. The weighting factor of this indicator concerning the weighting factor of the consistency and determines the direction of technological use of confectionery pastes, which is for PKV 0.4:0.2, VTSK – 0.3:0.3, IRF – 0.2:0.4.

Weights were determined by an expert method under the following conditions (1):

$$\sum_{i=1}^{n} m_{ij} = 1 \qquad (1)$$

Where:

$m_{ij}$ is the weighting factor of the i-th indicator of the j-th group (m and >0); n is the number of product quality indicators.

**Table 1** Score scale of organoleptic quality assessment of model compositions of legume butter with DDW.

| Complex indicators | Weighting coefficient | | | Individual indicators | Weighting coefficient | | | Characteristics | Quality level, points |
|---|---|---|---|---|---|---|---|---|---|
| | CPC | CPD | FPM | | CPC | CPD | FPM | | |
| Appearance | 0.2 | 0.2 | 0.2 | Color intensity | 0.9 | 0.1 | 0.9 | Strong | 5 |
| | | | | | | | | Weak | 4 |
| | | | | | | | | Noticeable | 3 |
| | | | | | | | | Barely noticeable | 2 |
| | | | | | | | | Absent | 1 |
| | | | | Transparency | 0.1 | 0.9 | 0.1 | Strong | 5 |
| | | | | | | | | Weak | 4 |
| | | | | | | | | Noticeable | 3 |
| | | | | | | | | Barely noticeable | 2 |
| | | | | | | | | Absent | 1 |
| Taste and smell | 0.2 | 0.2 | 0.2 | Distinctness (of the bean taste and smell) | 0.4 | 0.4 | 0.4 | Strong | 1 |
| | | | | | | | | Moderate | 2 |
| | | | | | | | | Weak | 3 |
| | | | | | | | | Barely noticeable | 4 |
| | | | | | | | | Absent | 5 |
| | | | | Saturation (of the bean taste and smell) | 0.3 | 0.3 | 0.3 | Strong | 1 |
| | | | | | | | | Moderate | 2 |
| | | | | | | | | Weak | 3 |
| | | | | | | | | Barely noticeable | 4 |
| | | | | | | | | Absent | 5 |
| | | | | Balance | | | | Excellent | 5 |
| | | | | | | | | Good | 4 |
| | | | | | | | | Satisfactory | 3 |
| | | | | | | | | Unsatisfactory | 2 |
| | | | | | | | | Bad | 1 |
| | | | | – | 1.0 | 1.0 | 1.0 | – | – |
| Consistence | 0.4 | 0.3 | 0.2 | Density, strength | 0.1 | 0.2 | 0.3 | Moderately compacted | 5 |
| | | | | | | | | Compacted | 4 |
| | | | | | | | | Dense | 3 |
| | | | | | | | | Very dense | 2 |
| | | | | | | | | Solid | 1 |
| | | | | Stickiness | 0.1 | 0.3 | 0.3 | Average | 5 |
| | | | | | | | | Moderate | 4 |
| | | | | | | | | Satisfactory | 3 |
| | | | | | | | | High | 2 |
| | | | | | | | | Too high | 1 |
| | | | | Softness | 0.1 | 0.2 | 0.2 | Moderately soft | 5 |
| | | | | | | | | Soft | 4 |
| | | | | | | | | Moderately solid | 3 |
| | | | | | | | | Solid | 2 |
| | | | | | | | | Too solid | 1 |
| | | | | Extensibility | 0.7 | 0.3 | 0.2 | Excellent | 5 |
| | | | | | | | | Good | 4 |
| | | | | | | | | Average | 3 |
| | | | | | | | | Weak | 2 |
| | | | | | | | | Very weak | 1 |
| | | | | – | 1.0 | 1.0 | 1.0 | – | – |
| Forming ability | 0.2 | 0.3 | 0.4 | Detection degree | 1.0 | 1.0 | 1.0 | Excellent | 5 |
| | | | | | | | | Good | 4 |
| | | | | | | | | Satisfactory | 3 |
| | | | | | | | | Unsatisfactory | 2 |
| | | | | | | | | Bad | 1 |
| | | | | – | 1.0 | 1.0 | 1.0 | 1.0 | – |

The weighting factor mij was determined by the formula (2):

$$m_{ij} = \frac{m_{ijcp}}{\sum_{i=1}^{n} m_{ijcp}} \quad (2)$$

Where:

$m_{ij}sr$ is the arithmetic mean of the experts' assessments of the i-th quality indicator of the j-th group.

The average value of mijsr was determined by the formula (3):

$$m_{ijcp} = \frac{1}{N}\sum_{z=1}^{N} m_{ijz} \text{ ,(z=1, 2, 3 ...N)} \quad (3)$$

Where:
N is the number of experts; $mi_{jz}$ – assessment of the i-th quality indicator of the j-th group, given by the z-th expert (z = 1, 2, 3… N).

The results of the analysis are presented in Table 1.

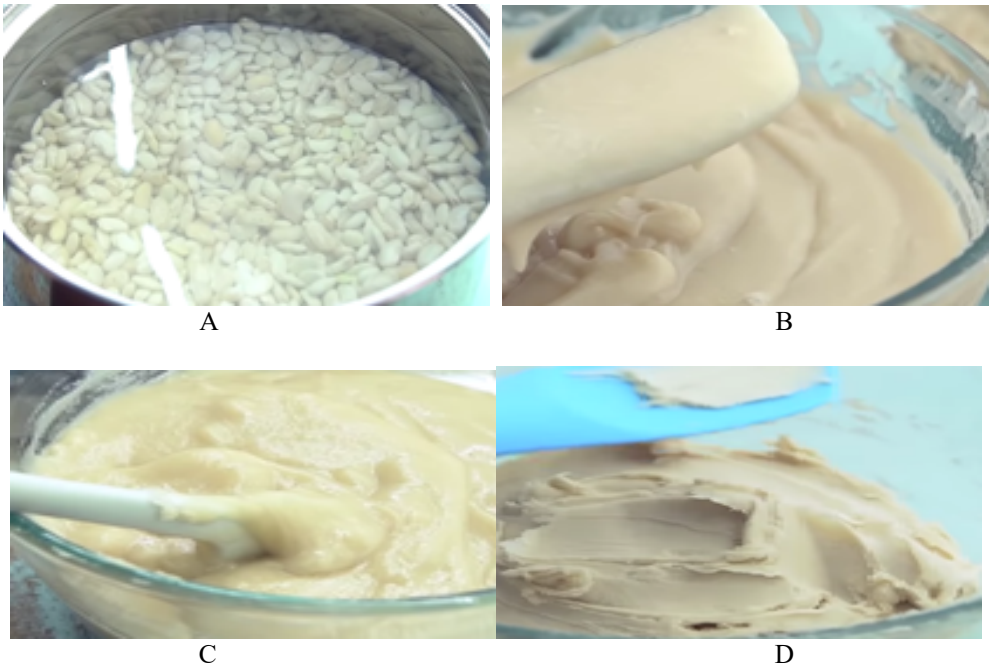
**Description of the Experiment**

Bean paste was prepared according to the developed technology. In the model compositions of the bean, pastes used white bean puree, obtained by boiling sugar and beans in a ratio of 1:1, resulting in a complex homogeneous system related to coagulation structures.

Since the beans are dried, they must be soaked in water for at least twelve hours. During this time, it will increase in size, and after soaking and washing the beans become lighter. Drain this water, fill the beans with fresh water and bring it to a boil. As soon as the foam appears on the surface of the water, drain the first water and fill the beans with fresh water so that it would completely cover the beans with water. Boil beans in this water until fully cooked. This time takes about two hours. If necessary, add water to the beans. The readiness of the beans was checked: first, by breaking some beans, and secondly, by rubbing between the fingers of one or two beans. Drain the remaining water from the beans, wash it once again in running water. Pouring back into the pan, cover with granulated sugar 1:1, and continue to cook our dish over high heat with constant stirring. You do not need to add water. A fully described process is presented in Table 2.

**Table 2** Technological system of making legume butter.

| Subsystem | Subsystem name | Subsystem tasks | Technological operations and parameters |
|---|---|---|---|
| **A** | Mechanical culinary processing | Preparation of prescription components | Beans are sorted and cleaned of extraneous impurities, put in the water (t = 15 °C) in a ratio of 1:2 ($\tau = 2.88.10^4$c), and then cleaned from the seed coat. DDW is dissolved in water (t = 20 – 21 °C) and filtered, the hydro module is 1:2. |
| **B** | Thermal processing | Formation of structural and mechanical properties | Thermal processing is carried out in several stages: *Stage I - getting beans mashed:* the prepared beans are cooked without draining until tender ($\tau = 3.6.10^4$c, t = 80 – 85 °C) and grounded to get a puree of homogeneous consistency. *Stage II - the process of butter plasticization:* sugar is added to the resulting puree and cooked ($\tau = 3.6.10^4$c, t = 60 – 65 °C). Sugar reduces the rate of starch clusterization, decreases the peak of viscosity, and gives plasticity to the butter. *Stage III - the introduction of DDW:* the prepared DDW is placed into the legume butter and boiled. |
| **C** | Cooling | Obtaining a homogeneous plastic mass | The resulting butter is cooled to t = 20 – 25 °C and mixed with coconut oil until a homogeneous plastic mass is obtained. |
| **D** | Infusing | Getting the finished product | Infusing legume butter for ($\tau = 8.64.10^4$c, t = 1 – 3 °C), which ensures the development of a constitutional and spatial structure and gives the butter certain functional and technological properties. |



A B C D

**Figure 2** Ilustration of procedures making legume butter.

**Number of repeated analyses:** All measurements of instrument readings were performed 5 times.
**Number of experiment replication:** The number of repetitions of each experiment to determine one value was also 5 times.

**Statistical analysis**
A directed complex of rheological studies was carried out during the development of the recipe composition of bean pastes. They were based on clarifying the role of whey dry demineralized and coconut oil. To do this, the planning and formulation of computational experiments with obtaining the appropriate regression equations.

The dependence of the structural and mechanical properties of the pastes on the content of DDW and coconut oil was constructed by the method of a planned experiment. According to the results of research of organoleptic and structural-mechanical characteristics by the method of the planned experiment the zones of rational concentrations of DDW and coconut oil in the prescription composition of bean pastes according to the direction of their technological use are established. Variable factors, optimization criteria, and the area of the definition of factors **(Stadnyk et al., 2018)** are defined (Table 3).

According to the experimental planning method, a complete factor experiment of type $2^1$ was implemented, with two factors, levels, and intervals of variation of factors (Table 4).

The dependences of the complex rheological index on the content of coconut oil and DDW are described by the following mathematical models:

$Y_{PKV}$ = 3.3 × 10-5x12 x22 + 0.00136 x1x22 + 0.00121 x12 x2 - 0.0106 x12 - 0.0188 x22 +0.4423 x1 + 0.6792 x2 - 0.9208

$Y_{VUK}$ = 2.92 × 10-5x12 x22 - 0.000515 x1x22 - 0.000692 x12 x2 + 0.0131 x1 x2 - 0.00091x22 +0.00841 x1 - 0.072 x2 + 4.886

$Y_{MFV}$ = 3.64 × 10-5x12 x22 - 0.002 x1x22 - 0.00065 x12 x2 + 0.0373 x1 x2 - 0.0287 x22 +0.00597 x1 - 0.546 x2 + 4.917

The obtained data made it possible to determine the optimal ratio of the components of the developed bean pastes (Table 5). It is established that all factors are statistically significant. The most statistical value in assessing their mutual influence is their joint use.

The optimal values are selected by rounding the optimized values within the compromise areas, to facilitate the dosing of the prescription components of bean pastes in the production environment

**RESULTS AND DISCUSSION**

It is revealed that the health transformation of future generations will depend on their way of life and the key position in this is occupied by nutrition **(Electronic resource, 2018)**. Virtually erased boundaries **(Ladodo et al., 2009)** that define ethnic traditions of food consumption, which were formed over the centuries under the influence of natural conditions, historical, religious and social, and economic factors.

The spread of bean paste is unusual because we are all used to seeing beans in first and second courses and salty. To use beans as the main ingredient of a sweet recipe, in our opinion, not much can be imagined. And yet half of the world's inhabitants use this legume as a sweet dessert.

The works of the authors **(Honda et al., 2020; Li et al., 2017; Balia, 2013)** reflect the use of beans by Eastern countries as a sweet filling for many of their dishes. Beans are used in these countries to make Anko paste.

**Table 3** Complex rheological indicator of the consistency of bean pastes with DDW.

| Samples | Meaning | | | |
|---|---|---|---|---|
| | Forming capacity | Strength | Elongation | Complex index |
| | $Y_1$ | Y2 | Y3 | Y |
| CPC | 0.40 | 0.20 | 0.40 | $Y_{cpc} = 0.4Y_1 + 0.2Y_2 + 0.4Y_3$ |
| CPD | 0.15 | 0.15 | 0.7 | $Y_{cpd} = 0.15Y_1 + 0.15Y_2 + 0.7Y_3$ |
| FPM | 0.60 | 0.25 | 0.15 | $Y_{fpm} = 0.6Y_1 + 0.25Y_2 + 0.15Y_3$ |

Note: $x_2$ – DDW content, %; $Y_{CPC}$ – complex rheological indicator for legume paste CPC, points; $Y_{FPM}$ – complex rheological indicator for legume paste FPM, points; $Y_{CPD}$-complex rheological indicator for legume paste CPD, points.

**Table 4** Levels and intervals of variation of factors of the complex rheological index of consistency of bean paste with DDW.

| Factors | Code symbols | Intervals of variation | Factors levels | | |
|---|---|---|---|---|---|
| | | | Main (0) | Upper (+1) | Lower (-1) |
| Cocoa oil, % | $x_1$ | 4 | 10 | 20 | 0 |
| DDW content, % | $x_2$ | 5 | 20 | 40 | 0 |

**Table 5** Parameters for optimizing the recipe components of legume butter.

| Samples | Coconut oil, % | | | DDW, % | | |
|---|---|---|---|---|---|---|
| | Minimum value | Maximum value | Optimal value | Minimum value | Maximum value | Optimal value |
| CPC | 9.8 | 10.3 | 10.0 | 19.4 | 22.5 | 20.0 |
| CPD | 9.7 | 10.1 | 10.0 | 9.8 | 10.5 | 10.0 |
| FPM | 9.8 | 10.2 | 10.0 | 29.4 | 31.1 | 30.0 |

The longest and most difficult moment in the process of preparing Anko pasta is the preparation of the main ingredient which is beans. Since the beans are dried, they must be soaked in water for at least twelve hours. We soak the tested beans in water for at least 6 – 8 hours. During this time, they increased in size and became quite soft.

In their research **(Kravchenko and Rybchuk, 2019a; Kravchenko, and Rybchuk, 2019b)** reveal the process of preparation of bean paste and its structural and mechanical properties. They note that cooking is as simple as possible, and most importantly, it includes a minimum amount of ingredients. Studies do not mention the effect of sugar on the structure and its amount.

The preparation and use of bean paste are presented by the authors **(Chen et al., 2012; Gagarina, 2006)**, where it is given as a filling in other desserts, such as daifuku Moti, or as a filling in sweet pies, as is done in Japan, China. and other related states.

In the works **(Krylova, 2000; Savenkova, 2012; Kuznetsova and Sydanova, 2002)** the possibility of using the foaming properties of legumes is substantiated. Their research is aimed at the technology of cheese desserts based on the study of the range and technological properties of dessert's whipped structure. The chemical composition of peas and beans was analyzed **(Sichkar, 2004)**, but the process of structure formation was not presented. The composition and structure of legume cells and the physical state of polymers have not been determined.

Studies **(Arseneva, 2007)** have established the influence of technological factors on the foaming properties of bean systems. It is established **(Gagarina, 2006; Bachinska, 2017)** that the optimal foaming properties of bean systems will be at hydro module 1:20; beating temperature 35 °C; duration 10 minutes; soaking beans – for 6 hours; cooking time 10 minutes Our data on the preparation of bean paste differ significantly.

There are food technologies using legume properties like foams. However, their foaming and properties remain unexplored. Little information about the change in taste properties The modern use of beans and raw milk in the technology of dessert production is limited. This gave impetus to the development of a new recipe and research on the use of beans.

Based on the developed technology and optimization of the recipe composition of legume butter, for the first time, we performed rheological and sensory studies aimed at substantiation of the features when using them. The effect of the DDW concentration on the adhesion strength of model compositions of legume butter was experimentally studied.

Studies of the dependence of the adhesion strength indicators made it possible to establish an increase in this indicator by 1.5 – 2 times compared to the control. The analysis data are confirmed by the results of organoleptic consistency analysis, as evidenced by a significant increase in stickiness. This leads to difficulties in work with this type of developed pastes. Taking into consideration that bean butter has a similar versatility of use to the marzipan one in confectionery production **(Stadnyk et al., 2020)**, it was considered appropriate to bring the adhesion strength indicators of bean butter with DDW closer to marzipan butter made using traditional techniques. Since bean butter is characterized by lower strength compared to marzipan butter, coconut oil was considered a promising component. It is conditioned by certain crystallization properties.

***The effect of coconut oil on organoleptic and structural-mechanical properties of model compositions of legume butter with dry demineralized whey.***

To determine the rational concentration of coconut oil in the recipe composition of legume butter with MSSD, model compositions have been developed. The effect of coconut oil on the organoleptic parameters of the model compositions of legume butter with DDW was determined following the developed score scale and presented in (Table 6).

According to the results of the analysis, a decrease in the stickiness of the model compositions of legume pastes CPC, CPD, FPM with an increase in the concentration of coconut oil was confirmed. Coconut oil helps to increase strength and density and gradually reduce extensibility indicators.

The result of organoleptic analysis of the consistency indicators of the model compositions of legume butter with DDW showed the best indicators at a coconut oil concentration of 10%. The overall score according to the developed descriptors was 2.98 for CPC, 2.92 for CPD, and 2.95 for FPM.

To confirm organoleptic parameters, experimental studies of the effect of coconut oil concentration in the amount of 4 – 12% on the adhesion strength indicators of the model compositions of legume butter with DDW concentration were conducted.

With an increase in the concentration of coconut oil, the adhesion strength indicators of legume pastes decrease from 64.4 to 23.3 $N.m^{-2}$ at the DDW concentration of 20%, from 55.7 to 21.8 $N.m^{-2}$ at the DDW concentration of 10%, and from 73.8 to 25.2 $N.m^{-2}$ at the DDW concentration of 30%. As a result of the regularities analysis of the effect of coconut oil on the adhesion strength of legume butter with DDW, a decrease in this indicator was found to be up to 3 times compared to the corresponding control samples. Experimental studies have shown that 10% of coconut oil in the formula of legume butter with DDW allows bringing the adhesion strength closer to the indicators of marzipan butter made using traditional techniques. The effect of coconut oil concentration on the relative deformation indicators of the corresponding control samples. Experimental studies have shown that 10% of coconut oil in the formula of legume butter with DDW allows bringing the adhesion strength closer to the indicators of marzipan butter made using traditional technology. The effect of coconut oil concentration on the relative deformation indicators of the model compositions of legume butter with DDW was determined. The results of studies of the effect of coconut oil concentration on the deformation characteristics of the model compositions of legume butter with DDW are presented in Table 7.

**Table 6** Organoleptic characteristics of model compositions of legume butter with DDW and coconut oil.

| Indicator name | Weighting coefficient | Characteristics | Descr. number | Weighting coefficient | Samples with the addition of coconut oil, % | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 4 | 6 | 8 | 10 | 12 |
| **CPC bean butter** | | | | | | | | | |
| Consistence | 0.4 | Density, strength | 1 | 0.1 | 5.0 | 5.0 | 5.0 | 5.0 | 4.6 |
| | | Stickiness | 2 | 0.1 | 4.4 | 4.6 | 4.8 | 5.0 | 5.0 |
| | | Softness | 3 | 0.1 | 4.4 | 4.6 | 4.8 | 5.0 | 5.0 |
| | | Extensibility | 4 | 0.7 | 5.0 | 5.0 | 5.0 | 4.8 | 4.2 |
| The total score for CPC descriptors | | | | | 4.88 | 4.92 | 4.96 | 4.93 | 4.4 |
| *The final assessment of the CPC indicator* | | | | | *1.95* | *1.96* | *1.98* | *1.93* | *1.76* |
| Forming ability | 0.2 | Detection degree | 1 | 1.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| The total score for CPC descriptors | | | | | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| *The final assessment of the CPC indicator* | | | | | *1.0* | *1.0* | *1.0* | *1.0* | *1.0* |
| **Overall CPC rating** | | | | | **2.95** | **2.95** | **2.98** | **2.98** | **2.76** |
| **CPD bean butter** | | | | | | | | | |
| Consistence | 0.3 | Density, strength | 1 | 0.2 | 4.6 | 4.8 | 5.0 | 5.0 | 3.8 |
| | | Stickiness | 2 | 0.3 | 4.4 | 4.6 | 4.8 | 5.0 | 5.0 |
| | | Softness | 3 | 0.2 | 4.4 | 4.6 | 4.8 | 5.0 | 3.8 |
| | | Extensibility | 4 | 0.3 | 4.4 | 4.4 | 4.2 | 4.2 | 4.0 |
| Total score by CPDdescriptors | | | | | 4.44 | 4.58 | 4.66 | 4.76 | 4.22 |
| *The final assessment of the CPD indicator* | | | | | *1.33* | *1.37* | *1.39* | *1.42* | *1.26* |
| Forming ability | 0.3 | Detection degree | 1 | 1.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Total score by CPD descriptors | | | | | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| *The final assessment of the CPD indicator* | | | | | *1.5* | *1.5* | *1.5* | *1.5* | *1.5* |
| **The general assessment of the CPD** | | | | | **2.83** | **2.87** | **2.89** | **2.92** | **2.76** |
| **Bean butter FPM** | | | | | | | | | |
| Consistence | 0.2 | Density, strength | 1 | 0.3 | 5.0 | 5.0 | 4.8 | 4.8 | 3.8 |
| | | Stickiness | 2 | 0.3 | 4.4 | 4.6 | 4.8 | 5.0 | 5.0 |
| | | Softness | 3 | 0.2 | 4.6 | 4.8 | 5.0 | 5.0 | 3.8 |
| | | Extensibility | 4 | 0.2 | 4.4 | 4.4 | 4.2 | 4.2 | 4.0 |
| The total estimate for FPM descriptors | | | | | 4.12 | 4.72 | 7.72 | 4.78 | 4.2 |
| *The final assessment of the FPM indicator* | | | | | *0.82* | *0.84* | *0.94* | *0.95* | *0.84* |
| Forming ability | 0.4 | Detection degree | 1 | 1.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| The total estimate for FPM descriptors | | | | | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| *The final assessment of the FPM indicator* | | | | | *2.0* | *2.0* | *2.0* | *2.0* | *2.0* |
| **Overall assessment of the FPM** | | | | | **2.82** | **2.84** | **2.94** | **2.95** | **2.84** |

**Table 7** Deformation characteristics of the model compositions of legume butter with DDW and coconut oil.

| Indicator name | Designation | | Samples with coconut oil addition, % | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 4 | 6 | 8 | 10 | 12 |
| Reversible deformation, $10^{-3}$ | $\gamma_{sv}$ | CPC | 340.0 | 325.7 | 302.9 | 301.0 | 287.1 | 281.4 |
| | | CPD | 358.6 | 348.6 | 337.1 | 328.6 | 305.2 | 291.1 |
| | | FPM | 334.3 | 328.1 | 312.9 | 305.0 | 289.1 | 276.4 |
| Irreversible deformation, $10^{-3}$ | $\gamma_{irrev}$ | CPC CPD FPM | 12.9 | 12.9 | 12.9 | 12.9 | 12.9 | 12.9 |
| General deformation, $10^{-3}$ | $\gamma_{gen}$ | CPC | 352.9 | 338.6 | 325.7 | 319.9 | 311.0 | 294.3 |
| | | CPD | 371.4 | 361.4 | 350.0 | 341.4 | 320.0 | 300.1 |
| | | FPM | 347.1 | 337.1 | 315.7 | 312.9 | 304.2 | 277.3 |

Note: (n = 5; $p \leq 0.05$).

An increase in the concentration of coconut oil in the recipe composition of legume butter contributes to a gradual reduction in overall deformation by 25 – 30% compared to the control. The irreversible deformation is stable for all prototypes, does not depend on the DDW concentration, and is equal to 12.9 notional units. The reversible deformation decreases parallelly to the general strain values.

Accordingly, the reversible deformation indicators of the CPD decrease from 358.6 to 291.1 notional units, CPC-from 340.0 to 281.4 notional units, FPM-334.3-276.4 notional units. The data obtained confirm the significant effect of coconut oil on the consistency of bean butter with DDW.

Features of the effect of coconut oil on the consistency of legume butter with DDW and conditions for achieving rational rheological parameters can be predicted by structural and mechanical characteristics. Clarification of the role of legume butter with DDW and coconut oil was carried out by setting up computational experiments to obtain the corresponding regression equations for the highly elastic modulus, conditionally instantaneous elastic modulus, pliability, and elastic aftereffect viscosity of the model compositions**.**

Based on the results of deformation characteristics, the main structural and mechanical characteristics of the coconut oil concentration range were determined. Mathematical dependences of the main structural and mechanical parameters of legume butter with DDW and coconut oil are determined in the form of:

***- Highly elastic module of the model compositions***

*DDW 10% $y=0.0315x^3-0.64x^2-2.1596x+198.11$*
*when $R^2=0.9998$*
*DDW 20% $y=0.0923x^3-1.5519x^2+0.1525x+190.75$*
*when $R^2=0.9944$*
*DDW 30% $y=0.1002^3-1.7972x^2+1.7426x+184.67$*
when $R^2=0.9911$

***- Conditionally instantaneous elastic modulus of the model compositions***

*DDW 10% $y=-0.00236x^3-0.023x^2-4.196x+317.64$*
*when $R^2=0.9992$*
*DDW 20% $y=-0.003x^3-0.0656x^2-5.3025x+312.99$*
*when $R^2=0.9963$*
*DDW 30% $y=0.0528x^3-1.1029x^2+1.0276x+294.54$*
*when $R^2=0.9929$*

***- Pliability of the model compositions***

*DDW 10% $y=-0.1581x^3+2.0264x^2-11.787x+874.21$*
*when $R^2=0.9932$*
*DDW 20% $y=0.375x^3-6.3333x^2+11.25x+829.85$*
*when $R^2=0.9993$*
*DDW 30% $y=0.111x^3-1.9884x^2-3.5323x+817.09$*
*when $R^2=0.9934$*

***- Elastic aftereffect viscosity of the model compositions***

*DDW 10% $y=-0.005x^3+0.041x^2-1.099x+12.46$*
*when $R^2=0.954$*
*DDW 20% $y=0.0368x^3-0.6149x^2-2.092x+388.15$*
*when $R^2=0.9992$*
*DDW 30% $y=0.0513^3-0.9526x^2+0.392x+364.28$*
*when $R^2=0.9969$*

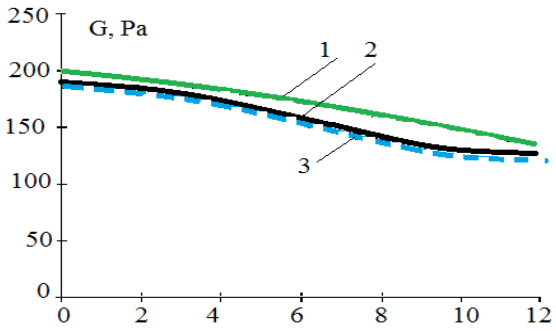



**Figure 3** Highly elastic modulus of the model compositions of legume butter with DDW and coconut oil: 1 – DDW 10%; 2 – DDW 20%; 3 – DDW 30%.

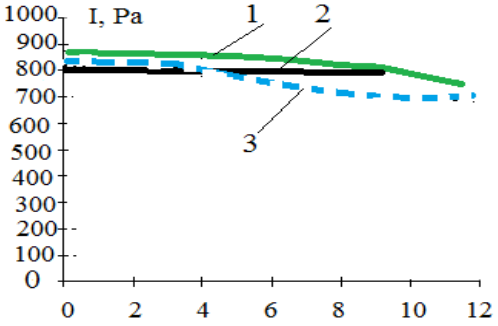



**Figure 4** The pliability of the model compositions of legume butter with DDW and coconut oil: 1 – DDW 10%; 2 – DDW 20%; 3 – DDW 30%.



**Figure 5** The products of model compositions.

Graphical dependences of the highly elastic modulus and pliability of the model compositions of legume butter with DDW and coconut oil are shown in Figure 3 and Figure 4.

With an increase in the concentration of coconut oil, the highly elastic modulus indicators (*Gel*) decrease by 1.5 times, amounting to 198.1 – 134.4 Pa for CPC, 190.5 – 128.2 Pa for CPD, and 184.5 – 120.1 PA for FPM (Figure. 2). It was found that the following elastic modulus indicators ($G_{pr}$), which are 313.2 – 254.2 Pa for CPC, 317.7 – 260.2 Pa for SC, and 296.3 – 240.1 Pa for FPM, decrease by 1.2 times. Elastic aftereffect viscosity, which is 381.2 – 341.1 Pa·c for CPD, 372.8 – 338.1 Pa·c for CPC, 353.5 – 320.2 Pa·c for FPM, pliability *(I)*, which is 7.96 –

7.53 $10^{-4}$ $Pa^{-3}$ for CPC, 8.50 – 7.50 $10^{-4}$ $Pa^{-3}$ for CPD, 7.80 – 6.81·$10^{-4}$ $Pa^{-3}$ for FPM (Figure 3).

When compared with other plastic confectionery masses used as finishing semi-finished products, the developed legume butter has significant advantages. Based on research **(Kravchenko and Rybchuk, 2019)**, it was found that the food energy of the developed confectionery legume butter is 2.7 times lower than that of sugar butter and 4.1 times lower than that of marzipan butter.

## CONCLUSION

The developed technology of confectionery legume butter based on DDW makes it possible to obtain high-quality low-calorie finishing semi-finished products for confectionery products. Results of the study confirm their high nutritional and biological value. A gradual decrease in the indicators of modular constants improves the forming ability, which ensures high processability of butter and is consistent with the results of the organoleptic analysis. According to the results of previous studies, the prospects of using DDW in legume butter technologies at a concentration of 10 – 30% and coconut oil at a concentration of 10% (Figure 5). Were confirmed, which makes it possible to obtain the desired structural and mechanical properties for finishing semi-finished products following the direction of their technological use.

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**Ethical Statement:**

This article does not contain any studies that would require an ethical statement.

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# HEAVY METALS ANALYSIS, GCMS-QP QUANTIFICATION OF FLAVONOIDS, AMINO ACIDS AND SAPONINS, ANALYSIS OF TANNINS AND ORGANOLEPTIC PROPERTIES OF POWDER AND TINCTURE OF *ECHINACEA PURPUREA* (L.) AND *RHAPÓNTICUM CARTHAMOÍDES*

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## ABSTRACT
Medicinal plants are one of the main sources of vitamins, minerals salts, macro-and microelements, and other biologically active substances that have a health and protective effect on the human body. The current study was aimed to appraise the heavy metals contents in the powder materials of two medicinally important plants *Echinacea purpurea* (L.) and *Rhapónticum carthamoídes* collected from the Semipalatinsk nuclear test site using atomic absorption spectrophotometer. Flavonoids, saponins, amino acid contents quantification were done both in raw materials as well as tincture prepared from both plants via GCMS-QP 2010 Ultra chromatomass spectrometer. Further, tannins concentrations and organoleptic properties of the tincture were elucidated using previously reported standard procedures. In the current study, the concentrations of heavy metals were within the permitted range i.e. lead (0.0027 mg.100g$^{-1}$), cadmium (0.00012 mg.100g$^{-1}$), arsenic (ND), mercury (ND). In the crude powder, flavonoids were observed to be in the highest concentration in *E. purpurea* (L.) (5.5 ±0.20 mg.100g$^{-1}$), whereas, its concentration was 3.1 ±0.346 mg.100g$^{-1}$ in *R. carthamoídes* powder. Tannin concentration was higher in *R. carthamoídes* (5.5 ±0.115 mg.100g$^{-1}$) and 3.1 ±0.46 mg.100g$^{-1}$ in *E. purpurea*. Likewise, saponins concentrations were 4.1 ±0.40 mg.100g$^{-1}$ and 5.6 ±0.17 mg.100g$^{-1}$ in *E. purpurea* and *R. carthamoídes* powder respectively. Concentrations of these active metabolites in the resultant tincture were flavonoids (7.6 ±0.23), tannins (7.5 ±0.28), and saponins (8.5 ±0.16) mg.100g$^{-1}$. In the current study, we observed highest concentrations of these essential amino acids in the tincture including leucine/isoleucine (78.00 ±1.15 mg.100g$^{-1}$), histidine (14.00 ±1.44 mg.100g$^{-1}$), lysine (49.33 ±2.02 mg.100g$^{-1}$), methionine (18.66 ±2.90 mg.100g$^{-1}$), cystine (29.00 ±0.57 mg.100g$^{-1}$), phenylalanine (24.16 ±1.87 mg.100g$^{-1}$) and threonine (32.50 ±1.22 mg.100g$^{-1}$) respectively. The resultant tincture has a pleasant agreeable taste coupled with acceptable herbal flavor which are important organoleptic properties for any product.

**Keywords:** heavy metals; GCMS-QP; flavonoids; tincture; tannins; saponins

## INTRODUCTION
Medicinal plants and their derived products are used by humans for a long for the management of various diseases **(Ovais et al., 2019; Ayaz et al., 2017a)**. Being natural, the majority of these products are safe and have comparatively fewer side effects. Though generally considered safe, natural products are sometimes contaminated with several hazardous heavy metals which can cause serious health consequences **(Ayaz et al., 2014; Khan et al., 2016)**.

Recent studies suggest that herbs contaminated with heavy metals might be potentially mutagenic, toxic, and carcinogenic if utilized for a prolonged time **(Schimmer et al., 1988; Higashimoto et al., 1993)**. In this regard, the World Health Organization (WHO) posted important recommendations regarding the use of herbal drugs. WHO recommends evaluating herbal agents for heavy metals contents before use to ensure their concentration is in the permitted range of human safety. In the East Kazakhstan region, one of the risk factors for disease, namely cancer, is the contamination of the environment, food raw materials, and foodstuffs with radioactive elements and heavy metals, including the consequences of the Semipalatinsk nuclear test site **(Kakimov et al., 2016a; Duyssembaev et al., 2014)**. Subsequently, contents of heavy metals including lead (Pb), cadmium (Cd), Arsenic (As), and mercury (Hg) were appraised via GC-MS in the current study.

Flavonoids are a highly assorted group of plant secondary metabolites having tremendous biological properties. They are predominantly present in green plants, vegetables, flowers, seeds, nuts, fruits, propolis, and honey. They are well-reputed for the management of numerous human disorders neurological disorders, microbial infections,

cardiovascular diseases, cancer, and inflammatory disorders **(Ahmad et al., 2015; Ayaz et al., 2019a; Ayaz et al., 2019b; Patel et al., 2018; Salaritabar et al., 2017)**. Dietary consumption of flavonoids and flavonoid-rich herbs are reported to exhibit significant beneficial effects on neurological health **(Sawikr et al., 2017)**. Thus scientific exploration of flavonoids contents of herbal products is the need of the day to signify their use in various disorders. Likewise, saponins represent another diverse group of pharmacologically active compounds that exist as triterpene or steroid glycosides. They are also reported to possess anti-cancer, anti-microbial, anti-diabetic, immunomodulating, anti-allergic, cardio-protective, hepatoprotective, and neuro-pharmacological potentials **(Lacaille-Dubois and Wagner, 2000; Thakur et al., 2011)**.

Echinacea *purpurea* L. also called purple coneflower (Figure 1a) belongs to the family Asteraceae and has a long history of use in traditional medicine in Europe, Australia, and North America **(Wills and Stuart, 1999; Stuart and Wills, 2000)**. Its medicinal uses include efficacy in bacterial respiratory infections, viral infections, immunomodulatory-



a) *Echinacea purpurea* L.



b) *Rhaponticum carthamoides*

**Figure 1** *Echinacea purpurea* L. and *Rhaponticum carthamoides*.

anti-inflammatory effects, urinary tract infections **(Speroni et al., 2002; Stanisavljević et al., 2009; Luo et al., 2003)**. Roots and aerial parts of the plant are used in the preparation of food supplements. Secondary metabolites isolated from the plant including phenolics, flavonoids, alkaloids exhibit considerable anti-radical's properties **(Stanisavljević et al., 2009)**. Likewise, *Rhaponticum carthamoides* also called maral root or Russian leuzea (Figure 1b), belong to the family Asteraceae and is found in alpine meadows of Kazakhstan, Siberia, and cultivated throughout Russia as well as eastern Europe **(Kokoska and Janovska, 2009)**. Its root preparations are reported to exhibit useful effects on the learning and memory of rodents **(Mosharrof, 1987)**. *R. carthamoides* and its metabolites are immunomodulators, act as a stimulant and improve humoral immunity **(Azizov, 1997; Seifulla et al., 1993)**. It also improves working capacity of skeletal muscles and shows adaptogenic as well as anabolic properties **(Bespalov et al., 1992; Ramazanov, 2006; Winston, 2019)**. Numerous compounds including flavonoids, glycosides, and lactones are isolated from the plant and its essential oils. Plant extracts and secondary metabolites are reported to possess cardiovascular, CNS, immunomodulatory, antioxidant, anti-cancer, antimicrobial, and repellent properties **(Kokoska and Janovska, 2009)**. The current study was designed to prepare a tincture from these two medicinally important medicinal plants and quantitatively evaluate their heavy metals, flavonoids, amino acids, tannins, saponins contents, and organoleptic properties.

**Scientific hypothesis**

As the selected medicinal plants are collected from the Semipalatinsk nuclear test site in the East Kazakhstan region, so we hypothesize that there might be a higher concentration of toxic heavy metals in these plants. The use of these plants without heavy metals analysis could be hazardous to users. Further, we hypothesize that the selected plants might be a good source of essential amino acids and flavonoids which play a significant role in the prevention and management of various diseases. Agreeable taste and other organoleptic properties are important parameters for any herbal preparation to be used by patients. Subsequently, there was a need for the preparation of tincture which exhibits desirable organoleptic properties and can accommodate maximum bioactive ingredients for the management of various disorders.

## MATERIAL AND METHODOLOGY

### Samples

***Plants collection and Processing***

Experimental work was carried out departmental laboratories of Technology of food and processing industries, biotechnology and standardization, the scientific center of radioecological research" of the Shakarim State University of Semey, Kazakhstan and Research Institute of Biotechnology" of Kemerovo State University Russia. The selected plants were collected in the East Kazakhstan region, Katon-Karagai district - levsey, and Shemonaikha district- echinacea. *Echinacea purpurea* (L.) was sampled from the Shemonaikha area of the East Kazakhstan region and *Rhaponticum carthamoides* root from the Katon-Karagay area of East Kazakhstan region. The authenticity of medicinal plant raw materials was carried out by a

specialist biologist on macroscopic (external) and microscopic (anatomical) features under the requirements of General Pharmacopoeia Article 1.5.3.0003.15 "Technique of microscopic and microchemical research of medicinal plant raw materials and medicinal plant preparations".

## Chemicals

### *Tincture preparation*

For the preparation of a tincture, an aqueous solution of ethyl alcohol of different concentrations was used. It is recommended to use a 35% aqueous solution of ethyl alcohol to make a medicinal plant tincture. To prepare the tincture used pharmacopeia medicinal plants that are not poisonous: for one part of the plant mass used 5 parts of the extractant. The infusion temperature of the infusion was within 20 – 25 °C **(Kowalczyk et al., 2012)**.

*Echinacea purpurea* (L.) and *Rhapónticum carthamoídes* root were used as plant materials. To prepare the tincture, selected plant materials were shade dried and grinded to small particles of 3 – 5 mm size.

Powdered material of both plants (100 g each) was added to an aqueous solution of 35% ethyl alcohol at herb: extractant 1:5 ratios. The resultant mixture was loaded into a mesh basket, which was placed in a percolator. The percolator was equipped with a jacket, for the possibility of maintaining the temperature of maceration in the range from 20 to 30 °C for 24 hrs. Subsequently, the temperature of the percolator was increased up to 40 – 50 °C with continuous stirring for at least one hr. The solvent was removed and a fresh extraction solvent was added to the treated herbs in the ratio of the herb: extractant 1:1. Infusion with periodic mixing was carried out for 12 hrs and drained all the extract into an intermediate container. The solvent extracts were combined in the tank. To purify the resulting mixture of ballast substances was carried out via the process of sedimentation at a temperature of 5 – 8 °C for 5 days, then carry out the process of filtration of alcoholic infusion through a cotton-grass filter. The resulting tincture was stored in a dark glass jar (Figure 2).

## Laboratory methods

### *Heavy Metals analysis*

Heavy metal contents in the tincture were quantitatively analyzed using atomic absorption spectrophotometer following the previously reported standard procedure **(Ayaz et al., 2014; Khan et al., 2016)**. All chemicals and reagents used were of high-quality analytical grade. Plants powdered material 0.2 g was added to a crucible and ignited at 55 °C for six hrs using a muffle furnace. The resultant ash was digested via concentrated HCL 0.5 mL and evaporated using hot plate apparatus. A small quantity of double distilled water was added to the mixture followed by filtration and the final volume was increase to 30 mL using double distilled water. The solution formed was analyzed using Shimadzu single quadrupole GCMS-QP2010 SE gas chromatograph-mass spectrometer (Shimadzu Corporation, Japan) for the number of heavy metals according to the standards available in our laboratory including lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg) following standard operating procedure and were compared with Permissible levels of Technical Regulations of the Customs Union in herbal preparation for human use.

### *Quantitative analysis of Flavonoids*

Flavonoid quantification was determined by previously reported standard protocol **(Ayaz et al., 2017b; Pandey and Tripathi, 2014; Rajani Kanaki, 2008; Ab Halim et al., 2012)**.

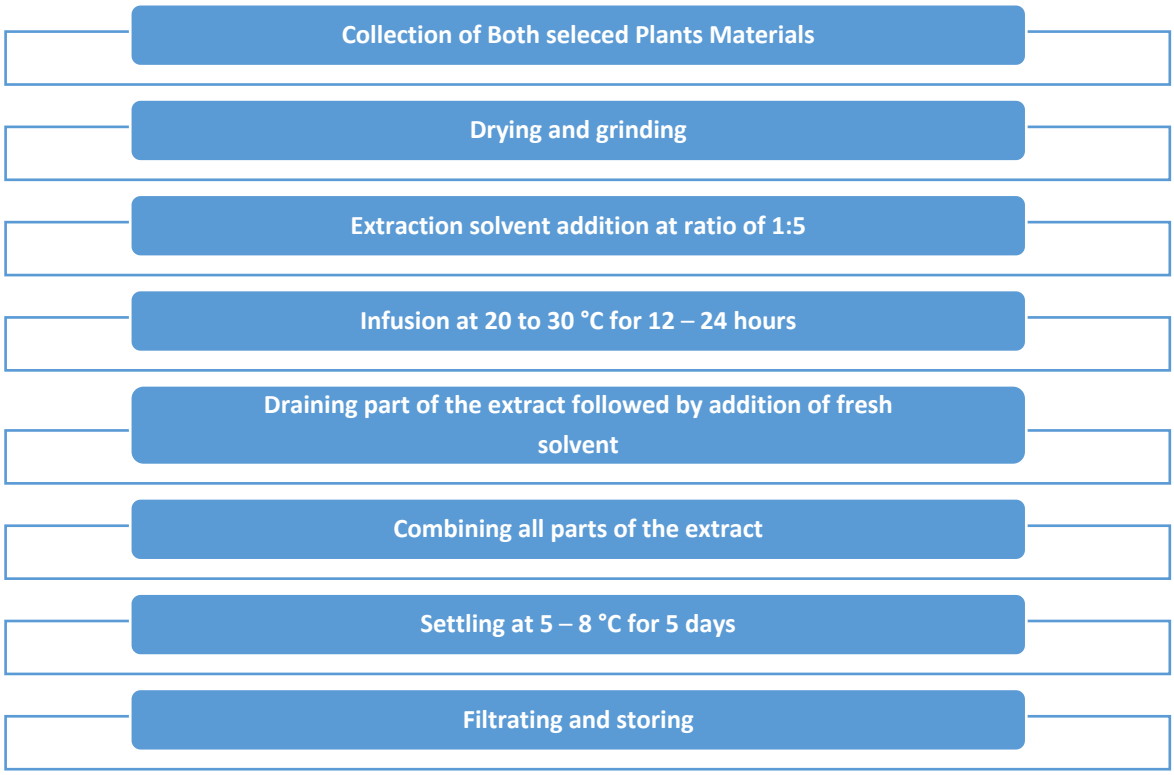


**Figure 2** Systematic flow chart of the extraction process to prepare tincture.

Briefly, 1 g of powdered (1 mm) sample was added to 100 mL grinding flask and 30 mL of 70% alcohol was added to it. Flask was heated in a boiling water bath for 30 minutes. Subsequently, the flask was cooled to room temperature under a shower of cold water and filtered through a paper filter into a measuring flask with a capacity of 100 mL. The filtrate was flushed with 70% alcohol and added the alcohol to increase volume up to the mark of the flask. The obtained solution was named "solution A". Thereafter, 4 mL of "solution A" was transferred to a 25 mL flask and 2 mL of 2% solution of aluminum chloride in 95% alcohol was added to it, followed by the addition of 95% alcohol into the flask until the mark. The resultant solution was kept for 20 min and optical density was measured using a spectrophotometer at a wavelength of 410 nm in a 10 mm thick cuvette. For comparison solution, 4 mL of "solution A" was added to a flask, volume was increased to 25 mL and 1 drop of diluted hydrochloric acid was added to it. Finally, volume increased using 95% alcohol. Flavonoids (X) was quantified using the formula (1);

$$X = \frac{D \times 100 \times 100 \times 25}{330 \times m \times (100 - W)} \quad (1)$$

Where:
D – optical density of the tested solution; 330 – Specific absorption index of the complex with aluminum chloride at 410 nm; m – a mass of the sample (g); W – mass loss on drying (%).

***Qualitative and quantitative analysis of Tannins***

For the qualitative assessment of tannins content, powder material (300 mg) of the plants was used following the previously reported procedure **(Ejikeme et al., 2014)**. The required powder material was transferred to a test tube and boiled in a water bath for 30 minutes using a water bath. After boiling, the mixture was filtered through Whatman filter paper 42 and a few drops of ferric chloride (0.1%) were added to the filtrate. A brownish-green color was resulted indicating the presence of tannins. Subsequently, quantitative analysis was done **(Geetha and Geetha, 2014)**. In 500 mL of conical flask put 2 g of shredded and sieved through a sieve with a hole diameter of 3 mm sample and added 250 mL of boiling water to it. Then the flask was weighted using an electronic scale and boiled the contents with periodic stirring. Thereafter, the flask was placed at room temperature to get the contents cool and weighted again, freshly boiled water was added to fill the missing weight. About 100 mL of the resulting extraction was filtered into a conical flask of 200 – 250 mL through absorbent cotton so that particles of samples do not get into the flask. Subsequently, 25 mL of filtered solution was poured into another conical flask with a volume of 750 – 1000 mL, then 500 mL of water was added, 25 mL of indigo-sulphonic acid and titrated against potassium permanganate solution (0.02 $mol.L^{-1}$; reagent grade 99.5%, LLP "Nikitin", Semey, Kazakhstan ) until golden yellow color appeared. At the same time, conducted a control experiment. The content of tannins (X) in percent converted to absolute dry raw materials, was calculated by the formula (2);

$$X = \frac{(V - V_1) \times 0.004157 \times 250 \times 100 \times 100}{M \times 25 \times (100 - W)} \quad (2)$$

Where:
V – the amount of potassium permanganate solution (0.02 $mol.L^{-1}$) used for titration of extraction (mL); V1 – the amount of potassium permanganate solution (0.02 $mol.L^{-1}$), used for titration in the control experiment (mL); 0.004157 – amount of tannins equivalent to 1mL of potassium permanganate solution (0.02 $mol.L^{-1}$) per tannin (g); M – sample weight (g); W – mass loss on drying (%); 250 – total extraction volume (mL); 25 – extraction volume took for titration (mL).

***Amino acids contents***

The amino acid composition was determined using GCMS-QP 2010 Ultra chromatomass spectrometer (Shimadzu Corporation, Japan), M-04-38-2009 test method (FR.1.31.2010.07015) **(Bashari et al., 2019)**. Plants sample weighing 1 g was transferred to a conical flask and a 1000 $cm^3$ extraction solution was added. The mixture was shaken for 60 min using a mechanical shaker and 100 $cm^3$ of supernatant liquid was transferred from it into a beaker. About 5.0 $cm^3$ of sulfosalicylic acid (reagent grade 99%, LLP "Kazbiohim", Almaty, Kazakhstan) was added to it and continued mixing with a magnetic mixer for 5 min. The mortar was filtered to remove the sludge. The pH of the obtained solution was adjusted to 2.20 using sodium hydroxide solution (assay percentage ≥98%, LLP "Kazbiohim", Almaty, Kazakhstan). The solution was transferred into a measuring flask and increased volume with a citrate buffer. The chromatography was performed following SOPs available for the equipment. It is important that the same amount (±0.5%) of standard solution and sample is entered into the column unless an internal standard is used (for chromatographic systems requiring a low sodium concentration) and unless the amino acid ratio in standard solutions and the sample should be as close as possible.

The amino acid content of the test sample, $g.kg^{-1}$, is calculated using the formula (3);

$$w = \frac{A_b \times c \times M \times V_b}{A_c \times m \times 1000} \quad (3)$$

Where;
$A_b$ – amino acid peak area in hydrolyzate and extract; c – molar concentration of amino acid in standard solution, $mol.dm^{-1}$; M – amino acid molecular weight; $V_b$ – total hydrolyzate volume or calculated total dilution volume of the extract cm; $A_c$ – amino acid peak area in a standard solution.

***Quantitative determination of saponins***

Previously reported standard protocol was used to quantitatively analyze saponins content **(Ayaz et al., 2016; Zeb et al., 2014)**. The sample was milled to the size of the particles passing through a sieve with 1 mm diameter holes. About 0.5 g of the sample was packed in a chuck from filter paper and was placed in the extractor of the Soxhlet device. Chloroform extraction (reagent grade 99%, LLP LenReaktiv, Moscow, Russia) was discarded and the holder with the plant sample was dried. The sample was placed on the cartridge in a round bottom flask with a capacity of 100 mL. Subsequently, 50 mL of 90% alcohol (LLP "Nikitin", Semey, Kazakhstan) was poured in and heated with the back cooler in a boiling water bath for 1 hour. The

extraction was filtered through a dry paper filter into a distillation flask with a capacity of 200 mL, the rest on the filter is twice washed with 90% alcohol in portions of 5 mL. Extractions were combined and the solvent was distilled under vacuum drying.

The residue was dissolved in 20 mL of 96% alcohol (LLP "Nikitin", Semey, Kazakhstan) during heating, cooled, quantitatively transferred with 25 mL of 96% alcohol into a flask with 50 mL capacity, filled up with 96% alcohol till the mark. Two mL of the solution obtained was placed into a 50 mL conical flask, 8 mL of concentrated sulfuric acid (mass fraction of $H_2SO_4$, 93.6 – 95.6%, LLP "Nikitin", Semey, Kazakhstan) was added to it and stirred. After 30 min the optical density of the obtained solution was measured on the spectrophotometer SF-46 (Lomo Company, Russia) at a wavelength of 405 nm in a cuvette with a layer thickness of 10 mm. The mixture consisting of 2 mL of 96% alcohol and 8 mL of concentrated sulfuric acid was used for comparison. Content of saponins (X) in 1 g of raw material was calculated by the formula (4);

$$x = \frac{D \times m_0 \times 50 \times 10 \times 6 \times 2 \times 100 \times 100}{D_0 \times m \times 1 \times 2 \times 100 \times 25 \times 6 \times 100 \times (100 - W)} = \frac{D \times m_0 \times 20}{D_0 \times m \times (100 - W)} \quad (4)$$

Where:
D – optical density of the test solution; $D_0$ – optical density of Pharmacopoeia standard sample of Escin; m – sample weight, g; $m_0$ – the mass of Pharmacopoeia Standard Sample Escin (g); W – mass loss on drying (%).

***Analysis of the organoleptic properties***

Organoleptic and physicochemical indicators including consistency, appearance, taste, color, taste, and odor were analyzed in the obtained tincture following American Herbal Products Association (AHPA) guidelines for organoleptic analysis of herbal ingredients **(Upton et al., 2016; Dentali, 2013)**.

## Statistical analysis

All experiments were performed in triplicate and results were expressed as mean ±SEM. One way ANOVA followed by multiple comparison Dunnett's test were applied for statistical differences among various study groups using GraphPad prism 7.0 software (USA). *p*-value <0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

Elevated levels of heavy metals particularly lead, cadmium, arsenic, and mercury which are usually acquired from the soil, household wastewater, irrigation systems used in agriculture, and other environmental sources. Being non-biodegradable these heavy metals present in herbal products can cause severe toxicological effects in humans if used chronically. Due to this reason, the WHO recommends that heavy metal contents of herbal products must be assessed before use to ensure that their concentration is within permitted limits. The majority of the heavy metals we assessed are non-essential elements and have no specific physiological function. Lead toxicity is manifested in the form of renal, hemopoietic, reproductive, and nervous system side effects **(Ara and Usmani, 2015; Papanikolaou et al., 2005)**. Likewise, chronic exposure to cadmium leads to cancer and toxicity of the respiratory, cardiovascular, CNS, urinary tract, and musculoskeletal system **(Rahimzadeh et al., 2017; Sun et al., 2020)**. Chronic exposure to arsenic skin lesions and cancer **(Ratnaike, 2003; Kakimov et al., 2016b)**. Mercury toxicity effect CNS, renal and gastro-intestinal system **(Bernhoft, 2012, Kuramshina et al., 2019)**. In the current study, we observed that the concentrations of heavy metals were within the permitted range i.e. lead (0.0027 mg.100g$^{-1}$), cadmium (0.00012 mg.100g$^{-1}$), arsenic (nondetectable), mercury (nondetectable) Table 1.

One of the mottoes beyond this analysis was contamination of the environment, food raw materials, and foodstuffs with radioactive elements and heavy metals as a result of Semipalatinsk nuclear tests and collection plants from that area.

Among the primary metabolites of medicinal plants are proteins, amino acids, chlorophyll, and some sugars, whereas secondary metabolites include phenolic compounds, flavonoids, alkaloids, saponins, tannins, terpenoids, and essential oils. The majority of these metabolites possess considerable therapeutic properties and medicinal plants are valued owing to the presence of these metabolites. Flavonoids exhibit strong anti-oxidant properties and thus ameliorate the progression of free radicals induced degenerative disorders **(Banjarnahor and Artanti, 2014; Rice-Evans and Packer, 2003; Imran et al., 2019)**. They are reported to inhibit the progression of tumors and halt cardiovascular diseases **(Rice-Evans and Packer, 2003; Hertog et al., 1993; Tijburg et al., 1997)**. Several medicinal plants rich in flavonoids demonstrate anti-inflammatory, anti-viral, hepato-protective, anti-allergic, neuroprotective, and anti-ulcer properties **(Rice-Evans and Packer, 2003; García-Lafuente et al., 2009, Akram et al., 2020)**. Saponins glycosides are present throughout the plant kingdom and contain diverse groups of compounds having steroidal aglycone and sugar molecules as basic components of their basic structure. Due to the diverse nature of compounds, this group of secondary metabolites is reported to exhibit numerous pharmacological properties including cytotoxic, antimicrobial, anti-oxidant, anti-diabetic, anthelmintic, antispasmodic, and anti-neuroplastic **(Yuan et al., 2010; Lu et al., 2005; Pal et al., 2009)**. Likewise, tannins are another important group of water-soluble, phenolic secondary metabolites that chemically belongs to proanthocyanidins **(Chung et al., 1998; Haslam, 1989)**. They possess anticancer, anti-viral, anti-inflammatory, anti-oxidant and enzyme inhibition properties **(Okuda et al., 1992; Scalbert, 1991)**.

In the crude powder, flavonoids were observed to be in the highest concentration in *E. purpurea* (L.) (5.5 ±0.20 mg.100g$^{-1}$), whereas, its concentration was 3.1 ±0.346 mg.10 g$^{-1}$ in *R. carthamoides* powder. Tannin concentration was higher in *R. carthamoides* (5.5 ±0.115 mg.100g$^{-1}$) and 3.1 ±0.46 mg.100g$^{-1}$ in *E. purpurea*.

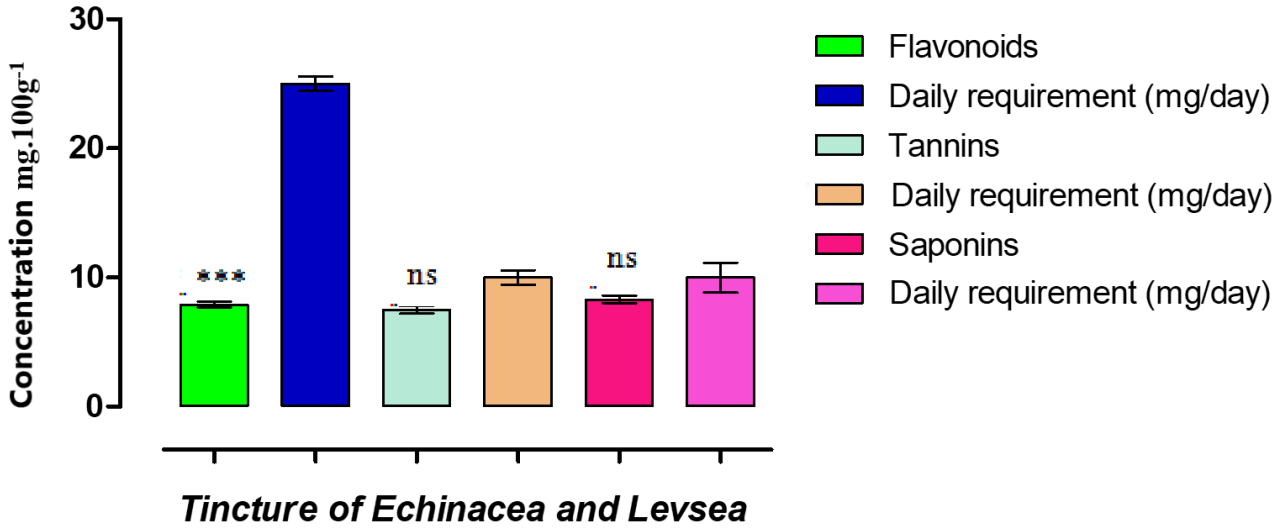


**Figure 3** Concentrations of various phytochemicals including flavonoids, tannins and saponins in tincture prepared from selected plants.

**Table 1** Concentration of heavy metals in tincture.

| Heavy Metals | Concentration ($mg.100g^{-1}$) | Permissible levels of Technical Regulations of the Customs Union |
|---|---|---|
| Lead | 0.0027 | 0.5 |
| Cadmium | 0.00012 | 0.03 |
| Arsenic | ND | 0.05 |
| Mercury | ND | 0.01 |

Note: Each value represents mean results of three independent experimental readings. ND – not detected.

**Table 2** Chemical composition of medicinal herbs.

| Name of medicinal herb | Group of Phytochemicals | $mg.100g^{-1}$ | Daily human requirements (mg) |
|---|---|---|---|
| ***Rhapónticum carthamoídes*** | Flavonoids | 3.1 ±0.346 | 25 |
| | Tannins | 5.5 ±0.115 | 10 |
| | Saponins | 5.6 ±0.17 | 10 |
| ***Echinacea purpurea*** **(L.)** | Flavonoids | 5.5 ±0.20 | 25 |
| | Tannins | 3.1 ±0.46 | 10 |
| | Saponins | 4.1 ±0.40 | 10 |

Note: Each value represents mean ± SEM of three independent experimental results.

Likewise, saponins concentrations were 4.1 ±0.40 $mg.100g^{-1}$ and 5.6 ±0.17 $mg.100g^{-1}$ in *E. purpurea* and *R. carthamoídes* powder respectively (Table 2).

To obtain the optimal ratio of medicinal plants in the composition at the first stage the chemical composition of the studied plants was determined. A mathematical simulation using MS Excel was performed to determine the ratio of levsea to echinacea. We enter data from Table 1 into MS Excel using a function;

$$F(x)= 0.5x_1+0.5x_2 \rightarrow const$$

Using the "Solution Finder" function of the solution we set limits. Based on the obtained mathematical model we obtain the ratio 1:1.

That is, it is established that to create a composition with a balanced composition of biologically active substances, we need a ratio of levsea and echinacea 1:1 respectively. The tincture was prepared using the above ratio of both plant powder materials and was assessed for the presence of flavonoids, saponins, and tannins. Concentrations of these active metabolites in the resultant tincture were flavonoids (7.6 ±0.23), tannins (7.5 ±0.28), and saponins (8.5 ±0.16) $mg.100g^{-1}$ (Figure 3). As discussed earlier, the plant powder as well as tincture consists of essential secondary metabolites and might be effective to use as a potential source of phytochemicals against various diseases.

Amino acids are highly abundant in medicinal plants and serve as a vital source of nutrition. Amino acids being the building blocks of proteins are grouped into essential and non-essential based on their synthesis inside the human body **(Kakimov et al., 2017; Garaeva et al., 2009)**. Briefly, an essential type of amino acid is synthesized only by medicinal plants whereas, a non-essential type of amino acid is prepared by both medicinal plants as well as humans **(Kumar et al., 2019)**.

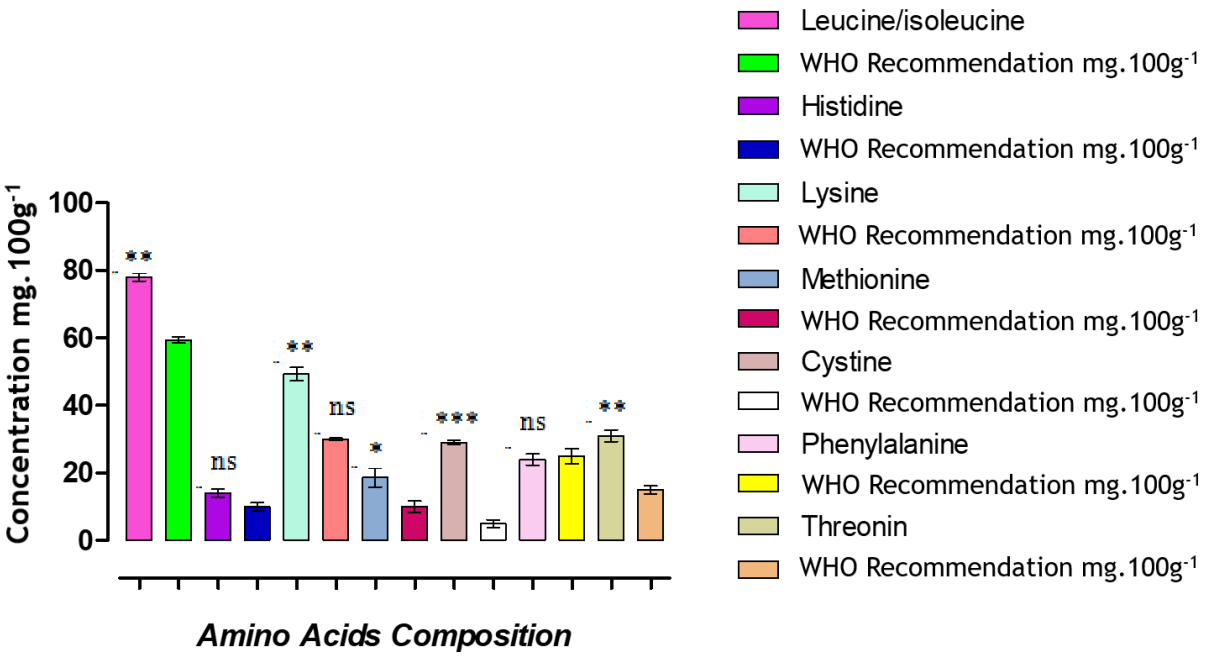


**Figure 4** Results of Essential Amino acids quantification in the tincture of *E. purpurea* and *R. carthamoides.*

**Table 3** Organoleptic indicators of tincture.

| Indicator | Description/Observations |
|---|---|
| Consistency and appearance | Liquid, homogeneous, transparent without sedimentation. |
| Taste and odor | Pleasant taste and typical herbal smell |
| Color | Light brown, uniform across the entire mass |

Due to the tremendous increase in the global population, there is a dire need for the development of alternative food sources. Among the alternative food sources, medicinal plants represent an important source of essential amino acids. These plants beside a source of nutrition are coupled to numerous pharmacological properties also which can be used both as a food source and therapeutic remedies **(Kumar et al., 2019)**.

In the current study, we observed the highest concentrations of these essential amino acids in the tincture including leucine/isoleucine (78.00 ±1.15 mg.100g$^{-1}$), histidine (14.00 ±1.44 mg.100g$^{-1}$), lysine (49.33 ±2.02 mg.100g$^{-1}$), methionine (18.66 ±2.90 mg.100g$^{-1}$), cystine (29.00 ±0.57 mg.100g$^{-1}$), phenylalanine (24.16 ±1.87 mg.100g$^{-1}$) and threonine (32.50 ±1.22 mg.100g$^{-1}$) respectively (Figure 4). Being a source of flavonoids, tannins, saponins, and essential amino acids, the *E. purpurea* and *R. carthamoides* based tincture might be a useful source of secondary metabolites for the prevention and management of various diseases and thus warrant further detailed studies.

The resultant tincture has a pleasant agreeable taste coupled with acceptable herbal flavor which are important organoleptic properties for any product (Table 4). Disagreeable taste in flavor might be a reason for the non-compliance of several medicinal preparations. Further, the tincture has pleasant homogeneous and transparent preparations without sediments and adulterants with a light brown color which aid in increasing individuals' acceptability of the preparations for oral use. These organoleptic, physicochemical indicators of the tincture correspond to the requirements of the State Pharmacopoeia of the Republic of Kazakhstan **(The State Pharmacopeia, 2008)**.

## CONCLUSION

In the current study, both plants exhibited low heavy metals contents which were within the WHO specified range for human use. Dried plant powder materials and resultant tincture exhibited considerably high contents of flavonoids, saponins, tannins, and essential amino acids. These metabolites own numerous pharmacological properties, thus both plants and their resultant tincture might be useful in the prevention and treatment of several diseases. Ideal organoleptic properties of the tincture yet another important aspect of the tincture for use in the general population. The outcomes of the current study will provide a scientific base for more systematic studies. Further detailed studies are required to signify the potential use of the selected plants in various disorders.

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# THE DIVERSITY OF FUNGAL POPULATION FROM GRAPE HARVEST TO YOUNG WINE IN SMALL CARPATHIAN WINE REGION

*Soňa Felšöciová, Jozef Sabo, Lucia Galovičová, Miroslava Kačániová*

## ABSTRACT

The study aimed to identify the filamentous fungi and yeast mycobiota found on the surface and in grapes, grape must, and wine obtained from four red grape varieties: Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon, and four white grape varieties: Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon. Grapes from vineyard Vrbové located in southwestern Slovakia were used for the research in 2020. The identification of filamentous fungi was performed using the macroscopic and microscopic observations and yeasts were identified by MALDI-TOF Mass Spectrometer. A total of 642 isolates were obtained. Grapes were rich in diversity of filamentous fungi (13 genera) and must on yeasts (8 genera). *Penicillium, Botrytis,* and *Hanseniaspora uvarum* were identified in both grapes and must. Three of the fungal genera identified by conventional or molecular techniques from the surface of red grape varieties were predominant: *Alternaria* (26%), *Botrytis* (21%), and *Issatchenkia terricola* (13%), two from endogenous mycobiota: *Hanseniaspora uvarum* (45%) and *Botrytis* (17%), four from the surface of white grape varieties: *Penicillium* (25%), *Botrytis* (21%), *Alternaria* (16%) and *Hanseniaspora uvarum* (15%), and three from endogenous mycobiota: *Botrytis* (44%), *Hanseniaspora uvarum* (23%) and *Alternaria* (20%). *Saccharomyces cerevisiae, Candida krusei, C. utilis,* and *Cryptococcus neoformans* were identified only in wine.

**Keywords:** yeast; filamentous fungi; *Penicillium;* must; mass spectrometry

## INTRODUCTION

Microfungi are ubiquitous microorganisms in the environment. If certain physical conditions, such as moisture level, temperature, and the presence of organic and inorganic substrates, are met in fungi, they can easily proliferate **(Andersen and Thrane, 2006)**. Wine grapes are no exception. Wine grapes (*Vitis vinifera,* L.) are an economically and culturally important agricultural commodity for which microbial activity plays key roles in grape and wine production and quality **(Barata, Malfeito-Ferreira and Loureiro, 2012; Swiegers et al., 2005)**. The grapevine harbors complex and diverse microbiota, such as bacteria, filamentous fungi, and yeasts **(Barata, Malfeito-Ferreira and Loureiro, 2012; Liu and Howell, 2020; Stefanini and Cavalieri, 2018)**, which substantially modulate vine health, growth, and crop productivity **(Gilbert, van der Lelie and Zarraonaindia, 2014; Müller et al., 2016)**.

Recently, winemakers have started to realize the potential contribution offered by the indigenous microbial population in producing a wine closely associated with geographical origin **(Tristezza et al., 2013)**. The geographical area, together with cultivar, climate, and vintage, is the major determinant for the microbiota of must at the beginning of the fermentation process **(Bokulich et al., 2014)**. Grapevine associated microbiota can be transferred to the grape must/juice and influence the production of secondary metabolites on wine composition, aroma, flavor, and quality **(Barata, Malfeito-Ferreira and Loureiro, 2012; Ciani et al., 2010; Calabretti et al., 2012; Morrison-Whittle and Goddard, 2018)**. Fine control of the composition of must microbiota is of paramount importance for the quality of the final product, since different components of must, the microbiota can contribute in contrasting ways to the aroma of the final product, giving either pleasant or undesirable aromatic notes to the wine. It is well known that several factors related to grape juice (i.e. grape composition and chemical characteristics, ethanol accumulation, and temperature) can affect the kinetics of yeast growth **(Fleet and Heard, 1993; Bisson, 1999; Zott et al., 2008)**. Autochthonous fermentation (also known as native or inoculated) is believed to display more complexity in aroma and mouthfeel characters than those conducted with a less rich and complex microbiota **(Boynton and Greig, 2016)**. In contrast, in many wine production regions, the grape juice

or must is immediately inoculated with a commercial strain of *Saccharomyces cerevisiae* **(Bisson, Joseph and Domizio, 2017)**. During fermentation, the microbiota can be affected by both microbial and chemical–physical factors. Some fungal species can either carry out an antimicrobial activity against certain other species/strains **(Oro, Ciani and Comitini, 2014)** or have a positive effect on the growth of other species **(Contreras, Curtin and Varela, 2014)**. Even though a part of this early stage microbiota does not survive the stressful conditions of late must fermentation, it still plays a role in shaping the entire process **(Heard and Fleet, 1988)**. Wine is the end product of the fermentative activity of yeast and bacteria. The microbiota of grape juice fermentation can vary significantly as over 40 genera and 100 different species of yeast have been isolated from grapes or wine **(Bisson, Joseph and Domizio, 2017)**. This paper reports on work to isolate and identify the filamentous fungi and yeast mycobiota from grape harvest from the Small Carpathian wine region of Slovakia to young wine.

## Scientific hypothesis

Most fermented products are generated by a mixture of microbes. Wine is no exception. Substantial yeast, fungal and bacterial biodiversity is observed on grapes, and in both must and wine.

## MATERIAL AND METHODOLOGY

### Samples

### Grape sampling

The grape samples were harvested during the 2020 vintage, from Sabo winery, Vrbové in the Small Carpathian wine region. The study area was described previously **(Felšöciová and Kačániová, 2019a)**.

**Table 1** Wine grape varieties used in the study, date of harvest, sugar content, and selected yeast.

| Grape variety | Date of harvest | Sugar content | Winemaking yeast |
|---|---|---|---|
| Alibernet | 20.10.2020 | 19 °NM | Laffort, Actiflor rosé |
| Dornfelder | 30.09.2020 | 19.5 °NM | reinoculated |
| Blue Frankish | 20.10.2020 | 20 °NM | Laffort, Actiflor rosé |
| Cabernet Sauvignon | 17.10.2020 | 20.5 °NM | Laffort, Actiflor rosé |
| Green Veltliner | 10.10.2020 | 21 °NM | Laffort, delta |
| Rheinriesling | 07.10.2020 | 20 °NM | Spontaneous |
| Pinot Blanc | 03.10.2020 | 20 °NM | Mycoferm, CRU611 |
| Sauvignon | 24.09.2020 | 20 °NM | Laffort, X5 |

In total, 8 grape samples without visual signs of fungal invasion were collected (Table 1).

Four samples of red grapes (Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon) and four samples of white grapes (Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon) were collected from multiple bunches of different grapevines, randomly distributed across the vineyard to assure the representativeness of the sampling. These samples were put into sterile plastic bags and transported to the laboratory chilled on ice and stored at – 20 °C until processing. Fresh grape must be prepared by crushing. Fermentation occurred in stainless steel tanks and was conducted with/without the addition of commercial yeasts (Table 1). Must and young wine of each wine grape variety were acquired and sampled in a winery, sent to the laboratory, and stored at 6 °C in the refrigerator.

### Chemicals

DRBC medium (Dichloran Rose Bengal Chloramphenicol, MERCK, Germany), MEA (Malt extract agar) **(Samson et al., 2010)**, CYA (Czapek yeast agar) **(Samson et al., 2010)**, CREA (Creatine-Sucrose agar) **(Samson et al., 2010)**, YES (Yeast extract agar) **(Samson et al., 2010)**, formic acid (Sigma-Aldrich), acetonitrile (Sigma-Aldrich), matrix α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich).

### Biological Material

Commercial yeasts: Laffort, Actiflor rosé, Laffort, delta, Mycoferm, CRU611, Laffort, X5.

### Grapevine Material

Four samples of red grapes: Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon and four samples of white grapes: Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon from Sabo winery, Vrbové in Small Carpathian wine region.

### Instruments

Stomacher easyMix®, MALDI-TOF-MS Biotyper (Bruker Daltonics, Bremen, Germany).

### Laboratory Methods

Plating methods with and without surface disinfection by the researcher **Magnoli et al. (2003)**, MALDI-TOF MS measurement by **Patel (2015)**.

### Description of the Experiment

### Mycological analysis

For each sample, the mycological diversity was analyzed at three stages: grapes must be obtained by the crushing of grapes and end of alcoholic fermentation as a young wine. The detection of fungi in grape samples was made by plating methods with and without surface disinfection. The individual samples were composed of 50 berries, plated on DRBC medium 7 – 8 berries per plate, and incubated for 5 – 7 days at 25 ±1 °C in the dark. Fungi were also isolated from the interiors of the grapes. Each grape surface (a total of 50 berries from each sample) was sterilized for 1 min in 1% NaClO and washed three times with sterile distilled water according to methods of **Magnoli et al. (2003)**, dried, plated onto DRBC, and incubated.

At a liquid stage, 200 mL of must and unfiltered wine were collected in sterile plastic bottles and stored at 6 ±1 °C in the refrigerator. Must sample in an amount of 20 mL were diluted with 180 mL of sterile physiological saline (0.85%) and shaken on a Stomacher easyMix®.

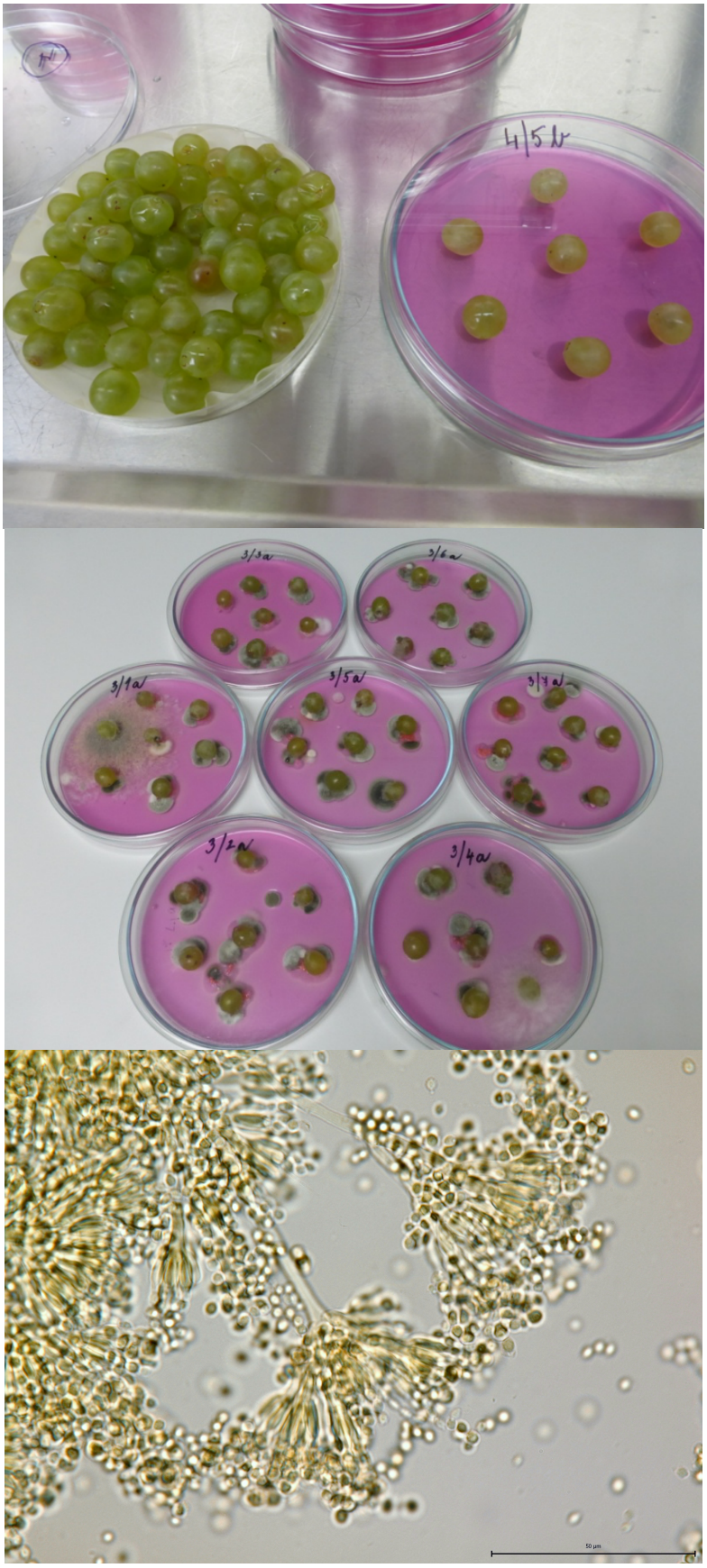

**Figure 1** Direct plating of grapes onto DRBC, Petri dishes after incubation, identification of *Penicillium* sp.

Dilutions of $10^{-1}$ and $10^{-4}$ in the double were surface inoculated in the amount of 0.1 mL on MEA agar plates and cultivated at 25 ±1 °C in the dark. An undiluted sample of wine was also applied in an amount of 0.1 mL to MEA and the plates were incubated at 25 ±1 °C for 5 days.

The developing filamentous fungi were counted and identified based on macro and microscopic characteristics

using the following references: **Pitt and Hocking (2009)** and **Klich (2002)**. *Penicillium* spp. were picked off onto MEA, CYA, CREA, and YES to obtain pure cultures and identify further species. The identification of isolated strains was carried out according to special mycological literature **(Pitt and Hocking, 2009; Samson and Frisvad, 2004; Samson et al., 2002; Samson et al., 2010)**. Yeasts were identified by MALDI-TOF Mass Spectrometry.

**MALDI-TOF MS measurement**

The colony of yeast that was cultivated on Petri dish was transferred to Eppendorf microtubes containing 300 µL of ultrapure water and then 900 µL of ethanol (99%) was added. The sample was centrifuged for 2 minutes at 14 000 rpm and the supernatant was discarded. 30 µL of 70% formic acid (Sigma-Aldrich) and 30 µL of acetonitrile (Sigma-Aldrich) were added to the pellet and the pellet was resuspended thoroughly. The Eppendorf microtubes were centrifuged again at 14 000 rpm for 2 minutes. From the sample thus prepared, we applied 1 µL of the supernatant to a MALDI-TOF-MS target plate and allowed the sample to dry at laboratory temperature. From the prepared sample, 1 µL of the supernatant was applied to a MALDI-TOF-MS target plate and the sample was dried at laboratory temperature. After drying, 1 µL of a matrix α-cyano-4-hydroxycinnamic acid (10 $mg.mL^{-1}$, Sigma-Aldrich), was added to the surface of the sample. After crystallization at laboratory temperature, the target plate was placed into the ionization chamber of the mass spectrometer. The samples were processed by linear and positive mode MALDI-TOF MicroFlex LT/SH (Bruker Daltonics, Germany) in the range 200 – 2000 m/z.

**Number of samples analyzed:** we have analyzed 8 grape varieties, 8 musts and 8 young wines.

**Number of repeated analyses:** 4 times.

**Number of experiment replication:** 3 times.

## Statistical Analysis

The obtained results were evaluated and expressed according to relative density (RD). 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:

$$RD\ (\%) = (ni/Ni) \times 100$$

Where:

ni –number of isolates of a species or genus; Ni – total number of isolated fungi.

## RESULTS AND DISCUSSION

The overall mycological colonization of 4 tested red grape varieties from the surface and endogenous mycobiota of grapes, must and wine are summarized in Table 2. A total of 249 strains of microscopic fungi belonging to 10 genera of filamentous fungi and 10 genera of yeasts were identified. Isolation of fungi colonized surface grapes resulted in the collecting of 53 fungal isolates (39 isolates from filamentous fungi and 14 isolates from yeasts). Data in Table 2 also show that 10 filamentous fungal genera and 4 yeast genera were identified from surface grape samples. *Alternaria* was the most abundant occurring genus which recorded 14 isolates with a relative density of 26% (RD) of all the isolates found. *Botrytis* was the second predominant genus which recorded a relative density of 21%, followed by *Issatchenkia terricola* with 13% RD, *Mucor* with 7% RD, and *Hanseniaspora uvarum* together with *Aureobasidium pullulans* (6%, each) of all the fungi found. The remaining 8 genera were detected in less than 5%. *Alternaria* (26% RD), *Penicillium* (23% RD), *Rhizopus* (15% RD), and *Cladosporium* (11% RD) were the main components of the 3 red grape mycobiota (Alibernet, Cabernet Sauvignon, and Blue Frankish) of the Vrbovský subregion at harvest time 2019 **(Felšöciová, Kačániová, and Vrábel, 2020)**. *Alternaria* (87% RD) was still the main component of Alibernet, Cabernet Sauvignon, and Dornfelder grapes mycobiota from Vrbové in 2018 **(Felšöciová and Kačániová, 2019c)**. The same 3 red grape samples plus Blue Frankish collected in 2017 were analyzed for the occurrence of yeasts by **Kačániová et al. (2018)**. Within 39 isolates of yeasts, the most abundant was *Rhodotorula*. This genus we identified only from must. *Saccharomyces*, the major wine yeast is not ubiquitous on the ripening grape and if present, only constitutes very small fractions of the yeast communities **(Setati et al., 2013; Bokulich et al., 2014; Taylor et al., 2014)**. *Saccharomyces* strains are more frequently isolated from heavily damaged grapes **(Mortimer and Polsinelli, 1999)**, where the juice of the grape became accessible to the yeasts through the skin lesions. Consistent with these earlier observations, no *Saccharomyces* was found in this study among the colonies when the samples were plated directly on the agar medium. Samples belonging to red grape varieties Alibernet and Blue Portugal from Suchá nad Parnou showed different abundance from surface colonization **(Felšöciová and Kačániová, 2019b)**. The most abundant genera were *Cladosporium* (28% RD), *Alternaria, Epicoccum* (26%, each), and *Botrytis* (24% RD) in the harvest year 2016 and 2017 *Cladosporium* (24% RD), *Penicillium* (23% RD), *Alternaria* (21% RD) and *Epicoccum* (14% RD) of all the fungal isolates found. **Medina et al. (2005)** referred to the diversity of filamentous fungi isolated from muscat grape varieties grown in Spain. *Cladosporium* was the most common strain isolated from two blue varieties Garnacha and Monastrell (78.2% and 92.2%, respectively) of all isolates. **Abrunhosa et al. (2001**) reported that *Alternaria* and *Cladosporium* were more often isolated from red grape varieties than white, regardless of the vineyard in Portugal, which can not be confirmed from our study. *Cladosporium* was detected only once.

All the previous fungal genera except *Acremonium, Aspergillus, Dipodascus,* and *Issatchenkia* were also isolated from endogenous colonization. Nevertheless, the relative abundances varied.

**Table 2** The occurrence and relative density of filamentous fungi and yeast identified from exogenous and endogenous mycobiota of 4 red grapes, must and wine from Small Carpathian wine region.

| Taxa | grapes exo | | grapes endo | | must | | wine | | Total | RD (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Filamentous fungi** | **No** | **RD (%)** | **No** | **RD (%)** | **No** | **RD (%)** | **No** | **RD (%)** | | |
| *Alternaria* | 14 | 26 | 3 | 4 | - | - | - | - | 17 | 7 |
| *Acremonium* | 1 | 2 | - | - | - | - | - | - | 1 | <1 |
| *Arthrinium* | 1 | 2 | 1 | 1 | - | - | - | - | 2 | <1 |
| *Aspergillus* | 2 | 4 | - | - | - | - | - | - | 2 | <1 |
| *Botrytis* | 11 | 21 | 11 | 17 | 3 | 2 | - | - | 25 | 10 |
| *Cladosporium* | 1 | 2 | 2 | 3 | - | - | - | - | 3 | 1 |
| *Mucor* | 4 | 7 | 7 | 11 | - | - | - | - | 11 | 4 |
| *Mycelia sterilia* | - | - | 1 | 1 | - | - | - | - | 1 | <1 |
| *Penicillium* | 2 | 4 | 1 | 1 | 72 | 57 | - | - | 75 | 30 |
| *P. crustosum* | - | - | 1 | - | - | - | - | - | 1 | - |
| *P. expansum* | - | - | - | - | 72 | - | - | - | 72 | - |
| *P. sp.* | 2 | - | - | - | | - | - | - | 2 | - |
| *Rhizopus* | 1 | 2 | 1 | 1 | - | - | - | - | 2 | <1 |
| *Trichoderma* | 2 | 4 | 1 | 1 | - | - | - | - | 3 | 1 |
| **Total of fil. fungi** | **39** | | **28** | | **75** | | | | **142** | |
| **Yeast taxa** | | | | | | | | | | |
| *Aureobasidium pullulans* | 3 | 6 | 6 | 9 | - | - | - | - | 9 | 4 |
| *Candida* | - | - | - | - | 6 | 5 | 2 | 67 | 8 | 3 |
| *C. catenulata* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. dubliniensis* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. krusei* | - | - | - | - | 2 | - | 2 | - | 4 | - |
| *C. oleophila* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. tropicalis* | - | - | - | - | 1 | - | - | - | 1 | - |
| *Cryptococcus* | - | - | - | - | 2 | 2 | 1 | 33 | 3 | 1 |
| *Cr. flavescens* | - | - | - | - | 1 | - | - | - | 1 | - |
| *Cr. neoformans* | - | - | - | - | 1 | - | 1 | - | 2 | - |
| *Dipodascus* | 1 | 2 | - | - | - | - | - | - | 1 | <1 |
| *Hanseniaspora uvarum* | 3 | 6 | 30 | 45 | 22 | 17 | - | - | 55 | 22 |
| *Issatchenkia terricola* | 7 | 13 | - | - | 7 | 5 | - | - | 14 | 6 |
| *Metschnikowia pulcherrima* | - | - | - | - | 13 | 10 | - | - | 13 | 5 |
| *Pichia occidentalis* | - | - | - | - | 1 | <1 | - | - | 1 | <1 |
| *Rhodotorula* | - | - | - | - | 1 | <1 | - | - | 1 | <1 |
| *Saccharomyces cerevisiae* | - | - | 2 | 3 | - | - | - | - | 2 | <1 |
| **Total of yeasts** | **14** | | **38** | | **52** | | **3** | | **107** | |
| **Total isolates** | **53** | | **66** | | **127** | | **3** | | **249** | |

Note: No – number of isolates; RD – relative density.

From endogenous colonization were reached 66 strains belonging to 8 genera and *Mycelia sterilia* of filamentous fungi (28 isolates) and 3 genera of yeasts (38 isolates). *Mycelia sterilia* is an unidentified microorganism – fungus without creation fruiting bodies. The most abundant genera were *Hanseniaspora uvarum* (45%), *Botrytis* (17%), and *Mucor* (11%) of all the isolates. The occurrence of *Botrytis,* as described **Felšöciová and Kačániová (2019b)**, was detected in 2% of the isolates from endogenous mycobiota in Alibernet and Blue Portugal in 2017 but in 2016 *Botrytis* was one of the most abundant genera (24% RD). The most abundant genera found by descending order except *Botrytis* were *Alternaria* (29% RD), and *Cladosporium* (23% RD) in 2016, and *Penicillium* (38% RD) and *Cladosporium* (29% RD) in 2017. *Penicillium* spp. in our red grape samples was generally low. Only *P. crustosum* was isolated. *Alternaria* (87% RD) was the most common genus not only in exogenous mycobiota but also in the endogenous mycobiota from Alibernet, Cabernet Sauvignon, and Dornfelder from Vrbové in 2018 **(Felšöciová and Kačániová, 2019c)**.

The highest number of yeasts was found from the must of red grape samples. A total of 52 strains of yeasts belonging to 7 genera were identified. The most abundant genera were *Hanseniaspora uvarum* (17% RD), *Metschnikowia pulcherrima* (10% RD), and *Candida* (5% RD) with 5 species of all the 127 fungi found. The apiculate yeasts usually predominate the early phase of fermentation and produce compounds, that enrich the aroma profile of the wine **(Zironi et al., 1993; Romano et al., 2003; Moreira et al., 2011; Sipiczki, 2016; Cioch et al., 2021)**. Pulcherrimin-producing *Metschnikowia* strains are common on ripe grapes. They are usually assigned to *M. pulcherrima* (*C. pulcherrima*) or less frequently to *M. fructicola* in the oenological literature **(Sipiczki, 2016)**. Species from the genus *Hanseniaspora, Candida, Pichia, Zygosaccharomyces,* and *Kluyveromyces* most desirably determine the diversity and complexity of the taste of wine **(Romano et al., 2003; Jolly, Varela and Pretorius, 2014)**. Most of them were identified from our samples of must. Must was riched on *Penicillium expansum* (57%). Except for *Penicillium expansum* (72 isolates), three

isolates of *Botrytis* (2%) were detected. Berries affected by *P. expansum* have an off-flavor and even a small amount of infected berries add a mouldy taste to the wine **(König and Fröhlich, 2017)**. **Felšöciová, Mašková and Kačániová (2018)** described 5 isolates of microfungi belonging to 3 genera of *Alternaria, Aspergillus* and *Cladosporium* and *Mycelia sterilia* from grape juice from red variety Dornfelder. Yeast counts in fresh grape juice were 2 x $10^5$ CFU.mL$^{-1}$. It is considered that *Dekkera/Brettanomyces* are the most important wine spoilage microorganisms **(Bartowsky et al., 2003; Beneduce et al., 2004; Cocolin et al., 2004)**. In this study, *Dekkera/Brettanomyces bruxellensis* was not detected, which is in line with the study of **Suárez et al. (2007)**, who reported that this spoilage yeast is mainly present in winemaking equipment with deficient cleaning, and is opposed to the findings reported by **Renouf and Lonvaud-Funel (2007)**.

In red wine were not detected any filamentous fungi what confirmed in their study also **Felšöciová, Mašková and Kačániová (2018)**.

A low number of yeasts was recorded, namely *Candida krusei* (2 isolates) and *Cryptococcus neoformans* (1 isolate). According to **Felšöciová, Mašková and Kačániová (2018**) yeast at the end of fermentation slightly decreased on 5.7 x $10^4$ CFU.mL$^{-1}$.

Fungal profiles of the various 4 white grape varieties, must and wine are summarized in Table 3. A total of 393

**Table 3** The occurrence and relative density of filamentous fungi and yeast identified from exogenous and endogenous mycobiota of 4 white grapes, must and wine from Small Carpathian wine region.

| **Taxa** | **grapes exo** | | **grapes endo** | | **must** | | **wine** | | **Total** | **RD (%)** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Filamentous fungi** | **No** | **RD (%)** | **No** | **RD (%)** | **No** | **RD (%)** | **No** | **RD (%)** | | |
| *Alternaria* | 11 | 16 | 23 | 20 | - | - | - | - | 34 | 9 |
| *Arthrinium* | 1 | 1 | - | - | - | - | - | - | 1 | <1 |
| *Asperillus* | 2 | 3 | - | - | - | - | - | - | 2 | <1 |
| *Botrytis* | 14 | 21 | 50 | 44 | 10 | 5 | - | - | 74 | 19 |
| *Cladosporium* | 1 | 1 | - | - | - | - | - | - | 1 | <1 |
| *Epicoccum* | - | - | 1 | <1 | - | - | - | - | 1 | <1 |
| *Fusarium* | 1 | 1 | - | - | - | - | - | - | 1 | <1 |
| *Mucor* | 2 | 3 | 3 | 3 | - | - | - | - | 5 | 1 |
| *Mycelia sterilia* | - | - | 1 | <1 | - | - | - | - | 1 | <1 |
| *Penicillium* | 17 | 25 | 5 | 4 | 119 | 65 | - | - | 141 | 36 |
| *P. expansum* | 17 | - | 5 | - | 36 | - | - | - | - | - |
| *P. glabrum* | - | - | - | - | 83 | - | - | - | - | - |
| *Phoma* | - | - | 1 | <1 | - | - | - | - | 1 | <1 |
| *Rhizopus* | 5 | 7 | 1 | <1 | - | - | - | - | 6 | 1 |
| **Total of fil. fungi** | **54** | | **85** | | **129** | | | | **268** | |
| **Yeasts taxa** | | | | | | | | | | |
| *Aureobasidium pullulans* | 1 | 1 | 1 | <1 | - | - | - | - | 2 | <1 |
| *Candida* | - | - | - | - | 15 | 8 | 1 | 5 | 16 | 4 |
| *C. catenulata* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. haemulonii* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. innocuum* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. krusei* | - | - | - | - | 2 | - | - | - | 2 | - |
| *C. lambica* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. lusitaniae* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. parapsilosis* | - | - | - | - | 1 | - | - | - | 1 | - |
| *C. tropicalis* | - | - | - | - | 6 | - | - | - | 6 | - |
| *C. utilis* | - | - | - | - | - | - | 1 | - | 1 | - |
| *C. valida* | - | - | - | - | 1 | - | - | - | 1 | - |
| *Cryptococcus* | - | - | - | - | 3 | 2 | - | - | 3 | <1 |
| *Cr. flavescens* | - | - | - | - | 1 | - | - | - | 1 | - |
| *Cr.neoformans* | - | - | - | - | 2 | - | - | - | 2 | - |
| *Dipodascus* | 3 | 4 | - | - | - | - | - | - | 3 | <1 |
| *Hanseniaspora uvarum* | 10 | 15 | 26 | 23 | 10 | 5 | - | - | 46 | 12 |
| *Kluyveromyces nonfermentans* | - | - | - | - | 1 | <1 | - | - | 1 | <1 |
| *Metschnikowia pulcherrima* | - | - | 1 | <1 | 33 | 18 | - | - | 34 | 9 |
| *Pichia* | - | - | - | - | 2 | <1 | - | - | 2 | <1 |
| *Pichia kluyveri* | - | - | - | - | 1 | - | - | - | 1 | - |
| *Pichia occidentalis* | - | - | - | - | 1 | - | - | - | 1 | - |
| *Saccharomyces cerevisiae* | - | - | - | - | - | - | 18 | 95 | 18 | 5 |
| **Total of yeasts** | **14** | | **28** | | **64** | | **19** | | **125** | |
| **Total isolates** | **68** | | **113** | | **193** | | **19** | | **393** | |

Note: No – number of isolates, RD – relative density.

strains of microscopic fungi belonging to 20 genera and *Mycelia sterilia* were obtained from surface and endogenous mycobiota of grapes, must, and wine. The filamentous fungi population was richer than the yeast population from surface mycobiota. A total of 68 strains belonging to 12 genera were identified (9 from filamentous fungi and 3 from yeasts). *Penicillium expansum* was isolated in large amounts (25% RD), followed by *Botrytis* (21% RD), *Alternaria* (16% RD), and *Hanseniaspora uvarum* (15% RD) of all the isolates. Our results corroborate the findings of **Felšöciová, Kačániová and Vrábel (2020)** in which 26 % of isolates were *Penicillium* and 21% *Alternaria* from 9 white grapes from Vrbové during the harvest 2019. *Alternaria* was also one of the main fungal genera isolated from Tunisian grape berries **(Melki Ben Fredj et al., 2007**), Spanish grapes **(Bau et al., 2005; Medina et al., 2005)**, Moravian grapes **(Ostrý et al., 2007)**. Yeasts as *Sporobolomyces roseus, Cryptococcus albidus, Rhodotorula rubra,* and *Candida* were part of the natural microbiota of certain varieties of grapes in southern Spain **(De la Torre et al., 1999)**. In Egypt, **Haridy (1994)** found that the most common spoilage yeast of grapes was *Hanseniaspora valbyensis*. Also, *Hanseniaspora* species were reported as common yeast constituents on grapes **(Phister et al., 2007)** as confirmed by our results.

A total of 113 isolates of microscopic fungi belonging to 10 genera and *Mycelia sterilia* were obtained from endogenous mycobiota (7 from filamentous fungi and 3 from yeasts). The highest relative density was reached by the genera *Botrytis* (44%), *Hanseniaspora uvarum* (23%), and *Alternaria* (20%). *Penicillium expansum* (4%) contributed a small proportion of all fungi in comparison with exogenous colonization. Interestingly, *Botrytis* from the same grape samples was detected less than 1% in the harvest year 2018 **(Felšöciová and Kačániová, 2019c)**.

The microbial community present in the must before fermentation was rich, especially the diverse biodiversity of yeasts. Data in Table 3 show that 2 filamentous fungal genera and 6 yeast genera were identified. The dominant genus across the entire microfungi population was *Penicillium* (65%) and in a small proportion *Botrytis* (5%). Two *Penicillium* species, namely *P. glabrum* (83 isolates) and *P. expansum* (36 isolates) were identified. From the yeast population, the dominant species was *Metschnikowia pulcherrima* (18%), followed by *Candida* (8%) with 9 species and *Cryptococcus* (2%) with 2 species. *Pichia* and *Kluyveromyces* were identified as less than 1%. *Cladosporium* (75.5% RD) and *Penicillium* (10.2% RD) were present with high abundance in 47 must samples collected from five Slovakian wine regions, representing on average 75.5% and 10.2%, respectively of the fungal populations **(Felšöciová, 2016)**. The *Penicillium* genus was made up of *P. bilaiae, P. brevicompactum, P. citrinum, P. crustosum, P. expansum, P. funiculosum, P. griseofulvum, P. chrysogenum, P. polonicum,* and *P.* sp. **Barboráková et al. (2011)** obtained information about the mycobiota of Slovak origin wines during the production process in the year 2009. Altogether thirty-three samples from the production process of 5 species of white Slovak origin wines were mycologically analyzed. The spectrum of isolated penicilia consisted of twenty-one species: *Penicillium aurantiogriseum, P. brevicompactum, P. citreonigrum, P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum, P. funiculosum, P. glabrum, P. griseofulvum, P. implicatum, P. oxalicum, P. paneum/carneum, P. pinophilum, P.polonicum, P. purpurogenum, P. restrictum, P. roqueforti, P. rubrum, and P. rugulosum*. **Pinto et al. (2015)** characterized and compared the diversity of the microbial communities during spontaneous wine fermentations from samples collected from six Portuguese wine regions. In general, the fungal populations of initial must were characterized by ubiquitous genera as *Aureobasidium, Rhodotorula, Hanseniaspora, Alternaria, Metschnikowia, Saccharomyces, Candida, Ramularia, Penicillium, Lewia, Filobasidiella, Leptosphaerulina,* and *Schizosaccharomyces*, forming the principal structure of the microbial populations. This is in line with the previous study reported by **Bokulich et al. (2014)**, where microorganisms as *Cladosporium* spp., *Aureobasidium pullulans, Hanseniaspora uvarum* were detected as the major eukaryotic population in the initial must samples. The high microbial biodiversity within initial must samples was mostly due to environmental microorganisms derived from vineyard. In the freshly crushed grape must/juice from Australia, fungal communities were highly diverse and characterized by ubiquitous genera such as *Aureobasidium, Cladosporium, Saccharomyces,* and *Rhodotorula,* deriving from the vineyard ecosystem **(Liu et al., 2021)**. On the other hand, filamentous fungi were surprisingly missed in fresh grape juice from the white variety.

Palava on DRBC agar medium according to **Felšöciová, Mašková and Kačániová (2018)**, but the initial yeast diversity rapidly evolved in extremely stressful conditions, dominated by high sugar and low initial temperatures and the concentration of yeasts was 1 x $10^4$ CFU.mL$^{-1}$.

In wine were not detected any filamentous fungi but 2 genera of yeasts were recorded. The entire microbial community was mostly characterized by *Saccharomyces cerevisiae* (95%), and *Candida utilis* (5%). **Felšöciová, Mašková and Kačániová (2018)** referred that at the end of the fermentation process only a few strains of yeasts survived (7.4 x $10^3$ CFU.mL$^{-1}$) in wine sample from white variety Palava.

## CONCLUSION

Four red grape varieties Alibernet, Dornfelder, Blue Frankish, Cabernet Sauvignon, and four white grape varieties Green Veltliner, Rheinriesling, Pinot Blanc, Sauvignon, from Small Carpathian wine growing region were analyzed by direct plating methods and must and wine by plate dilution method. In total, 410 isolates of filamentous fungi were identified by morphological analyses and 232 isolates of yeast by MALDI-TOF. From red grape varieties, 249 microbial isolates and 393 from white grape varieties were isolated, among which the filamentous fungi represented 64% of all isolates. The most abundant genera were *Penicillium, Hanseniaspora uvarum* and *Botrytis* from the total fungi accounted.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE STUDY OF SOLUBLE SOLIDS CONTENT ACCUMULATION DYNAMICS UNDER THE INFLUENCE OF WEATHER FACTORS IN THE FRUITS OF CHERRIES

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## ABSTRACT

High tasting assessment of the fruit of sweet cherry is due to the favorable soluble solids content (SSC). The weather parameters and varietal features during the formation of fruit have the dominant influence on the accumulation of soluble solids. This issue has gained new relevance in the context of global climate change. The research aimed to develop a dependence of the accumulation of soluble solids of the various sorts of sweet cherries on the weather conditions of the South Steppe zone of Ukraine. Statistical analysis of the values of soluble solids in sweet cherry fruit was performed according to the average indicators of three groups of cultivars. To achieve this goal, the laboratory, factor, correlation, and regression analyses were carried out. The mathematical model was built with the application of factor and regression analysis methods, with the principal component analysis being used. The factor and regression analysis methods became the basis for the linear regression model of dependence of SSC fund accumulation on the influence of climatic parameters for the cultivar types of the three ripening terms. Based on the constructed regression models, we analyzed the degree of influence of the weather parameters on the SSC indicator by calculating the coefficients of $\Delta_i$ relative influence. The largest influence was set for the group of temperature and humidity parameters with the maximum share of $\Delta_i \geq 9.50\%$. It was mathematically substantiated that the weather parameters of the last month of fruit formation had the greatest influence on the accumulation of SSC in the sweet cherry fruit, regardless of the period of ripening. For early and medium ripening sweet cherries, those were the weather parameters for May, and for those of late-term of ripening June parameters were of the maximum value.

**Keywords**: pomological variety; ripening terms; principal component analysis; factor analysis; biochemical indicator; cherry

## INTRODUCTION

Sweet cherry is in demand in many countries of the world. The share of sweet cherry fertile plantations in the world is more than 3.1%, which makes about 12.5 thousand hectares. The majority of these plantations – 42.6% are concentrated in the Middle East and European countries **(Durau et al., 2012)**. Ukraine ranks tenth in the world in terms of sweet cherry fruit production. In 2018, the gross output of the fruit amounted to 84.6 thousand tons, which is about 3.3% share of the world's production **(Zheplinska et al., 2019)**. According to FAO data, the world market for sweet cherries is undersupplied. According to scientifically based standards, the annual consumption of sweet cherry fruit per capita should be 2 kg **(Winkler and Knoche, 2019)**. The global production of sweet cherries should reach 14 million tons to ensure such volumes. Now it is 4 million tons.

The fruit of the sweet cherry cultivar, being the first to open the season, is a source of easily digestible monosaccharides and contains a powerful immunostimulatory complex of several valuable nutrients. This is the reason for their popularity not only in Ukraine but also in Hungary, Bulgaria, Turkey, etc. **(Blagov et al., 2009)**. Studies by Canadian and Russian scientists have shown that each variety is characterized by a certain genetically determined biochemical composition **(de Carvalho et al., 2019)**. High testing assessment of sweet cherries is due to favorable soluble solids content. Most dry matter is represented by soluble solids content (SSC), which includes carbohydrates, acids, nitrogen compounds, enzymes, soluble forms of pectin and vitamins, phenolic compounds, mineral salts, etc. **(Ivanovych, 2018)**.

The ratios of soluble solids and insoluble solids according to Ukrainian and foreign researchers **(Ivanova, Herasko and Dolhova, 2019)** range within the scope of 14.61 – 18.98% and 2.15 – 2.65%, respectively. The results of many scientific studies proved that the level of soluble solids content affected the intensity and direction of oxidation-reduction processes during the storage of fruit and

determined the choice of process specifications **(Basanta et al., 2014)**. Provided there is a low soluble solids content and excess moisture in the fruit, transpiration processes are intensified **(Mikhailik et al., 2014)**.

Several scientific research traditionally used regression and correlation analyses to assess the degree of influence of parameters on the experimental indicators. However, in conditions when the number of factors for analysis significantly exceeds the number of experimental research, it is not possible to apply a traditional least squares method to build a regression model. Therefore, it has been proposed to build a model using the method of factor analysis – principal component analysis **(Chigozie, 2012)**.

## Scientific Hypothesis

Evaluation of climatic conditions during cultivation and selection of rational parameters for obtaining juice from cherry berries will increase the efficiency of the processing process and improve the method of determining the content of soluble solids in various fruit and berry raw materials, which in turn will intensify the process.

## MATERIAL AND METHODOLOGY

### Samples

To determine the SSC, a sample was taken for each pomological cultivar which consisted of 100 fruit from 6 trees that came into full fruiting. Trees typical of a certain pomological cultivar, of the same age, with a medium intensity of fruiting, were selected for the research. The fruit was weighed and counted in the process of harvesting. The sweet cherries of each commercial cultivar were carefully harvested in the state of consumer ripeness. They were stored and transported to the laboratory provided that the fruit in the period of the indicator determination had the appearance and the taste inherent in the pomological cultivar.

### Chemicals

Chemical reagents were not used for scientific research.

### Biological Material

The study included sweet cherry fruit of 33 varieties, which were divided into three groups of ripening terms: Group1 – cultivars of the early termination of ripening – Sweet Erlise, Merchant, Bigaro Burlat, Rubinova Rannya (Figure 1a), Valeriy Chkalov (Figure 1b), Kazka, Zabuta; Group 2 – cultivars of the medium-term of ripening – Cordia, Octavia, Vinka (Figure 1c), Pervistok (Figure 1d), Temp, Uliublenytsia Turovtseva, Talisman, Dilemma, Melitopolska Chorna, Orion, Chervneva Rannya, Dachnytsia, Prostir; Group 3 – cultivars of the late-term of ripening – Karina (Figure 1e), Regina (Figure 1f), Mirazh (Figure 1g), Krupnoplidna, Udivitelna, Zodiac, Siurpryz, Kolkhoznytsia, Kosmichna (Figure 1h), Prazdnichna, Anons, Temporion, Meotyda.

The agricultural background in the areas of the research within the whole period of the research met the requirements of agricultural technology. The accumulation of moisture in the soil mainly takes place in autumn, and partly in winter, and the early spring. The soils of the experimental plots for the cultivation of the crop are the light loamy black humus earth. The forest is the basis for soil formation. According to the aggregate size distribution, this type of soil has a high content of physical sand.

Horticultural farms of the region of research are located in the South Steppe subzone of Ukraine. The landscape of the territory is flat with the Atlantic-continental climate and a high-temperature scenario. The range of average annual air temperature is 9.1 – 9.9 °C. The average monthly air temperature in the warmest months ranges from 20.5 – 2.1 °C. The sum of active temperatures above 10 °C from April to October is 33 to 16 °C. The average annual rainfall is 475 mm. In terms of rainfall, the region belongs to the area with insufficient moisturization. The average annual relative humidity is within 73%. The average annual wind speed is 3.7 $m.s^{-1}$. The climate is arid with the dominance of dry east, north-east winds. The value of the hydrothermal coefficient is within the range of 0.22 – 0.77.

### Instruments

Refractometer (ABBE AR12, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Laboratory hydraulic press (LHP – 25, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

The meteorological data provided by the meteorological station at Melitopol.

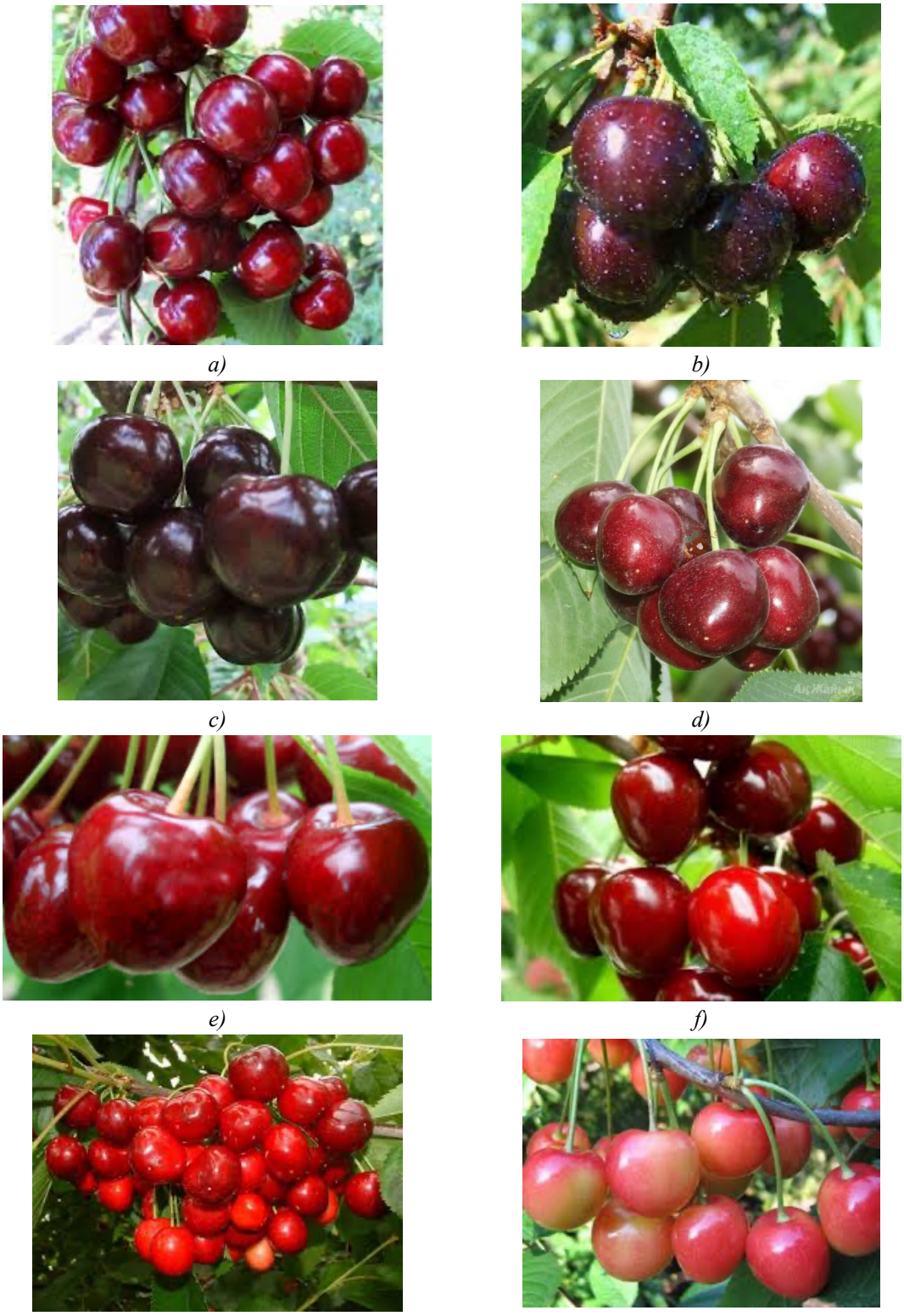
### Laboratory Methods

The SSC was determined by the refractometric method. The essence of the method is based on determining the mass fraction of SSC with the help of a refractive index. The refractive index of the solution under analysis was measured at the temperature of 20.0 ±0.5 °C on the ABBE AR12 refractometer. In the process of sampling, care was taken to ensure that the sample received for the research was representative and was not subject to spoilage during the transportation and storage. The juice was squeezed from the selected average sample with the help of a press. The resulting juice was used for determination. The mass fraction of soluble solids was determined by direct readout of the instrument scale in percent. In case of temperature deviation in the process of measurement, the values of temperature corrections were taken into account. The quality assessment of each pomological cultivar was performed five times **(Serdiuk et al., 2020a)**.

As it is known, the analysis of matching correlation coefficients is an initial but rather rough estimate of the degree of influence of a single factor on the indicator under research. Other instruments of statistical analysis, such as regression and factor analysis, the principal component analysis, should be used to further investigate the issue **(Palamarchuk et al., 2019)**.

However, since the number of considered factors $x_{j,j} = 1.25$ in this case, significantly exceeds the number of observations (Y_1i,Y_2i,Y_3i, where i = 1 – 12 years of research) it is not possible to apply the classical pattern of regression analysis. Moreover, the factors themselves are strongly correlated with each other, as many interfactor matching correlation coefficients are close to ±1. This indicates the presence of multicollinearity effect.

Therefore, it is proposed to conduct the research of the degree of influence of each of the factors and identify the most significant ones using the method of principal component analysis (PCA). The principal component analysis method allows reducing a large number of variables by constructing artificial factors (principal components – $(PC_i, i = 1...n)$.



*a)* *b)* *c)* *d)* *e)* *f)* *g)* *h)*

**Figure 1** Photos of individual samples of cherries: *a* – Rubinova Rannya; *b* – Valeriy Chkalov; *c* – Vinka, *d* – Pervistok; *e* – Karina; *f* – Regina; *g* – Mirazh; *h* – Kosmichna.

The main components are a linear combination of the original factors $x_i$ and do not correlate with each other. At the same time, a set of such principal components is distinguished, which extracts the maximum variance from the variables **(Palamarchuk et al., 2020; Ming-ming and Jing-lian, 2015; Chen et al., 2018)**.

Description of the Experiment. The research was conducted during 12 consecutive vegetation periods from 2008 to 2019. During the experiment, we used the meteorological data provided by the meteorological station at Melitopol, Ukraine.

The construction of models of sweet cherry SSC dependence on weather parameters was carried out following the following pattern:

1. Determination of SSC using the above method.

2. Systematization of information and creation of a database of weather conditions in the years of research. At this stage, the following indicators were selected: average minimum air temperatures, average air temperatures, average maximum air temperatures, absolute minimum air temperatures, absolute maximum air temperatures, precipitation total, average relative humidity, minimum relative humidity, absolute minimum relative humidity.

3. Based on the indicators selected above, the following indicators were calculated: hydrothermal coefficient, temperature differences for certain periods, sums of active temperatures, sums of effective temperatures.

4. Based on the correlation analysis, the weather factors that significantly affect the accumulation of SSC for the sweet cherry of the early, medium, and late-ripening terms were identified.

5. Analysis of the factors defined in Paragraph 4 by methods of factor and regression analysis to determine the degree of influence of each factor on the SSC for the cultivar groups of 3 ripening terms **(Ivanova, Herasko and Dolhova, 2019)**.

**Statistical Analysis**

The following pattern of statistical data analysis is proposed:

1. Using the principal component analysis of the given factors (parameters $x_{ij}, i = 1 \ldots n$ – number of the weather parameter, $j = 1 \ldots m$ – number of the year of research) the set of principal components is composed $(PC_i, i = 1...n)$. Then the initial principal components are determined $(PC_i, i = 1...k, k \prec n)$, which provide the maximum variance – more than 90% **(Chigozie, 2012)**.

2. Using the least-squares method, the regression dependence of the indicators $Y_1$, $Y_2$, $Y_3$ on the principal components $(PC_i, i = 1...k)$ is formed such that:

$$\hat{Y}_i = b_0 + \sum_{j=1}^{k} b_j \cdot PCA_j, \quad i = 1 \ldots 3, \tag{1}$$

3. The expressions of the principal components through the initial values of the weather factors $(x_i, i = 1...n)$ are substituted into the regression model (1). After such a transformation, we obtain the regression dependences of the SSC indices on the initial factors for the three groups of cultivars such that:

$$\hat{Y}_i = a_0 + \sum_{j=1}^{k} a_j \cdot x_j, \tag{2}$$

Where:

$x_j$ shall be factors; $a_j$ – shall be model parameters; $(\hat{Y}_i, i = 1...3)$ shall be SSC indicators for the sweet cherry of the three-cultivar groups.

4. The constructed regressions are analyzed and the degree of influence of each of the factors on the resulting indicators are evaluated. The analysis of the constructed regression is performed based on $\Delta_i$ coefficients. These coefficients characterize the relative degree of influence of each of the considered factors $(x_j, j = 1...n)$ on the researched indicator $(y_i, i = 1...3)$ for the 3 groups of cultivars. The $\Delta_i$ coefficients are calculated according to the formula:

$$\Delta_i = \left| \frac{\tilde{a}_i \cdot r_{yx_i}}{R^2} \right| \tag{3}$$

Where: $\tilde{a}_i$ – shall be the parameters of the standardized regression model; $r_{yx_i}$ – shall be the matching correlation coefficients; $R^2$ – shall be the determination coefficient.

To perform the statistical analysis, the instruments of a modern Data Mining computer technology with RStudio v. 1.3.959 software environment was used.

## RESULTS AND DISCUSSION

The level of soluble solids accumulation can change under the influence of soil and weather conditions, yield, and the degree of fruit ripeness. In the south of Ukraine, the fruit of the best cultivars of sweet cherry contain 12.1 – 19.9% of soluble solids, whereas in the central part of the country this indication is only 11.3 – 12.8% **(Slavin and Lloyd, 2012; Bublyk et al., 2014; Ivanova et al., 2019)**. The foreign scientists established the change in the biochemical composition of sweet cherry cultivars of different ripening terms depending on the region of cultivation **(Cittadini et al., 2008; Basanta et al., 2014; Chen et al., 2018)**. The dominant influence of weather factors on the accumulation of SSC in comparison with the cultivar characteristics was established in our previous research **(Serdiuk et al., 2020b)**. With that said, the soluble solids content in sweet cherries depends on many factors. However, the decisive influence of weather factors is noted **(Sansavini and Lugli, 2008)**. Given this, the issue of predicting the soluble solids content of sweet cherries depending on the share of weather factors is relevant for the sake of further improvement of transportation and processing technology **(Zheplinska et al., 2021)**.

Year, treatment, and their interaction had significant effects on fruit size, thickness, and weight. Fruit height, the height: width ratio and SSC were not influenced by year and treatment, though the interaction of these factors significantly affected SSC **(Bobos et al., 2019)**.

The difference between daytime and night-time temperatures positively influenced the accumulation of dry solids and sugar content in sweet cherry fruits, whereas the amount of precipitation had a negative impact, forming fissures **(Caprio and Quamme, 2006)**. No direct influence of the amount of rainfall and mean daily temperature on SSC was observed **(Sheiko et al., 2019)**.

Low temperatures during the blooming cause reduction of the yield capacity, but winter frosts and relatively low temperatures during the pollination might also negatively affect yields **(Smetanska et al., 2021; Hedhly et al., 2004)**.

The SSC (consisting mostly of sugars) varies between 11 and 25 °Brix in sweet cherry **(Serrano et al., 2005)**. In general, American consumers preferred sweet cherries with SSC >16% **(Crisosto et al., 2003)**.

The results of twelve years of research suggest that the average content of CPP in cherries grown in the analyzed region was at the level of 16.7%. The average content of CPP in the fruits of cherries of the early maturing varieties was at the level of 15.3% (Table 1), ie it was 8.4% lower compared to the average varietal value.

In the fruits of cherries of groups of varieties of medium and late maturity, the average content of SSC exceeded the average varietal value by 1.8 and 7.2%, respectively (Tables 2 and Table 3). Thus, among the studied varieties, the maximum content of SSC was characterized by cherries of the late-ripening group.

The variety of early maturity, which according to the results of twelve years of research was characterized by the largest mass fraction of SSC was Rubinov early, and the smallest – Merchant (Table 1).

Among the varieties of the group of medium maturity, the maximum average content of CPP was recorded in the fruits of the varieties Talisman and Dachnytsia, and the groups of the late group - in the fruits of the variety Udivitelna.

These research results indicate significant variability in the content of SSC over the years of research in the group of varieties of early maturity. The greatest influence of abiotic factors on the content of CPP in the fruits of this group was found for varieties Kazka and Valeriy Chkalov with coefficients of variation of 22.8 and 22.7%, respectively. The most stable in terms of SSC are varieties Zabuta, Merchant, Bigaro Burlat, as evidenced by the corresponding coefficients of variation of 18.4, 19.3, and 19.6%. The variability of these varieties under the influence of weather factors is considered average.

The variability of the SSC content over the years of research in the fruits of cherries of the groups of varieties of medium and late maturity was average ($V_p$ = 19.6 and 17.7%, respectively). Among the group of varieties of medium maturity, the most stable dry matter content was in the fruits of the variety Dachnytsia ($V_p$ = 14%), and the most variable - in the variety Space ($V_p$ = 29%). In the group of late-maturing varieties, the greatest variability in the content of SSC was recorded in the fruits of the varieties Kolkhoznytsia ($V_p$ = 21.5%) and Anons ($V_p$ = 21.6%), the smallest - in the varieties Krupnoplidna ($V_p$ = 13.4%) and Prazdichna ( $V_p$ = 13.7%).

Thus, in terms of dry matter content and variability of their formation under the influence of weather factors in the analyzed region, the most promising from a technological point of view were varieties Dachnytsia, medium ripening, and large-ripening varieties Krupnoplidna. These varieties differed in the high content of SSC, and their low variability over the years of research. Among the group of varieties of early maturity of the most promising variety is not identified. All studied varieties with a high content of CPP were characterized by high variability. Varieties Zabuta, Merchant, Bigaro Burlat, in which the variability of SSC content over the years of research was average, were characterized by their low content.

The average cultivar content of SSC in the sweet cherry of 33 researched cultivars grown in the South Steppe subzone of Ukraine was at the level of 16.7%. In the group of cultivars of early ripening, the maximum average SSC is recorded in the fruit of Rubinova Rannya sweet cherries. It was at the level of 16.8%.

Among the cultivars of the other two groups, the fruit of Talisman and Dachnytsia, Krupnoplidna, and Udivitelna cultivars were characterized by the highest average SSC. They accumulated 18.5 – 18.6% of SSC. The fruits of cultivars Prostir and Anons, collected in 2015, were characterized by the lowest quantity of dry solutes. The quantity of the dry solutes was lower than the average cultivar value, by 59.8 and 61.7% accordingly. The highest mass fraction of the dry solutes was recorded in fruits of the Dachnytsia and Karina cultivars, collected in 2012 **(Serdiuk et al., 2020a)**.

To establish the share of the influence of the weather parameters and cultivar characteristics on the formation of SSC of sweet cherry fruit, a two-factor analysis of variance was performed. Its results showed that for all groups of cultivars, regardless of ripeness, the weather parameters of the year of research has the dominant influence on the formation of SSC fund with the share of influence for the cultivars of early ripening being 74.5%, for those of medium ripening – 61.9% and for the groups of late-ripening – 69.4%.

The influence of the characteristics of the cultivar was less significant. The results of the study are presented in more detail in the research of **Serdiuk et al. (2020b)**.

Thus, the results obtained earlier proved the feasibility of predicting the SSC in sweet cherries following

the average values for the certain group of cultivars, and not separately for each pomological cultivar. Medium dependence was found between the fruit weight and the fruit SSC (correlation coefficient 0.53 – 0.59) **(Fatih et al, 2014; Kishchak, 2014; Polat et al., 2009)**. Subsequently, a search was made for the presence of correlations between the index of accumulation of soluble solids in the sweet cherries of early ($Y_1$), middle ($Y_2$), late ($Y_3$) ripening, and the complex of hydrothermal parameters (factors) – $X_i$. There were selected 25 indicators of weather factors ($X_i$) that within the specified growing period can significantly affect the accumulation of SSC in the fruit of sweet cherry of early ($Y_1$), medium ($Y_2$), late ($Y_3$) ripening (Table 1). These thermal indicators of air (°C) shall be the average temperature in May ($X_1$), June ($X_2$); absolute minimum temperature in May ($X_3$), the absolute maximum temperature in May ($X_4$) June ($X_5$), the average maximum temperature of April ($X_6$), May ($X_7$), June ($X_8$); average minimum temperature in May ($X_9$), June ($X_{10}$); the annual sum of effective temperatures over 15 °C ($X_{11}$); the difference between the average maximum and minimum temperatures of April ($X_{12}$); the sum of active temperatures during the vegetation period ($X_{13}$); the average temperature at blossom ($X_{14}$); the average minimum temperature at ** The influence of the factor is insignificant, $\Delta_i \prec 0.5\%$ blossom ($X_{15}$); the average maximum temperature at blossom ($X_{16}$); hydrothermal coefficient at blossom ($X_{17}$); the sum of effective temperatures at blossom ($X_{18}$). The indicators of humidity (%) shall be: average relative humidity in May ($X_{19}$), June ($X_{20}$); absolute minimum relative humidity in May ($X_{21}$), June ($X_{22}$), at blossom ($X_{23}$); average minimum relative humidity at blossom ($X_{24}$), the average relative humidity at blossom ($X_{25}$).

**Table 1** Dry matter content in cherries cultivars of the early termination of ripening, % (2008 – 2019 yy.), $\bar{x} \pm s\bar{x}$ , n = 5.

| Pomological variety | Average content SSC, % | min content SSC, % | max content SSC, % | Variation by years, $V_p$, % |
|---|---|---|---|---|
| *Rubinova Rannya* | 16.8 ±3.9 | 11.7 | 22.6 | 23.3 |
| *Valeriy Chkalov* | 16.5 ±3.7 | 11.4 | 21.2 | 22.7 |
| *Sweet Erlise* | 16.2 ±3.3 | 11.7 | 21.2 | 20.4 |
| *Merchant* | 14.0 ±2.7 | 11.5 | 18.7 | 19.3 |
| *Kazka* | 14.8 ±3.4 | 10.1 | 20.5 | 22.8 |
| *Bigaro Burlat* | 13.5 ±2.7 | 10.3 | 18.9 | 19.6 |
| *Zabuta* | 14.9 ±2.7 | 11.4 | 21.0 | 18.4 |
| **Average value** | 15.3 ±3.3 | 10.1 | 22.6 | 21.9 |
| **HIP $_{05}$** | 0.416 | - | - | - |

**Table 2** Dry matter content in cherries cultivars of the medium-term of ripening, % (2008 – 2019 yy.), $\bar{x} \pm s\bar{x}$ , n = 5.

| *Pomological variety* | Average content SSC, % | min content SSC, % | max content SSC, % | Variation by years, $V_p$, % |
|---|---|---|---|---|
| *Vinka* | 16.8 ±3.4 | 10.9 | 21.7 | 20.0 |
| *Pervistok* | 16.7 ±3.5 | 10.5 | 21.1 | 21.1 |
| *Temp* | 17.4 ±3.1 | 12.1 | 22.3 | 17.6 |
| *Uliublenytsia Turovtseva* | 15.3 ±3.0 | 12.3 | 22.1 | 19.9 |
| *Talisman* | 18.5 ±2.9 | 15.1 | 23.2 | 16.1 |
| *Dilemma* | 17.8 ±3.3 | 12.5 | 22.5 | 18.3 |
| *Melitopolska Chorna* | 17.2 ±3.8 | 12.3 | 22.4 | 21.4 |
| *Cordia* | 16.6 ±2.6 | 12.0 | 20.0 | 15.9 |
| *Octavia* | 16.3 ±3.2 | 11.8 | 20.2 | 19.5 |
| *Orion* | 17.6 ±3.8 | 12.0 | 22.8 | 21.5 |
| *Chervneva Rannya* | 17.3 ±3.3 | 11.9 | 22.1 | 19.0 |
| *Dachnytsia* | 18.5 ±2.6 | 15.1 | 23.3 | 14.0 |
| *Prostir* | 15.7 ±4.6 | 10.0 | 22.0 | 29.0 |
| **Average value** | 17.0 ±3.3 | 10.0 | 23.3 | 19.6 |
| **HIP $_{05}$** | 0.455 | - | - | - |

**Table 3** Dry matter content in cherries cultivars of the late-term of ripening, % (2008 – 2019 pp.), $\bar{x} \pm s\bar{x}$ , n = 5.

| Pomological variety | Average content SSC, % | min content SSC, % | max content SSC, % | Variation by years, Vp, % |
|---|---|---|---|---|
| *Krupnoplidna* | 18.5 ±2.5 | 13.9 | 22.5 | 13.4 |
| *Karina* | 18.2 ±3.8 | 12.4 | 23.1 | 20.9 |
| *Regina* | 17.1 ±3.2 | 12.9 | 22.8 | 18.7 |
| *Mirazh* | 18.2 ±3.5 | 12.8 | 23.0 | 19.3 |
| *Udivitelna* | 18.6 ±3.5 | 12.8 | 22.8 | 18.5 |
| *Zodiac* | 17.0 ±2.5 | 12.9 | 19.7 | 14.8 |
| *Siurpryz* | 17.9 ±3.4 | 13.0 | 23.0 | 19.0 |
| *Kolkhoznytsia* | 17.8 ±3.8 | 11.9 | 23.0 | 21.5 |
| *Kosmichna* | 17.7 ±2.9 | 12.7 | 21.3 | 16.3 |
| *Prazdnichna* | 16.8 ±2.3 | 11.9 | 19.5 | 13.7 |
| *Anons* | 17.6 ±3.8 | 10.3 | 21.5 | 21.6 |
| *Temporion* | 18.3 ±3.4 | 11.2 | 21.5 | 18.7 |
| *Meotyda* | 18.4 ±2.9 | 11.0 | 22.3 | 15.9 |
| **Average value** | 17.9 ±3.2 | 10.3 | 23.1 | 17.7 |
| **HIP $_{05}$** | 0.635 | - | - | - |

For these factors the presence of the weak, medium, and strong linear correlations, as evidenced by the values of matching correlation coefficients between values of each factor and the resulting rate $r_{Y_1X_i}, r_{Y_2X_i}, r_{Y_3X_i}$ . According to the hypothesis testing of significance of correlation coefficients by Student's test at the significance level of 0.05 significant matching correlation coefficients are within the limit of $r_{Y_jX_i} \geq 0.55, i = 1 \ldots 25, j = 1 \ldots 3$ .

Thus medium ripening cultivars, we identified 17 and, for early and the late-ripening cultivars, there were 16 weather parameters, for which a strong or medium linear correlation dependence with the analyzed indicator (SSC) was established. Further research was carried out according to the pattern given above.

**Table 4** Matching correlation coefficients and meanings of relative share of influence $(\Delta_i)$ for weather parameters ($X_i$,) and soluble solids content (SSC) in the fruit of sweet cherry of Early $(r_{Y_1X_i})$, Medium $(r_{Y_2X_i})$, and Late $(r_{Y_3X_i})$ terms of ripening.

| Conventional Sign of a Parameter ($X_i$,) | Matching Correlation Coefficients $r_{Y_jX_i}$, of the Share of $(\Delta_i)$ Factors Influence for Cultivar Groups | | | | | |
|---|---|---|---|---|---|---|
| | early | | medium | | late | |
| | $r_{Y_1X_i}$ | $\Delta_i$, % | $r_{Y_2X_i}$ | $\Delta_i$, % | $r_{Y_3X_i}$ | $\Delta_i$, % |
| $X_1$ | 0.965 | 14.71 | 0.958 | 13.02 | 0.615 | 1.87 |
| $X_2$ | 0.401* | ** | 0.464* | ** | 0.864 | 13.85 |
| $X_3$ | 0.746 | 5.00 | 0.811 | 8.17 | 0.683 | 2.98 |
| $X_4$ | 0.772 | 14.23 | 0.730 | 0.86 | 0.342* | ** |
| $X_5$ | 0.402* | ** | 0.472* | ** | 0.831 | 8.02 |
| $X_6$ | 0.568 | 1.26 | 0.656 | 9.62 | 0.531* | 1.47 |
| $X_7$ | 0.950 | 16.06 | 0.958 | 5.45 | 0.604 | 2.83 |
| $X_8$ | 0.504* | ** | 0.540 | ** | 0.823 | 13.98 |
| $X_9$ | 0.934 | 15.94 | 0.901 | 14.18 | 0.701 | 3.11 |
| $X_{10}$ | 0.375* | ** | 0.371* | ** | 0.713 | 9.49 |
| $X_{11}$ | 0.769 | 4.58 | 0.818 | 12.92 | 0.477* | ** |
| $X_{12}$ | 0.304* | ** | 0.440* | ** | 0.630 | 4.56 |
| $X_{13}$ | 0.621 | 0.53 | 0.705 | 3.84 | 0.192* | ** |
| $X_{14}$ | 0.714 | 3.48 | 0.743 | 0.83 | 0.414* | ** |
| $X_{15}$ | 0.557 | 0.87 | 0.492* | 3.22 | 0.225* | ** |
| $X_{16}$ | 0.785 | 2.83 | 0.851 | 2.18 | 0.546 | ** |
| $X_{17}$ | -0.581 | ** | 0.653 | ** | 0.589 | 3.85 |
| $X_{18}$ | 0.624 | 0.12 | -0.638 | 1.07 | -0.480* | 0.84 |
| $X_{19}$ | -0.507* | 0.83 | -0.590 | 0.53 | -0.068* | ** |
| $X_{20}$ | -0.296* | ** | -0.366* | ** | -0.571 | 6.99 |
| $X_{21}$ | -0.606 | 9.54 | -0.638 | 12.16 | -0.155* | ** |
| $X_{22}$ | -0.551 | 0.57 | -0.555 | 2.73 | -0.229* | ** |
| $X_{23}$ | -0.300* | 6.84 | -0.380* | 8.00 | -0.763 | 11.48 |
| $X_{24}$ | -0.658 | ** | -0.720 | ** | -0.685 | 7.94 |
| $X_{25}$ | -0.588 | 1.15 | -0.637 | 1.33 | -0.729 | 9.69 |

Note: * Insignificant matching correlation coefficients $(r_{Y_jX_i} \geq 0.55, i = 1\ldots25, j = 1\ldots3)$ (according to the relevance hypothesis verification by the Student's test with the level of relevance being 0.05).

1. Five main components were identified by the principal components method $(PC_i, i = 1...5)$. These five main components provided more than 90% of the cumulative proportion of variance.
2. Regression models of the dependence of the soluble solids index for each group of cultivars on the selected main components $(PC_i, i = 1...5)$ of the form (1) were constructed. The values of the determination coefficient (Adjusted R-squared) for all three regressions are close to one, which indicates a strong influence of independent variables on the dependent variable (Table 5). The *p*-value <0.05, which indicates the adequacy of models based on Fisher's test at a significance level of 0.05.
3. After the transition to the initial factors, we obtain a regression model of the form (2). This regression model characterizes the dependence of the SSC index (for $\hat{Y}_1$, $\hat{Y}_2$, $\hat{Y}_3$) on weather factors (in standardized factors $\tilde{x}_i$)

$$\hat{Y}_1 = 11.9533 + 0.30626\tilde{x}_1 + 0.1346\tilde{x}_3 + 0.3707\tilde{x}_4 + \\ + 0.0446\tilde{x}_5 + 0.3397\tilde{x}_7 + 0.34266\tilde{x}_8 + 0.1195\tilde{x}_{10} - \\ - 0.0172\tilde{x}_{12} + 0.0980\tilde{x}_{14} - 0.3792\tilde{x}_{15} - 0.0345\tilde{x}_{16} + \\ + 0.1120\tilde{x}_{18} + 0.003184\tilde{x}_{19} - 0.0329\tilde{x}_{21} - 0.0190\tilde{x}_{22} - \\ - 0.3481\tilde{x}_{15} - 0.2086\tilde{x}_{24} - 0.2238\tilde{x}_{25}$$

$$\hat{Y}_2 = 12.8530 + 0.2431\tilde{x}_1 + 0.1800\tilde{x}_3 + 0.0242\tilde{x}_4 + \\ + 0.2355\tilde{x}_5 + 0.1486\tilde{x}_7 + 0.2646\tilde{x}_8 + 0.2562\tilde{x}_{10} + \\ + 0.0838\tilde{x}_{12} - 0.0211\tilde{x}_{14} + 0.03209\tilde{x}_{15} - 0.0676\tilde{x}_{16} - \\ - 0.6099\tilde{x}_{18} - 0.03234\tilde{x}_{19} - 0.0148\tilde{x}_{21} - 0.3914\tilde{x}_{22} + \\ + 0.09913\tilde{x}_{15} - 0.2207\tilde{x}_{24} - 0.2531\tilde{x}_{25}$$

$$\hat{Y}_3 = 13.1095 + 0.0580\tilde{x}_1 + 0.3055\tilde{x}_2 + 0.083\tilde{x}_3 + \\ 0.1839\tilde{x}_6 - 0.0526\tilde{x}_7 + 0.0891\tilde{x}_8 + 0.3236\tilde{x}_9 + \\ + 0.0844\tilde{x}_{10} - 0.2534\tilde{x}_{11} + 0.1379\tilde{x}_{13} + 0.1245\tilde{x}_{17} - \\ - 0.0332\tilde{x}_{18} - 0.2331\tilde{x}_{20} - 0.2869\tilde{x}_{23} - 0.3914\tilde{x}_{22} + \\ + 0.09913\tilde{x}_{15} - 0.2207\tilde{x}_{24} - 0.2531\tilde{x}_{25}$$

**Table 5** Main regression characteristics of the ssc index from the main components.

| | Coefficients of Regression | | | | | | Adjusted R-squared | *p*-value |
|---|---|---|---|---|---|---|---|---|
| | $b_0$ | $b_1$ | $b_2$ | $b_3$ | $b_4$ | $b_5$ | | |
| $\hat{Y}_1$ | 11.9533 | -0.5675 | 0.23137 | -0.4717 | 0.50313 | 0.17735 | 0.9498 | 0.0001224 |
| $\hat{Y}_2$ | 12.85308 | -0.5177 | 0.15739 | -0.1426 | 0.28085 | 0.41546 | 0.9305 | 0.000342 |
| $\hat{Y}_3$ | 13.1096 | -0.6491 | 0.27885 | -0.1925 | 0.27026 | 0.01668 | 0.8854 | 0.00862 |

Based on the constructed models, the coefficients $\Delta_i, (i=1\ldots25)$ are calculated for each factor according to formula (3). The coefficients $\Delta_i$ determine the share of each factor in the total variance of the SSC indicator. The results of the calculation are shown in Table 1.

For the cultivars of early and medium ripening terms $\Delta_i$ varies within 0.12 – 16.06% (Table 1), for the late-ripening group this index was 0.84 – 13.98%. For the further analysis of the research results, the factors depending on the values of coefficients $\Delta_i, (i=1\ldots25)$ were divided into three groups.

The first group includes the factors that have a strong influence on the accumulation of SSC with the value of $\Delta_i \geq 9.50\%$. For the cultivars of early ripening sweet cherries, these include the average monthly, absolute maximum, average maximum, and minimum air temperature in May and absolute minimum relative humidity in May. For the fruit of the medium ripening group, these are the average monthly and average minimum air temperatures in May, the average maximum temperature in April, the sum of annual effective temperatures above 15 °C, and the absolute minimum relative humidity in May. For the fruit of late-ripening, the average monthly, average maximum, and average minimum temperature on June, the absolute minimum, and the average relative humidity in the period of blossom. The second group includes the factors that have an average influence on the accumulation of SSC on the sweet cherry fruit with the meaning of $\Delta_i$ from 2.00 – 9.48% (Table 4). The third group includes other factors that have a weak influence on the accumulation of SSC with the meaning of $\Delta_i \leq 2.00\%$ (Table 4). The total percentage of the influence of the factors of this group on the cultivars of early ripening is 5.3%, for the cultivars of medium ripening, it is 4.62%, for the late-ripening group it is 4.18%. It was found out that SSC extremely correlated with the time of the blooming, proving the key role of the spring temperatures in the monitoring of the time of flowering **(Caprio and Quamme, 2006; Guo et al., 2013; Habib et al., 2017; Menzel et al., 2006; Miller-Rushing et al., 2007)**.

As a result, the weather conditions of the last month of fruit formation have the greatest influence on the accumulation of SSC in sweet cherry fruit, regardless of the ripening term. For the sweet cherry cultivars of early and medium ripening, these are the weather conditions of May, for the late-ripening cultivars it is June.

## CONCLUSION

1. A correlation analysis was performed among 25 weather factors ($X_i$,) and the SSC in the sweet cherry of early ($r_{Y_1X_i}$), medium ($r_{Y_2X_i}$), late ($r_{Y_3X_i}$) terms of ripening. Strong and average linear correlation among 17 weather factors ($X_i$,) and SSC for early, medium ripening sweet cherries and 16 climate parameters for the late-ripening $\left(r_{Y_jX_i} \geq 0.55, i=1\ldots25, j=1\ldots3\right)$.

2. Based on the methods of factor and regression analysis, linear regression models of the dependence of SSC fund accumulation on the influence of weather factors on the groups of cultivars of the early, medium, and late-ripening terms.

3. Based on the constructed regression models, the analysis of the degree of influence of weather factors on the SSC indicator was performed by calculating the coefficients of relative influence $\Delta_i, \%$. The greatest influence is established for the group of temperature and humidity indicators with the maximum share of participation $\Delta_i \geq 9.50\%$.

4. It was mathematically substantiated that the weather conditions of the last month of fruit formation have the greatest influence on the accumulation of SSC in the sweet cherry fruit, regardless of the ripening term. For the sweet cherry cultivars of early and medium ripening, these are the weather conditions of May, for the late-ripening cultivars it is June.

5. It is established that in terms of dry matter content and variability of their formation in regions with climatic conditions of the Southern steppe subzone of Ukraine the most promising from the technological point of view were varieties Dachnytsia, medium maturity, and variety Krupnoplidna, late maturity. These varieties differed in the high content of dry soluble substances and their low variability over the years of research.

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# BIOLOGICALLY ACTIVE SUBSTANCES OF *CAMELLIA SINENSIS* IN A HUMID SUBTROPICAL CLIMATE OF RUSSIA

***Oksana Belous, Natalia Platonova***

## ABSTRACT

We did a comparative analysis of tea and raw tea materials. There is an increase in the content of carotenoids and flavonoids (thearubigins and theaflavins) in June, a decline in July, and August, and consequently a slight increase again in other months. The increase is due to unfavorable conditions – drought during these periods. In raw new variety forms No. 855 and No. 582, as well as in black tea variety form No. 582 (0.09 $mg.g^{-1}$), we determined the high value of theaflavins (0.10; 0.11 and 0.09 $mg.g^{-1}$, respectively). The highest content of thearubigins was found in variety forms No. 582 and No. 3823 (1.33 $mg.g^{-1}$ and 1.17 $mg.g^{-1}$). Ascorbic acid is significantly disintegrated (on average 96 – 97%) in the production of black tea. In green tea, ascorbic acid disintegrates to a lesser extent, leaving about 13% of its initial amount in the raw material. The dynamics of GPOD activity in a 3-leaf sprout are variety-specific. At the beginning of the growing season (May), the activity of the enzyme was low – in the range of 0.363 to 0.607 g-unit in sec. In June, there is a decrease in activity, which, however, is not significant ($p$ <0.05) and is due to the biological characteristics of the tea culture. In green tea, the ruthine is on average 3 times more than in black tea (on average about 38.09 and 12.12 $mg.100g^{-1}$, respectively). We have identified 11 amino acids; the highest percentage accounted for proline (from 30 to 70%), valine (17 – 30%), and serine (about 10%). We have identified 11 amino acids in Krasnodar tea, a large proportion of these amino acids has proline, valine, and serine. There was a variation in the content of biologically active substances depending on genotype characteristics. Studies have identified some controversial issues that require explanation and further study.

**Keywords:** fresh sprout; biologically active substances; plant antioxidative system; growing and processing conditions; genotype characteristics; Camellia sinensis

## INTRODUCTION

The antioxidant system of tea contains components that determine the taste of the finished product, and the importance of these substances for humans is undeniable. The composition of these components, as in the raw tea materials and the finished product is rather well studied: we studied the influence of environmental factors on biochemical components, considered the effect on the quality of the tea micro-, and macro, raised the issues of changes of biochemical characteristics with the different methods of collecting leaves and the timing of rejuvenating pruning **(Willson, 1975; Marimuthu and Kumar, 2001; Wright, 2005; Khan and Mukhtar, 2007; Wang et al., 2014)**.

The essence of black tea technology is to accelerate the transformation of substances by activating the action of enzymes and the oxidation process. This causes changes in biologically active substances (BAS) that are part of the tea raw materials **(Wright, 2005; Skhalyakhov et al., 2019)**. In the production of green tea, enzyme activity is suppressed, which is achieved by steaming flushes. Therefore, in green tea, retains almost the entire volume of vitamins contained in the raw material (tea leaf sprout), and it`s number of several times more than in black tea.

The composition of the tea leaf includes no less important components – ascorbic acid (AA) and Ruthin **(Wright, 2005; Konnov et al., 2019; Zhang et al., 2020)**. AA is one of the water-soluble antioxidants, participates in all types of metabolism, in the regulation of cell division and growth, protects biomolecules from free radical oxidation, and serves as a cofactor of many enzymes. Ruthin is the main vitamin of tea, in combination with vitamin C, it enhances its effectiveness, promotes the accumulation and retention of ascorbic acid in the body.

The composition of tea contains about 17 amino acids, which together with proteins is about 16 – 25% soluble extractives **(Belous, 2013; Gai et al., 2019)**. Tea amino acids contribute to the formation of the bouquet and aroma of the beverage since the interaction with the tannin and catechin in high temperatures in the tea production process to form aldehydes.

For many decades based on the Subtropical Scientific Centre of the Russian Academy of Sciences the control of quality indicators of tea was conducted, which revealed a significant variation in the basic biochemical parameters

(the content of tannin and extractive substances) depending on the area where tea plants were grown, weather conditions, varieties, agriculture, leaf maturity, processing, storage, and many other factors **(Argunova et al., 1994; Prokopenko and Tuov, 1994; Belous and Pritula, 2010; Belous, 2013; Platonova et al., 2017; Belous and Platonova, 2017; Belous and Platonova, 2018; Konnov et al., 2019; Platonova et al., 2019; Gvasaliya, 2019)**. However, complex fundamental studies of the biochemical characteristics of raw tea materials, in particular, the finished tea (with an emphasis on the antioxidant complex) at the present world level has not been previously carried out. The purpose of our research is to study regularities within the formation of antioxidant components in the tea system that was grown on the Krasnodar region plantations. One of many tasks is to conduct a comparative biochemical analysis of tea and raw materials with the establishment of varying the content of substances of the antioxidant group depending on the varietal characteristics and to determine the changes in the main biochemical components in the processing of raw materials in the finished black and green tea.

### Scientific hypothesis

The content of photosynthetic pigments has genotypic features.

There is a direct relationship between the quality of raw materials and the content of flavonoids in tea.

There are significant losses of ascorbic acids during processing raw materials to finished tea, despite the equal conditions of collection.

The dynamics of GPOD activity in a 3-leaf sprout are variety-specific. There is a direct relationship between increased enzyme activity and hydrothermal factors.

The largest number of amino acids is synthesized in May, and in the future, AAs are included in the synthesis, the processes of transformations, and mutual conversions to other compounds.

## MATERIAL AND METHODOLOGY

**Samples**

The finished product of tea was made in the plant physiology and biochemistry laboratory of the Federal Research Centre the Subtropical Scientific Centre of the Russian Academy of Sciences using classical technologies.

Black tea (Figure 1) – in the production of black tea, the raw material passed the stages of withering, twisting (moisture loss up to 55%), fermentation (oxidation of polyphenols with the formation of theaflavins and thearubigins), drying (at a temperature of about 95 °C to a final water content of not more than 5 – 6%).

Green tea: steaming (temperature about 95 – 100 °C), twisting, drying (at a temperature of about 95 – 105 °C to the final water content of no more than 3 – 5%).

**Chemicals**

Ethanol (96% ethyl alcohol), U.S.P. – JSC LenReactiv (St. – Petersburg, Russia).

Potassium iodide (KJ), Reagent – JSC LenReactiv (St. – Petersburg, Russia).

Hydrochloric acid (HCl), U.S.P. - JSC LenReactiv (St. – Petersburg, Russia).

2,6-dichlorophenol indophenol (C12H6Cl2NNaO2xH2O), Reagent – LLC TD "Himmed" (LLC Sigma-Aldrich, USA.

Hydrogen peroxide (H2O2), N.F. - JSC Reachim (Moscow, Russia).

Guaiacol (C7H8O2), Reagent – LLC TD "Himmed" (LLC Sigma-Aldrich, USA).

Ninhydrin (C9H4O3*H2O), Reagent – JSC Interhim (St. – Petersburg, Russia).

**Animals and Biological Material:**

The research began in 2016, the targets are samples from raw material (3-leaf fresh sprout) of cultivars Kolkhida and Sochi, and forms 3823, 582, 855, and 2264 (Sochi, Lazarevsky district, Krasnodar region, Russia) (Figure 2a, b).

**Instruments**

We using a PE-5400VI spectrophotometer, manufactured by EKROSCHEM (Russia) and capillary electrophoresis system «Kapel 105-M», manufactured by LUMEX (Russia).

**Laboratory Methods**

The main component determination of the antioxidant system was carried out in the plant physiology and biochemistry laboratory of Subtropical Scientific Centre. Contents of photosynthetic pigments using method invented by Shlyk and the calculated formulas of Smith and Benitez **(Shlyk, 1971)**.

The determination of ascorbic acid was performed using the classical iodometric method **(Vorobyov et al., 2013)**.



**Figure 1** Black tea from experimental plants.



a b

**Figure 2** Experimental tea plantation (a), and raw materials (b), Sochi, Russia.

Flavonoid compounds with P-vitamin activity were determined by titration following the method of vitamin analysis **(Voskresenskaya et al., 2006)**.

The content of free amino acids amount in samples was determined by the ninhydrin dyeing method and then use a high-performance liquid chromatography method **(Speckman et al., 1958)**.

The activity of guaiacol peroxidase (GPOD) was determined by the spectrophotometric method **(Vorobyov et al., 2013)**.

**Description of the Experiment**

**Sample preparation:**

**The content of chlorophyll and carotenoids:** The exact weight (100 mg) of the crushed raw material was placed in a mortar and ground with 96% ethyl alcohol. The resulting extract was drained through a glass filter no. 3. The extract and was transferred to a 25 mLvolumetric flask and brought to the required volume with pure 96% ethyl alcohol. The optical density of extracted pigments was measured 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.

**The determination of ascorbic acid:** The raw material sample (2 g) was ground with 4 mL of 2% hydrochloric acid, transferred to a 25 mL flask and 0.001 n is titrated with a solution of 2,6-dichlorophenolindophenol to a persistent blue staining.

**Flavonoid compounds:** Standard solutions of the studied flavonoids in 95% ethyl alcohol with a concentration of 1 $mg.mL^{-1}$ were prepared according to an exact suspension. Working alcohol solutions were prepared by diluting the standard ones and stored in the refrigerator for no more than 7 days. To determine the TFs and the TRs, the method of spectrophotometry was at a wavelength range of 665 nm for TFs and 825 nm for TRs in contrast to water used as a blind sample.

**Determination of the activity of guaiacol peroxidase:** The tissue sample after extraction of weakly bound peroxidases was placed in 5 mL of cold citrate-phosphate buffer, and rubbed. The resulting homogenate was centrifuged at 3 thousand rpm for 15 minutes. The activity of peroxidases in leaves was determined in a reaction mixture of the following composition: 0.5 mL of 0.1 M citrate-phosphate buffer (pH 6.7), 0.5 mL of 0.3% hydrogen peroxide, 0.5 mL of 0.05% guaiacol, and 0.5 mL of the sample (1 g of the sample was ground in 10 mL of a pH 5.5 citrate-phosphate buffer). The peroxidase activity was determined at 25 °C immediately after the isolation of the enzymes from the samples. 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).

**The content of free amino acids:** conducted using the capillary electrophoresis system «Kapel 105-M». The raw material of the leaves (100 mg) was dried, 1 mL of water was added, and placed in a thermostat (-20 °C) for 2 – 3 hours. Centrifuged (13,000 rpm) for 20 min. The eluate was evaporated at 65 °C, 100 µL of NaOH (0.15 M), 0.2 mL of phenylisothiocyanate (PITC) was added, and incubated for 1 hour.

**Number of samples analyzed:** All experiments include six analyzed samples.

**Number of repeated analyses:** All experiments include three repeated analyses.

**Number of experiment replication:** All experiments include 2 replications of the experiment.

## Statistical Analysis

Statistical processing of the experimental data was carried out using the ANOVA package in STATGRAPHICS Centurion XV (version 15.1.02, StatPoint Technologies) and MS Excel 2007. Statistical analysis included univariate analysis of variance (method of comparing averages using variance analysis, *t*-test) and variance analysis (ANOVA). The significance of the difference between the means of the least significant difference (LSD) results with $p < 0.05$ was considered statistically significant. All experiments were performed in triplicate and the values were expressed as mean ±*SD*. The differences between the samples were assessed using the unpaired *t*-test.

## RESULTS AND DISCUSSION

### The content of pigments in tea

At the beginning of the active growing season (in May), the largest amount of total chlorophylls is synthesized (1.335 μg.g$^{-1}$), followed by a smooth drop by August (1.018 μg.g$^{-1}$). We found another pattern when studying the content of carotenoids and flavonoids (thearubigins and theaflavins). There is an increase in the content of these groups of pigments in June, a decline in July and, by August, and a slight increase again. The increase is due to unfavorable conditions – drought during these periods. Also, it was noted that the content of photosynthetic pigments shows genotypic features (Table 1).

The main photosynthetic pigment is chlorophyll *a*; carotenoids transfer additional energy to chlorophylls, performing a light-absorbing function, and divert excess energy from them, performing a light-protecting function **(Endo et al., 1985; Nafissatou et al., 2011; Li et al., 2016)**. We noted that a significant accumulation of chlorophyll in the leaves is typical for the control cv. Kolchida. At that time, as the studied forms contain chlorophyll a is significantly lower (Table 1).

The content of chlorophyll *b* indicates the level of adaptation of plants to low light. 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 the sun's rays, and many of the leaves of the side surfaces are in the shade. In this case, the increased content of chlorophyll *b* is preferable for the photosynthetic activity of the leaves of this tier. We noted the same pattern as with chlorophyll *a*: more chlorophyll *b* is accumulated by the control variety; moreover, the differences are significant or on the border of materiality, as in the case of forms 3823 and 2264. It is important not only the content of a particular pigment but also their ratio. In all the tea plants studied, the *a/b* ratio ranges from 2.813 mg.g$^{-1}$ to 3.362 mg.g$^{-1}$, and the differences between the forms are insignificant (Table 1).

Photosynthetic pigments are not only components of the antioxidant protection of the plant itself, but also are part of the biochemical components of the drink since the preparation of black and green tea pigments to some extent pass into the infusion **(Lu et al., 2009; Li et al., 2018)**. Thermal processing induced the formation of degradation product: changes occur in their content: a significant part of the chlorophyll is destroyed during steam treatment, as well as during the drying process **(Castillon et al., 1949; Nafissatou et al., 2011; Ošťádalová et al., 2014; Xu et al., 2018; Dini et al., 2019)**. Therefore, there is less chlorophyll in green tea than in raw materials, and there are even fewer green pigments in the production of black tea than in green tea. But this is good since the presence of chlorophyll in the finished tea affects its quality – the tea acquires an herbal smell and an uncharacteristic taste. The smallest amount of this pigment in green and black tea contains form 855 (0.33 mg.g$^{-1}$), which is preferred. Carotenoids provide not only the plant's resistance to exogenous stress but also its value as a source of antioxidants for humans and their content in ready-made (black and green) tea is a positive fact. However, we have not detected any changes in their content, and, as a rule, carotenoids are not taken into account in the qualitative assessment of tea.

**Table 1** Content of photosynthetic pigments.

| Variety/Form | Pigment content in μg.g$^{-1}$ of raw weight | | | | |
|---|---|---|---|---|---|
| | ***Chl. a*** | ***Chl. b*** | ***Chl.a / Chl.b*** | ***ΣCar.*** | ***Σ Chl./ΣCar.*** |
| cv. Kolchida | 0.878 ±0.150 | 0.307 ±0.086 | 3.151 ±0.201 | 0.302 ±0.057 | 3.922 ±0.798 |
| f. 3823 | 0.732 ±0.197 | 0.279 ±0.076 | 2.813 ±0.227 | 0.285 ±0.057 | 3.549 ±0.536 |
| f. 582 | 0.726 ±0.261 | 0.257 ±0.100 | 2.958 ±0.339 | 0.269 ±0.091 | 3.650 ±0.359 |
| f. 855 | 0.689 ±0.143 | 0.237 ±0.084 | 3.362 ±0.210 | 0.256 ±0.047 | 3.621 ±0.475 |
| f. 2264 | 0.732 ±0.113 | 0.258 ±0.063 | 3.088 ±0.135 | 0.273 ±0.041 | 3.630 ±0.342 |
| LSD ($p \leq 0.05$) | 0.11* | 0.05* | 0.84 | 0.04 | 0.21* |

Note: *$p$ <0.05 with the controlled comparison.

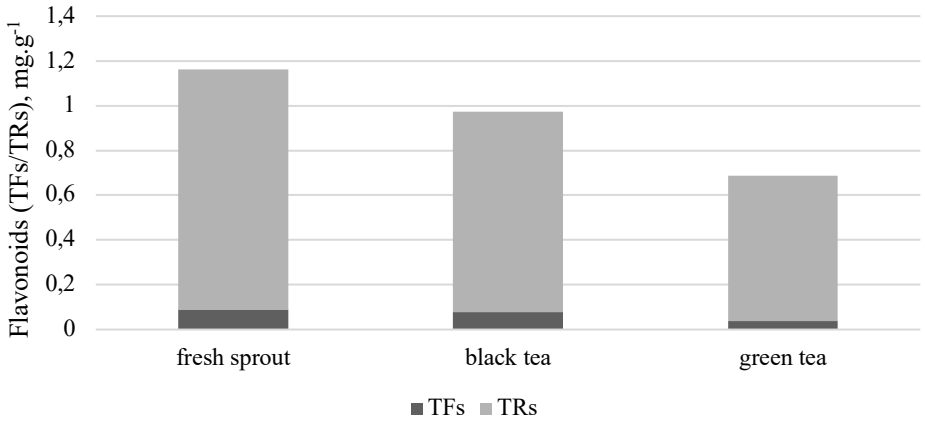


**Figure 3** Content of flavonoids in Krasnodar tea, mg.g$^{-1}$.

Changes occur in the content of chlorophyll: during steam treatment, as well as during drying, a significant part of it is decayed. In the manufacture of black tea, green pigments remain even less than in green.

However, it should be noted that the presence of chlorophyll in the finished tea adversely affects the quality of the product. The smallest amount of this pigment in green and black tea contains form 855 (0.33 mg.g$^{-1}$), which is preferred. In respect of carotenoids, such profound changes are not observed by us, which is confirmed by the literature data, besides, carotenoids are not taken into account in the qualitative assessment of tea. However, they are an important component of the antioxidant system and they, therefore, required no less comprehensive study.

In the production of black tea, catechins are disintegrated by oxidation with the formation of flavonoids –TFs and TRs (Figure 3).

We were revealed that between the quality of raw materials and the content of flavonoids in tea there is a direct relationship (Table 2).

As we show, green tea TFs are slightly less than in sprouts and black tea. TFs are unstable compounds and during enzymatic oxidation, they easily pass into TRs, so in black tea, TRs are much more. In the varietal section, it can be noted that the raw materials of forms 855 and 582 (0.10 mg.g$^{-1}$ and 0.11 mg.g$^{-1}$ respectively), as well as black tea produced from form 582 (0.09 mg.g$^{-1}$), showed the highest values in the content of theaflavins, while the highest content of TRs was found in forms 582 and 3823 (1.33 and 1.17 mg.g$^{-1}$).

Currently, there is no single standard for the content of these pigments in the finished product. But, according to international rules, any blend of tea should have a ratio of TRs: TRs are not lower than 1:16 and in the highest quality tea 1:10. According to this indicator, tea made from raw materials collected from experimental plants meets international requirements **(Kang et al., 2017; Li et al., 2018; Palanivel et al., 2020)**.

**Table 2** Content of flavonoids in tea and raw materials (sprout), mg. g$^{-1}$

| Sample | Sprout | | Green tea | | Black tea | |
|---|---|---|---|---|---|---|
| | TFs | TRs | TFs | TRs | TFs | TRs |
| cv. Kolchida | 0.07 ±0.02 | 0.84 ±0.30 | 0.04 ±0.01 | 0.72 ±0.28 | 0.07 ±0.02 | 1.03 ±0.28 |
| cv. Sochi | 0.07 ±0.02 | 0.88 ±0.47 | 0.04 ±0.02 | 0.71 ±0.39 | 0.07 ±0.01 | 0.95 ±0.19 |
| f. 3823 | 0.09 ±0.03 | 1.17 ±0.60 | 0.04 ±0.01 | 0.60 ±0.23 | 0.08 ±0.02 | 1.08 ±0.17 |
| f. 582 | 0.11 ±0.04 | 1.33 ±0.65 | 0.04 ±0.02 | 0.70 ±0.32 | 0.09 ±0.02 | 1.18 ±0.32 |
| f. 855 | 0.10 ±0.02 | 1.13 ±0.39 | 0.04 ±0.02 | 0.63 ±0.41 | 0.07 ±0.01 | 0.10 ±0.12 |
| f. 2264 | 0.09 ±0.02 | 1.10 ±0.36 | 0.03 ±0.01 | 0.53 ±0.13 | 0.08 ±0.02 | 1.03 ±0.15 |

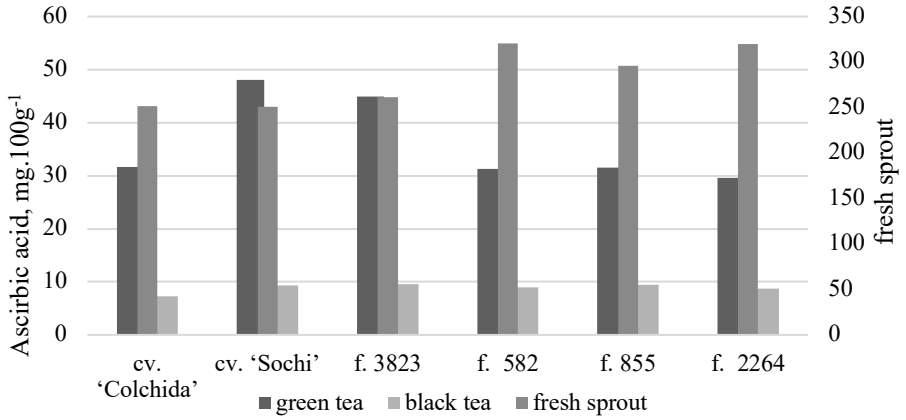


**Figure 4** Changes in the content of ascorbic acid during processing, mg.100g$^{-1}$.

## Determination of ascorbic acid

The least amount of AA is contained in the raw material of forms 2264 and 582 (319 and 320 mg.100g$^{-1}$, respectively) (Figure 4).

In black tea, the differences between varieties are insignificant, a slightly higher content of AA is noted in black tea made from forms 855 and 3823 (13.73 and 13.07 mg.100g$^{-1}$, respectively).

Vitamin C is a necessary nutrient in the production of collagen. Vitamin C deficiency results in the lost formation of collagen fibers, weakening vascular walls, and causing scurvy. Ascorbic acid is also being a tool in the biosynthesis of several secondary polyphenols such as theaflavins thearubigin **(Sharangi, 2009; Palanivel et al., 2020)**. AA also acts as an antioxidant and is believed to play an important role in the prevention of lifestyle-related diseases such as cancer **(Nafissatou et al., 2011; Palanivel et al., 2020)**. The native level of AA is very less in tea leaves and negligible in processed tea **(Palanivel et al., 2020; Zimmermann and Gleichenhagen, 2011)**.

Our research show, that during processing raw materials (fresh sprouts) to finished tea (Figure 1), there are significant losses (up to 90%) of AA, despite the equal conditions of collection. In the production of black tea, due to the development of oxidative reactions during fermentation, disintegrated a significant part of this vitamin on average 96 – 97% (preserved only 3 – 4%).

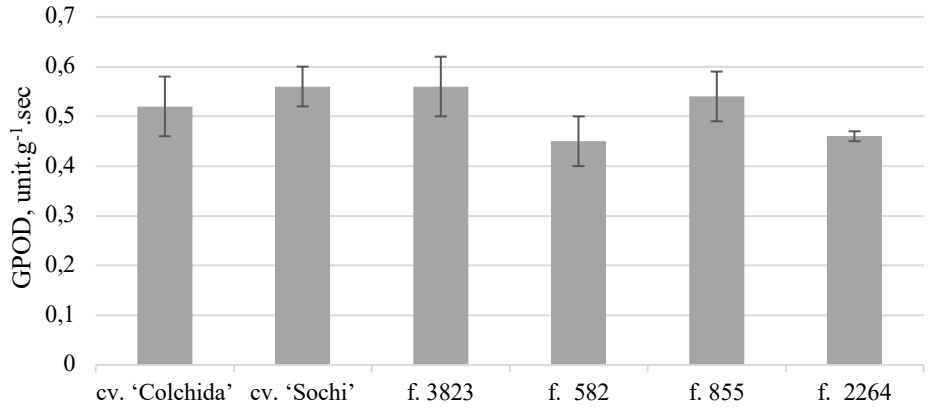


**Figure 5** The activity of GPOD, g-unit in sec; LSD ($p \leq 0.05$) = 0.05.

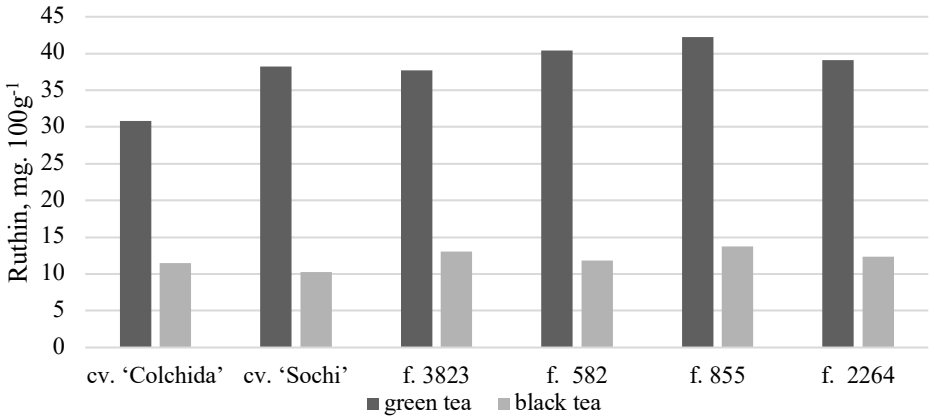


**Figure 6** Content of ruthin in green and black tea, mg.100g$^{-1}$.

In green tea, where fermentation is excluded, AA is decayed to a lesser extent. As a result, green tea is about 13% of the initial amount of AA in raw materials (sprout).

This issue requires additional research, which is planned by us in the future.

## Determination of the activity of guaiacol peroxidase

At the beginning of the growing season (May), the activity of the enzyme was low – in the range of 0.363 to 0.607 g-unit in 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.

The highest activity of GPOD during the entire vegetation period is characterized by the cultivar Sochi and form 582 (about 0.56 g-unit in sec) (Figure 5). 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.

Peroxidase is considered a marker of the physiological state of plants **(Marinescu et al., 1999; Pandey et al., 2017)**. Several scientists note that during a period of stress, the isoenzyme system is reorganized, which ensures the plant's resistance to external factors and the regulation of homeostasis **(McRae and Ferguson, 1985; Castillo, 1992; Sharangi, 2009)**. For example, researches have demonstrated that the expression level of peroxidases and superoxide dismutase increased after the heat treatment of creeping plants **(Khan and Robinson, 1993; Huang et al., 2006)**.

We were shown that after May's 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 period under a stressful hydrothermal condition, 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 GPOD, as a non-specific reaction to a stress factor **(Mittler, 2002; Thongsook and Barrett, 2005)**. However, the dynamics of enzyme activity in a 3-leaf sprout are variety-specific.

We performed a correlation analysis that showed a direct relationship between increased enzyme activity and hydrothermal factors. The most significant correlation was found between the activity of GPOD in freshly harvested 3-leaf tea sprouts and the amount of precipitation (r = 0.86).

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 **(Kareska, 2009; Skhalyakhov et al., 2019)**. In the process of technological processing of raw materials to ready-made the oxidases that ensure the normal course of redox processes are important. Thus, peroxidase catalyzes the oxidation of polyphenols and certain aromatic amines using oxygen, hydrogen peroxide, or organic peroxides.

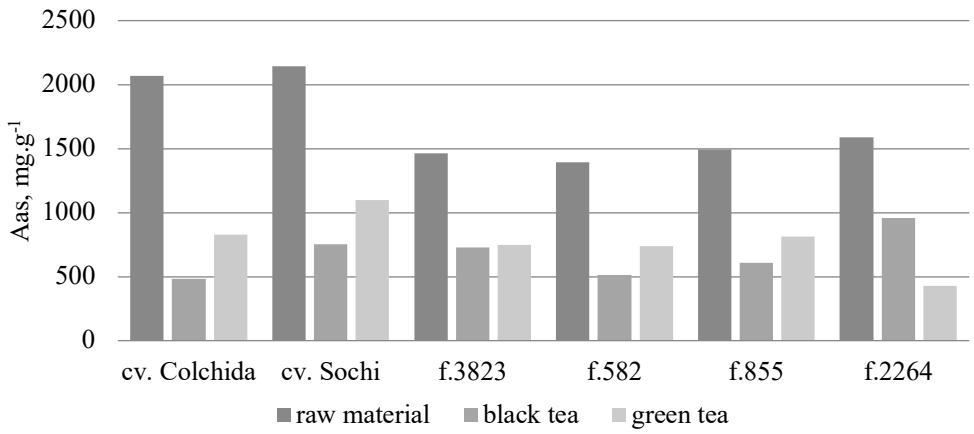


**Figure 7** Content of amino acids in Krasnodar tea, $mg.g^{-1}$.

Given this fact, a lower level of activity of GPOD is a negative phenomenon, both for the stability of the plant itself and for the quality of the finished tea **(Wright, 2005; Huang et al., 2006)**.

## Flavonoid compounds with P-vitamin activity

Studies have shown depending on the season of tea leaf collection, the content of ruthin in green tea ranges from 36 $mg.100g^{-1}$ to 41 $mg.100g^{-1}$, and in black tea-in the range of 17 $mg.100g^{-1}$ – 20 $mg.100g^{-1}$, which is lower than in green tea. Thus, a lower content of ruthin is characterized by the cv. Sochi (on average 10 $mg.100g^{-1}$ in black tea and 34 $mg.100g^{-1}$ in green), a higher-form 582 (about 23 $mg.100g^{-1}$ in black tea and 46 $mg.100g^{-1}$ in green).

Ruthin, namely tea catechin, is very significant for human health. In the absence of it, the permeability of capillary blood vessels increases, and their strength decreases **(Vinson, 2000; Sinija, Mishra, 2008; Sharangi, 2009; Ferreira et al., 2020)**. When acting together with vitamin C (with which it is often found in food), the latter is better absorbed and retained in the body and more successfully prevents scurvy **(Skotnicka et al., 2011)**. Ruthin is an equally important component of the antioxidant system of tea plants, it not only takes part in the main redox reactions but also increases the absorption of ascorbic acid **(Gulati and Ravindranath, 1996)**. The content of ruthin in black and green tea changed as the content of ascorbic acid. The ruthin on average is 3 times more in green tea than in black (on average about 38.09 and 12.12 $mg.100g^{-1}$ respectively). At the same time, the variability of the indicator during the season is important: a more stable content of vitamin P in tea forms 2264, 3823, 582, and 855. A variation was revealed in the content of vitamin C and P depending on genotypic characteristics (Figure 4 and Figure 6).

## Content of amino acids in tea

We identified 11 AAs in Krasnodar tea, a large proportion of which is proline (30 – 70% of the total AAs content in the sample), valine (17 – 30%), and serine (about 10%) (Figure 7).

AAs are the component in tea that contributes full-bodied flavor and sweetness. Of these AAS, more than 60% is theanine, which is unique to tea. Theanine has a structure similar to that of glutamine, with its particular trait being a refined, rich flavor and sweetness **(Karori et al., 2007)**. AAs other than theanine present in tea leaves include proline, asparagine, arginine, serine, and others **(Kumar et al., 2015)**.

We were shown that the largest number of AAs is synthesized in May, and in the future, the processes of their transformations and mutual conversions to other compounds are probably included in the synthesis.

It is necessary to note a strong variability in the content of AAs, and only Pro is stable enough (V, % = 4 – 26% during vegetation). When processing raw materials into ready-made tea, the number of AAs falls, which is due to oxidative deamination and their conversion into protein compounds that are involved in the formation of tea aroma.

Most of the AAs in the raw material are contained in the cv. Kolchida and Sochi. During processing, there are not only general changes – there are slightly fewer AAs in black than in green, which is understandable since it is during the fermentation of black tea that active processes of oxidative deamination take place. There are varietal differences in the metabolic reactions of converting AAs: in the black tea the AAs composition of the cv. Kolchida is more susceptible to changes, while in green tea, the composition of form 2264 changes a little bit.

## CONCLUSION

We conducted a comparative biochemical analysis of the content of antioxidant substances in tea raw materials (fresh sprout) and finished black and green tea. There was a significant change in the content of flavonoids depending on the type of finished product, as well as ascorbic acid and ruthine in the processing of tea raw materials. The differences are due to the lack of fermentation process in the production of non-fermentation (green) tea, which is the most important technological method in the production of black tea. This allows green tea to retain almost all water-soluble vitamins that inhibit lipid peroxidation in cell membranes. There is a significant variation in the content of biologically active substances (flavonoids, vitamins, and amino acids), due to the genotypic characteristics of tea plants. It is important to research the rules about the accumulation and distribution of biological substances by tea plants, which will give us some scientific ideas about how to control the contents of it and increase biological active substances in the new tea cultivar.

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# THE ATTITUDE OF CZECH UNIVERSITY STUDENTS TO BEER CONSUMPTION FROM MICROBREWERIES

*Vratislav Kozák, Vendula Lapčíková*

## ABSTRACT

The questionnaire survey was focused on the relation of university students of generation Z in the Czech Republic to beer from microbreweries. The survey involved 1,103 respondents from among Czech university students. 1,004 respondents complied with the survey conditions (they had to belong to university students and consume beer). Although 98% of beer consumed in the Czech Republic is from industrial breweries, it has turned out that the proportion of students who consume beer from industrial breweries and microbreweries is almost the same. As for beer from microbreweries, students prefer classic beers of the Pilsen type. So far, these do not circulate in beer tourism in larger quantities. The respondents agree that beer tourism will compete successfully with wine tourism. For beer tourism to develop successfully, microbreweries must also offer catering, accommodation, and other services; beer baths are in high demand at present. The economic benefits of microbreweries are in several areas – they integrate into tourist destinations, use local raw materials for beer and food preparation, create new jobs, enhance abandoned breweries and contribute to the development of rural areas.

**Keywords:** millenniums; microbreweries; beer preferences; beer tourism; microbreweries offer

## INTRODUCTION

This study deals with the feelings of Czech university students about beer with a focus on beer consumption from microbreweries. This is an important topic because, according to the Scopus database, this issue has not been qualified in the past two years (October 2020). In the first 400 links on scopus.com, there are only two scientific studies, namely **Rivarol, Kozák and Spadoni (2019)**, which examines the attitude of Czech and foreign university students in the Czech Republic toward the consumption of craft beers, and the second study by **Kozák, Jeřábek and Šefčík (2018)** that analyzes breweries in the Zlín Region in the Czech Republic. They pay more attention to those microbreweries that offer both specialty beers and regional culinary specialties and possibly other services such as catering, accommodation, or even a beer spa.

These microbreweries are involved in creating higher quality culinary tourism in the region. It is, therefore, clear that comprehensive research on consumer preferences of beer drinkers from the group of university students has not been conducted in the Czech Republic.

University students are an important target group for producers of beverages, both alcoholic and non-alcoholic. In the research year (2019), they were in the age range of 18 – 26, and at that age, their consumption habits regarding the consumption of beverages had been reserved or slightly avoided. Either alcoholic beverages are not consumed at all, or they choose between spirits, wine, and beer. This research focused on beer consumers and investigated the attitude of Czech university students toward beer from microbreweries. The secondary objective of the research is to assess how many microbreweries can operate in the Czech Republic. There have been 43 industrial breweries for several decades, the number of active microbreweries was estimated at 550 at the end of 2019, and the new ones are still emerging. Based on this research, the authors create a qualified estimate of whether the number of microbreweries will find their place on the Czech beer market.

According to **Veisová (2017)**, university students belong mostly to generation Z (with some overlap with the generation Y – millenniums), only part of which, i.e. persons born between 1993 and 2001, was used for this research.

The millennium generation at the time of research (2019) was aged 25 – 39 and is well mapped by marketing research. Generation Y in the age range 5 – 24 does not appear in the research results. The research thus focused on the millennium generation. The millennium's buying habits have been influenced by the Internet, and this generation has experienced the gradual launch of Facebook. The exchange of Facebook opinions on healthy lifestyle, including the consumption of beverages, has become an integral part of virtual discussions. Unlike generation X (born 1966 – 1979), they came into the world in a surplus economy.

**Molinillo, Vidal-Branco and Japutra (2020)** explored the attitude of millenniums to the purchase of bio food in Spain and Brazil.

support the inter-generational theory, which states that each



**Figure 1** Beer spa Černý Orel Kroměříž **(Cerny-orel.eu, 2021)**.

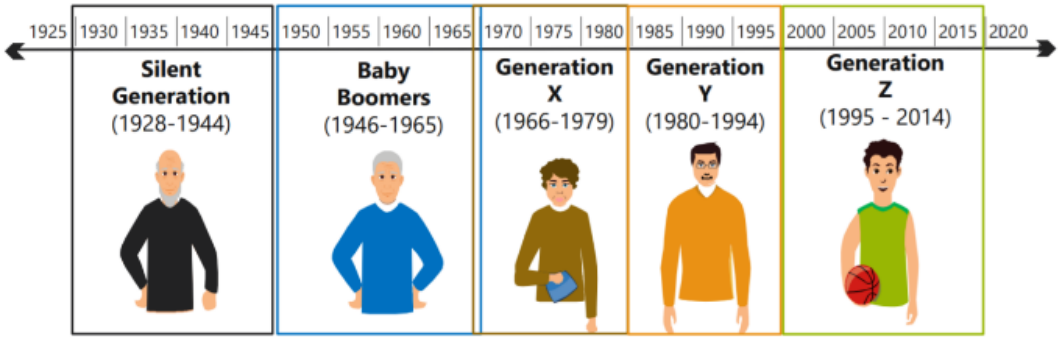


**Figure 2** Division of individual generations **(Veisová, 2017)**.

Although these are culturally different regions, they conclude that millenniums are willing to pay extra for this kind of food and those respondents in both countries show a higher level of health awareness than previous generations. The authors **Gallenti et al. (2019)** note in their research that while the Italian Millennium is willing to accept a premium price for high-quality wines, they also follow the carbon footprint and ethical attributes of wine production, including the effective communication of these attributes. A team of Italian researchers **Galati et al. (2019)** concluded similarly that the "natural" wine that has recently appeared on the market satisfies the needs of healthy and environmentally friendly consumers. Millenniums pay more often for the "natural" wine. **Wolf et al. (2019)** also concluded that different generations have different preferences for wine and wine purchasing habits. People of the Baby Boomers generation (1946 – 1965) spend a lot of money on wine, are wine enthusiasts, and get information from specialized wine magazines. The experiment results of **Zúñiga and Torres (2019)** provide further evidence to generation cohort has different ethical ideologies, in this case in response to alcohol advertising.

It deals with the names of craft beer produced by American microbreweries. **Nuessel (2018)** observes the following points: (1) beer history, (2) increase in the number of microbreweries in the US, (3) definition of microbrewery, (4) discussion of beer and craft beer in culture (film, television, beer collecting) and (5) selecting a list of brands of craft beer. A possible overproduction of beer from microbreweries in Italy is being considered by the authors **Fastigi, Viganò and Esposti (2018)**. In their research, they found that local supply chains (from barley cultivation to malting) were created and new types of microbreweries were created – an agricultural microbrewery. Research on the integration of microbreweries and cluster cooperation in Porto Alegro, Portugal **(Monticelli et al., 2018)** confirmed their integration into the strategic dynamics of the cluster.




**Figure 3** Beer from microbreweries **(Mistoprodeje.cz, 2021)**.

They consider cooperation to be a process that involves close strategies of parallel cooperation in various areas, such as sharing resources, information, especially in the distribution and sales phases. The research has shown synergies between identified mechanisms and common objectives, demonstrating the dynamics of these small businesses. Research of the authors **Cortese et al. (2017)** analyzed the craft beer industry and the position of microbreweries in a specific area of Piedmontese in the province of Biella (Italy). Research has provided information on the importance of good raw materials, maintaining a high standard of quality, and creating more value, even for the territory itself. The data also enriches the current literature on the production and position of microbreweries in the emerging beer country. The authors **Holtkamp et al. (2016)** investigated the use of neo-localism in the marketing of microbreweries in the USA and they tested its effectiveness. Three primary indicators of neo-local business practice have been identified in the development of this tool: (1) the use of local names and images in labeling and marketing; (2) environmental sustainability; and (3) social and community engagement. These indicators are important for both consumers and businesses to create a sense of place and engage in their community. **Maier (2016)** strived to find out the reasons for an increase in the number of microbreweries in the Czech Republic. The analysis compares various brewing indicators in selected historical countries with traditional alcohol consumption. Research questions are focused on relations between the number of microbreweries and demographic aspects, aspects of market concentration, and some other indicators of the beer market. Simulations were also carried out - under what conditions there will be 400, 500 and 1,000 microbreweries in the Czech Republic. A statistically significant dependence on the number of microbreweries is the concentration on the beer market (Gini index), a percentage of the total population of the age group 25 – 39, and the share of domestic beer consumption in cans. Since the beginning of the economic crisis, the number of Czech microbreweries has been growing exponentially and the results suggest that the microbreweries' boom will continue. This author also deals with the problem of the number of microbreweries in the Czech Republic in further research **(Maier, 2013)**. Two research teams of Ellis and Bosworth **(Ellis and Bosworth, 2015)** and Danson and colleagues **(Danson et al., 2015)** were developing microbreweries in the UK. Researchers have concluded that the economic benefits of microbreweries are in multiple areas, besides beer production, these creating new jobs, upgrading the listed buildings, and developing rural tourism.

## Scientific Hypothesis

H0: University students are expected to deviate from beer consumption, so beer is consumed by a maximum of 75% of university students.

Ha: University students are expected to move away from beer consumption, so more than 75% of university students consume beer.

The 75% limit was chosen based on earlier studies, particularly in the research used by **Rivaroli, Kozák and Spadoni (2019)**. What motivates Czech and international "millennial-aged" university students to consume craft beers? Do microbreweries have websites?

## MATERIAL AND METHODOLOGY

The research aims to deepen scientific knowledge in the field of beer consumer preferences among university students. More extensive and comprehensive research in this area has not been carried out in the Czech Republic yet. The research was carried out at a time when there was a steep increase in the number of microbreweries in the Czech Republic, which is currently approaching the limit of 550 (December 2019). The results will help to create a qualified estimate of whether there is still space for other microbreweries on the Czech beer market. The prospective age limit of 18 – 26, which includes university students, is very important for further estimation of beer consumption. The attitude of Czech university students to beer with a focus on beer consumption from microbreweries was examined by a meusingestionnaire survey. 1,103 respondents, Czech university students who were beer consumers, took part in it. The data were statistically processed first by descriptive statistics and then the data were evaluated using the confidence interval.

The data were collected from September to November 2019. In the questionnaire survey, 13 questions examined the feelings of university students about beer. The survey revealed that 99 respondents out of the total number of university students said they were not university students and then completed the questionnaire. All 1,004 respondents then answered the remaining 12 questions. Except for the first question, the remaining 12 questions were scored from 1 – completely agree to 5 – completely disagree.

**Table 1** Questions and answers **(own processing)**.

| | | **Yes** | **No** | | | |
|---|---|---|---|---|---|---|
| 1 | Are you a university student (20 – 32 years old) and do you consume beer? | 1,004 | 99 | - | - | - |
| | | **1** | **2** | **3** | **4** | **5** |
| 2 | I almost exclusively consume beer produced in industrial breweries | 81 | 443 | 314 | 123 | 43 |
| 3 | I almost exclusively consume beer produced in microbreweries | 27 | 156 | 360 | 387 | 74 |
| 4 | I consume approximately the same amount of beer from industrial breweries as well as microbreweries | 131 | 298 | 135 | 316 | 124 |
| 5 | From the offer of microbreweries, I mainly search for classic beers (lagers and draft beers of the Pilsen type) | 150 | 348 | 200 | 242 | 64 |
| 6 | From the offer of microbreweries, I mainly search for specials (stronger beers, beers with IPA, APA flavor) | 165 | 304 | 150 | 271 | 114 |
| 7 | I prefer to consume beer from microbreweries in their bars (restaurant) | 188 | 354 | 247 | 168 | 47 |
| 8 | I prefer to consume beer from microbreweries in the home environment | 77 | 226 | 301 | 296 | 104 |
| 9 | I prefer beer from one microbrewery | 14 | 58 | 123 | 375 | 434 |
| 10 | I taste beer from a larger number of microbreweries | 206 | 394 | 166 | 169 | 69 |
| 11 | I go around microbreweries and get to know new kinds of beer | 38 | 95 | 135 | 315 | 421 |
| 12 | As for the development of beer tourism, microbreweries must provide catering and accommodation facilities | 123 | 373 | 246 | 182 | 80 |
| 13 | I consider beer tourism as a promising part of tourism that will compete successfully with wine tourism | 230 | 427 | 181 | 124 | 42 |

Note: 1 – completely agree; 2 – rather agree; 3 – neither agree nor disagree; 4 – rather disagree; 5 – completely disagree.

**Table 2** Descriptive statistics of questions **(own processing)**.

| n | average | modus | max | min | median | Sd |
|---|---|---|---|---|---|---|
| Question 1 | 1,103 | 1.089755213 | 1 | 2 | 1 | 1 |
| Question 2 | 1,004 | 2.605577689 | 2 | 5 | 1 | 2 |
| Question 3 | 1,004 | 3.323705179 | 4 | 5 | 1 | 3 |
| Question 4 | 1,004 | 3.003984064 | 4 | 5 | 1 | 3 |
| Question 5 | 1,004 | 2.72310757 | 2 | 5 | 1 | 3 |
| Question 6 | 1,004 | 2.865537849 | 2 | 5 | 1 | 3 |
| Question 7 | 1,004 | 2.531872510 | 2 | 5 | 1 | 2 |
| Question 8 | 1,004 | 3.123505976 | 3 | 5 | 1 | 3 |
| Question 9 | 1,004 | 4.152390438 | 5 | 5 | 1 | 4 |
| Question 10 | 1,004 | 2.502988048 | 2 | 5 | 1 | 2 |
| Question 11 | 1,004 | 3.982071713 | 5 | 5 | 1 | 4 |
| Question 12 | 1,004 | 2.724103586 | 2 | 5 | 1 | 3 |
| Question 13 | 1,004 | 2.323705179 | 2 | 5 | 1 | 2 |

The questionnaire survey data were first processed using descriptive statistics, where data were examined in terms of average, standard deviation, variance, median, and mode. To reject the null hypothesis or not, the non-reject confidence interval was used.

### Statistical Analysis

Another method we used to reject or not reject the hypothesis $H_0$ is the confidence interval. Since the confidence interval is not at the level of 0.75, we reject the null hypothesis which claims that university students can be expected to move away from beer consumption. So, beer is consumed by a maximum of 75% of university students and we accept an alternative hypothesis that more than 75% of university students are expected to deviate from beer consumption. For statistical analyses was used statistical program SPSS.

## RESULTS AND DISCUSSION

The questionnaire survey was carried out among 1,103 university students; this survey took place in the period of September – October 2019.

In the questionnaire survey, it was found out that 1,004 respondents are university students, i.e. the generation of 18 – 20 years old who consume beer. Only 99 respondents out of a total of 1,103 respondents confirmed that they were not university students or did not drink beer.

If the respondent replied that he/she belongs to the category of a university student and a beer consumer, it was possible to continue with the questionnaire answering further twelve questions that had a scale from 1 – completely agree to 5 – completely disagree.

The second question from the questionnaire survey dealing with drinking beer from industrial breweries was answered by 81 respondents "fully agree". The highest number of responses were "rather agree" and "neither agree nor disagree". The respondents thus drink more beer from industrial breweries or do not take a stand on this issue. 43 respondents disagree with this question.

The respondents were asked whether they consumed beer exclusively from microbreweries. Only 27 respondents said that they only consume beer from microbreweries. Most respondents replied that they do not consume beer exclusively from microbreweries and 74 of those surveyed replied that they do not consume beer exclusively from microbreweries.

Another question was to find out whether students consume as much beer from industrial breweries as from microbreweries. The most common answer was "rather agree", but in terms of data, it is interesting that the second most common answer was "rather disagree". There is a difference of only 16 respondents.

As for another question, the respondents most often replied "agree" regarding searching for microbreweries offering beer of the Pilsner type – 348 respondents. Concerning the question of whether they drink mainly specials such as IPA, most respondents answered "agree" – 304 respondents.

In the survey, the respondents were asked whether they drink beer from one microbrewery mainly. The vast majority of respondents replied "rather disagree" or "completely disagree". It is clear therefore that the respondents do not prefer one microbrewery only. The vast majority of respondents also do not travel to microbreweries and do not taste new types of beer.

When it comes to the question of whether it is important for the development of beer tourism that microbreweries provide both catering and accommodation facilities, most respondents tend to answer "agree". Due to catering and accommodation facilities, microbreweries can lure other customers, for example, to have something to eat with beer, and the respondents tend to agree that beer tourism is a promising part of tourism that will compete successfully with wine tourism.

The data were further investigated by descriptive statistics in terms of average, modus, median, maximum, minimum, standard deviation, and variance. N is the number of respondents. For questions 2 – 13, there are 1,004 respondents.

For question 1, the number n of respondents is 1,103, including those who answered the first question that they do not drink beer and no longer continue with the questionnaire.

It can be noted that the largest average is related to question number 9 – 4.1552. The highest number of answers were "rather disagree" and "completely disagree", which were on a scale of 4 and 5, i.e. the respondents do not prefer beer from microbreweries. The lowest average, except for questions 1 and 13, when the most frequent answers were "fully agree" and "rather agree", i.e. 1 and 2, here the

respondents agree with the statement that they consider beer tourism to be a promising part of tourism that will compete successfully with wine tourism. Here the average is 2.323. The maxima and minima for all questions are the extreme values of the scale1- "totally agree" or the scale 5 – "totally disagree". The highest standard deviation regards question number 6, where the standard deviation value is 1.2. This suggests that there were large differences in these data. The lowest value of the standard deviation concerns question number 3, where, on the contrary, the data were the least different from each other. The highest variance number as for the data surveyed is for question number 6 and the lowest value for question number 3, except for question 1. The confidence interval was chosen to reject or not to reject the null hypothesis.

$H0: \pi = 0.75 \quad HA: \pi \neq 0.75 \quad \pi = 1004/1103 = 0.91$

$\alpha = 0.05$

$p \pm Z1 - \propto/2 \cdot \sqrt{((p\ (1-p))/p)} \quad 0.91 \pm 1.96 \cdot 0.0086$

Confidence interval: <0.894; 0.9268>

Since the confidence interval is not at the level of 0.75, we reject the null hypothesis which claims that university students can be expected to move away from beer consumption. So, beer is consumed by a maximum of 75% of university students and we accept an alternative hypothesis that more than 75% of university students are expected to deviate from beer consumption.

This research focused on beer consumers and investigated the attitude of Czech university students toward beer from microbreweries. There have been 43 industrial breweries for several decades, the number of active microbreweries was estimated at 550 at the end of 2019, and the new ones are still emerging. Based on this research, the authors made a qualified estimate that the number of microbreweries will find their place on the Czech beer market. According to information from the daily press, only 3 microbreweries were closed in 2019 due to property conflicts between shareholders.

The results of the survey in question 9 refuted Michael Short's assertion (2004 – 2009: CEO of Plzeňský Prazdroj) that Czechs drink the same brand of beer from birth to death. This is not the case for university students; only 7% of them adhere to this principle. Another conclusion is that respondents prefer beer from industrial breweries. This fact can be influenced by the aspect that 98% of beer in the Czech Republic is produced by industrial breweries and is, therefore, more accessible. Nevertheless, the question of whether the students consume the same amount of beer from industrial breweries as from microbreweries was most answered "rather agree", and then "rather disagree". A similar discrepancy was in the answers whether students drinking beer from microbreweries prefer classic or beer specials. The number of responses was slightly higher for classic beers. Most respondents do not have their preferred microbrewery. There is a consensus on the last two questions, namely that for the development of beer tourism, it is appropriate that microbreweries offer catering, accommodation and other services, and that beer tourism will compete successfully with wine tourism.

It gave similar conclusions **(Jaeger et al., 2021)**, which identified the existence of two preferential segments. One segment (lovers of artisanal style) favored characteristic complex and bold new flavors of many craft beers, while the second segment (lovers of traditional style) prefer milder and less complex flavors associated with traditional beer. A survey of 323 beer drinkers was performed in Italy **(Lerro et al., 2020)**. The study findings show that Italian craftsmen are young, educated, and employed. Drink craft beer more than once a month, especially in the pub with friends and colleagues. Craft beer is perceived higher quality and different flavors, while in comparison with commercial beer is the result of tastier, more authentic, and natural. In terms of beer attributes, Italians prefer to taste, fermentation process, and color, while trade, brand, and price are the least important. Similar conclusions have been reached **(Jaeger et al., 2020; Garavaglia, 2020; Carbone and Quici, 2020)**, which found that artisanal beer is a product category that is constantly expanding, and artisanal beer drinkers generally differ from traditional (or regular) drinkers in terms of their preferences for innovative beers with new and complex taste profiles and greater involvement in the beer behavior and activities focused on the product. This study examines the existence of segments-controlled flavor throughout the segment craft beer where a declared craft beer drinker show characteristic preference for craft beer (thicker and more complex flavors), while others prefer less complex and more traditional flavors.

On the contrary, **(Pachura, 2020)** emphasized the importance of business models in retaliatory microbreweries. Microbreweries as a research focus can be considered unique, not only in terms of growth trends and social causes but also because of the very strong links to the local context space. The interdisciplinary research methodology included a literature survey, a quantitative description of the sector, and ultimately an empirical survey of local breweries and local communities' craft beer consumers. The result of the research is the development of the original design model of a particular business model for microbreweries.

This model focuses on the importance of the local area in the context of the values offered by small breweries, and the role of local networks. A completely different view of the world of microbreweries brings research **(Wojtyra et al., 2020)**, which dealt with clustering breweries in the Czech Republic, Poland, and the Slovak Republic. Their findings suggest that the brewers in central Europe clump reasons related to income and total well-being, education level, economic activity and creativity age population, and distance from cities. The authors of this study confirm the findings of the research **(Milburn and Guertin-Martín, 2020)**, which dealt with the negative impact of increasing the number of microbreweries. However, this growth is promising for the industry and beer lovers, cases of environmental degradation at the hands of breweries remained. This article draws on interviews with American craftsmen who study the problem of environmental damage in the brewing industry. The authors focus on the consumption of drinking water and sewage disposal, particularly in the brewing facilities, but also across the supply chain of beer. The role of consumer awareness of the environmental impact has been deemed a mechanism to deal with environmental damage in the beer industry.

To attract more clients, it seems appropriate to offer a richer assortment than just beer. As stated in the research, especially breweries outside the tourist centers should use neo-localism in business practice - the use of local names and symbols, in compliance with the principles of environmental sustainability and social and community engagement.

These conclusions are also accepted **(Beckman et al., 2020)**, who investigated whether persistent engagement theory plays a role in predicting the experience of visitors to the festival of craft beer and food to the festival stage. Throughout the world a growing number of craft breweries producing more unique styles of beer, as growing demand for craft beer. Consumers craft beer attend many of these breweries and attract the craft beer festivals, where they can taste several local, regional, national, and international craft beers. The results revealed that the continued involvement is important in predicting all four factors festival landscape (quality of the food/beverage, convenience, personnel, equipment, and Festival). Practical implications: Craft Beer Festival organizers should appeal to the clubs, breweries, and craft beer publications to attract those who are committed to their action to the industry of Craft beer. People with a permanent and lasting commitment to craftsmanship beer are more likely positive experiences with the festival stage at the event. Finally, the organizers should focus on devices festival of factors, festival staff, and quality of food and drink to influence satisfaction at the event. Meetings of artisanal beer fans at food and drink festivals are also mentioned **(Ikäheimo, 2020; Dense, 2020)**. Italian researchers **(Beckman et al., 2020)** even say that in the last decade there has been a flourishing agricultural cooperatives and social sectors exponential growth of craft beer in Italy. They began to emerge social microbreweries (social cooperatives, which operate in the craft beer). This research suggests that the craft beer industry provides significant opportunities for social innovation in social cooperatives with a special focus on the labor integration of vulnerable people. Also, this document highlights the different paths for scaling social microbreweries, including a focus on organizational growth (increasing the size of the company), scaling (influencing a larger number), and scaling the depth (influencing cultural roots). Different approaches to scaling combine common strategies: building networks and partnerships. This seems like a fundamental measure to increase the impact of the social microbrewery. The economic benefits of microbreweries are in several areas – they integrate into tourist destinations, use local ingredients for brewing and food, create new jobs, enhance abandoned breweries, and contribute to the development of rural areas. The authors intend to repeat a similar survey regularly in the coming years. The subject of the survey will not only be the relation of university students to beer from microbreweries, but also the development of microbreweries and the focus of their services.

Completely original research into the influence of music and etiquette on beer selection took place in Brazil **(de Paula et al., 2020)**. According to non-sensory characteristics, industrial beer showed a better rating. The use of a song from the field of pop-rock together with the label positively influenced the acceptance and intention to buy craft beer. This study shows the R&D industry of craft and industrial breweries that they can use the influence of these non-sensory characteristics in the preparation of new brands and use the influence of songs on consumer acceptance to offer higher value-added products to the store. **(O'Brien, 2020)** examines the use of names (brewery and beer) in brewing and the association of names (signs and symbols) embedded in their graphic identity. This research, based on visual content analysis, aims to better understand the ubiquity of local geography in Surrey craft beer brands. The authors **(Matthew et al., 2021)** relied on the literature to suggest that younger Chinese consumers support the growth of artisanal beer. A generational approach to artisanal beer was tested during the research. The answers to the open-ended questions revealed a lack of previous experience, awareness, and knowledge, which suggests that traders were unable to distinguish Chinese artisanal beer from other beer products. Although interest in Chinese artisanal beer is growing, the Chinese artisanal beer industry is still asleep and is at an early stage of development, hence the title of the chapter. The Sleeping Dragon needs to be awakened, with merchants using an integrated marketing communication strategy to help the Chinese craft beer industry realize its potential and move forward in the craft beer revolution.

Similar conclusions, which confirm the results of our research, were reached **(Belmartino and Liseras, 2020; Schroeder, 2020; Apardian and Reid, 2020; Mello and da Silva, 2020; Wojtyra, 2020; Wojtyra, Grudzien and Lichota, 2020; Poelman and Ostyn, 2020)**. They state that neolocalism is highly valued in the industry by both producers and consumers. They identify two groups of craft beer consumers: beginners and craft beer lovers. Among the attributes that consumers value most are quality, taste, and bitterness. The authors **(Jaeger et al., 2020)** focused their research on the development of taste preferences. They concluded that there was a shift in the declared preferences of the artisanal drinker from the lighter flavors of traditional beers to which they were accustomed, to newer and more robust flavors of artisanal beers. This shift in taste preferences is preliminarily attributed to the same exposure effects that are responsible for the development of taste preferences in other foods and beverages. The preferences of craft beer consumers in each EU country are addressed **(Cabras, 2020)**. They examined the gender nature of craft beer (CB) consumption in Italy and Germany **(Rivaroli et al., 2020)**. The results of the study provide evidence that the difference in the consumption behavior of craft beer is not very significant.

Interesting inspiration for further research is the conclusions **(Mellor et al., 2020)**. The authors point out that beer contains several potentially beneficial bioactive substances, but the benefits are limited by the negative consequences of its alcohol content. There is the potential to increase the bioactive properties of beer while reducing the alcohol content through new brewing approaches, which are often used in artisanal brewing, in terms of ingredients, brewing methods, and type of fermentation. Another topic for research is to deal with the relationship of consumers to special types of beer produced with the addition of fruit in the production process. They are becoming particularly popular in the field of artisanal breweries **(Fanari et al., 2020)**.

## CONCLUSION

The questionnaire survey was focused on the relation of university students of generation Z in the Czech Republic to beer from microbreweries. The survey involved 1,103 respondents from among Czech university students. 1,004 respondents complied with the survey conditions (they had to belong to university students and consume beer). Although 98% of beer consumed in the Czech Republic is from industrial breweries, it has turned out that the proportion of students who consume beer from industrial breweries and microbreweries is almost the same. Students most preferred the beer of the classic Pilsen type, which does not circulate so much in beer tourism yet. Respondents in the research agreed that beer tourism will successfully compete with wine tourism. The economic benefits of microbreweries are in many areas and also contribute to the development of local areas and regions. The authors will further intensively examine the topic of the relationship between beer and a specific generation.

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# INFLUENCE OF EIGHT CHOSEN ESSENTIAL OILS IN THE VAPOR PHASE ON THE GROWTH OF *RHIZOPUS STOLONIFER* AND *RHIZOPUS LYOCOCCUS*

***Dana Tančinová, Miroslava Hlebová, Denisa Foltinová, Zuzana Mašková, Zuzana Barboráková***

## ABSTRACT

This study aimed to evaluate the fungicidal effect of eight essential oils against five strains of the genus *Rhizopus*. Strains were obtained from various moldy foods, namely *Rhizopus stolonifer* KMi 383 from chestnut, *R. stolonifer* KMi 510 from strawberry, *R. stolonifer* KMi 511 from nectarine, *R. stolonifer* KMi 524 from cherry tomatoes, and *R. lyococcus* KMi 512 from blackberry. The essential oils (EO) used in this study were jasmine EO (extract from *Jasminum officinale* L.), bergamot EO (*Mentha aquatica* L. var. *citrata* (Her.) Fresen), bitter orange EO (from *Citrus aurantium* L.), grapefruit EO (*Citrus paradisi* Macfady), sweet flag EO (East Asian Calamus, from *Acorus calamus* L. var. *angustatus* Bes), star anise EO (from *Illicium verum* J.D.Hook), geranium EO (from *Pelargonium graveolens*), and lemongrass EO (from *Cymbopogon citratus* DC). The semi-quantitative composition of the essential oil samples was determined by gas chromatography coupled with mass spectrometry (GC-MS). The antifungal activity of essential oils against the strains of *R. stolonifer* and *R. lyococcus* was determined during 7 days, using the micro-atmosphere method (0.625 μL.mL$^{-1}$ of air). Two essential oils, geranium and lemongrass, completely inhibited the growth of all isolates. Bitter orange essential oil inhibited the growth of all isolates of *Rhizopus stolonifer*, but isolate of *Rhizopus lyococcus* began to grow after four days of cultivation. In conclusion, certain essential oils are highly effective in the vapor phase. These could be used in further tests of their antifungal activity and could be used in the control of *Rhizopus* spp. or other fungal pathogens.

**Keywords:** essential oils; *Rhizopus stolonifer*; *Rhizopus lyococcus*; antifungal activity; vapour phase

## INTRODUCTION

*Rhizopus* species including *R. stolonifer* are naturally found in soil, debris, and air. Fungal dispersal mechanisms of *R. stolonifer* are wind, air currents, and some invertebrates such as mites and insects, among others **(Bautista-Baños et al., 2014)**. *Rhizopus stolonifer* is a ubiquitous fungus and can be isolated from many kinds of foods. It grows rampantly at 25 ºC, filling a Petri dish with sparse, dark mycelium in 2 days. It produces barely macroscopic aerial fruiting structures which are at first white, then become black. Given seven undisturbed days, it sheds dry black spores outside the Petri dish, providing an effective inoculum for a continuous chain of future contamination **(Pitt and Hocking, 2009)**. *Rhizopus* rot is common on soft fruits, more abundant in warm humid climates than in cool climates. In several fruits and crops such as strawberries, peaches, avocados, tomatoes, cucumbers, and table grapes, *Rhizopus* rot causes soft rot during transport and storage **(Kassemeyer and Berkelmann-Löhnertz, 2009)**. Infection usually starts from wounding after the cracking of fruits. At first, the lesions (soak with water) are rapidly softened and diseased lesions gradually expanded. The mycelia grow vigorously on the surface of fruits and formed stolons. Colonies are white cottony at first, becoming heavily speckled by the presence of sporangia and then brownish-black. They spread rapidly using stolons fired to various points of the substrate and attach by rhizoids. The color of sporangia is white at first and then turns black with many spores **(Kwon et al., 2001)**. The postharvest handling operations are the main reason *R. stolonifer* succeeds in entering and infecting most horticultural commodities **(Bautista-Baños et al., 2014)**.

Significant postharvest losses occur during the supply chain on fresh produce. Postharvest decay is one of the main factors that determine losses and compromises the quality of fruits and vegetables. Traditionally, postharvest decay control is achieved using chemical fungicides; however, the important concerns relating to environmental and human health require the development of novel methods for the control of postharvest decay **(Mari, Bautista-Baños and Sivakumar, 2016)**. The growing awareness of consumers concerning the relationship between food and health revolutionized the food industry. New techniques such as high pressure, nanotechnology, irradiation, etc., are increasingly used to maximize the

nutritional properties of foods, while new ingredients with functional properties contribute to improving health. The ‘‘elimination” of additives used in a wide variety of foods is demanded, while ‘‘natural” additives are seen as a benefit for both quality and safety **(Viuda-Martos et al., 2008)**. Over the past few years, consumers demand safe, environmentally-friendly, and natural products. It has driven the search for preservation techniques that improve product quality and safety without causing nutritional or sensory losses. Natural antimicrobial essential oils have the potential to provide quality and safety benefits and have fewer impacts on human health **(Ju et al., 2019)**. The development of natural crop protective products as alternatives to synthetic fungicides is currently in the spotlight **(Combrinck, Regnier and Kamatou, 2011; Stević et al., 2014)**.

The present research aimed to determine the inhibitory effect of chosen essential oils on the growth of different *Rhizopus stolonifer* and *Rhizopus lyococcus* strains.

## Scientific hypothesis

The chosen essential oils can affect the growth of *Rhizopus* strains.

## MATERIAL AND METHODOLOGY

### Samples

Essential oils – Hanus Nitra (www.hanus.sk).

### Chemicals

Potato dextrose agar (PDA, HIMEDIA India).

Hexane Sigma-Aldrich (HPLC Plus, for HPLC, GC, and residue analysis, ≥95%).

Dimethylsulfoxide (DMSO) Sigma-Aldrich (Hybri-Max™, sterile-filtered, BioReagent, suitable for a hybridoma, ≥99.7%).

### Biological Material:

Strains of *Rhizopus* spp. – Collection of microscopic fungi of the Department of Microbiology, SUA in Nitra.

### Instruments

Agilent 6890 GC-FID (Agilent Technologies, Palo Alto, CA, USA.

Agilent 7890A GC coupled to an Agilent MSD5975C MS detector (Agilent Technologies, Palo Alto, CA, USA).

EVE™ Automatic cell counter (NanoEnTek, Korea).

### Laboratory Methods

Adapted from **Guynot et al. (2003)**.

### Fungal culture

We used the strains of *Rhizopus stolonifer* and *Rhizopus lyococcus* obtained from the Collection of Microorganisms of the Department of Microbiology of the Slovak Agricultural University in Nitra. The strains were obtained from moldy plant sources; *Rhizopus stolonifer* KMi 383 (GenBank ID MF461020.1) from moldy chestnut, *R. stolonifer* KMi 510 from moldy strawberry, *R. stolonifer* KMi 511 (GenBank ID KU554577.1) from moldy nectarine *R. stolonifer* KMi 524 (GenBank ID AM933546.1) from moldy cherry tomatoes, and *R. lyococcus* KMi 512 (GenBank ID JN206375.1) from moldy blackberry.

### Plant essential oils

The following essential oils were used in the research: jasmine (extract from *Jasminum officinale* L.), bitter orange (from *Citrus aurantium* L.), bergamot EO (*Mentha aquatica* L. var. *citrata* (Her.) Fresen), grapefruit (*Citrus paradisi* Macfady), sweet flag (East Asian Calamus, from *Acorus calamus* L. var. a*ngustatus* Bes), star anise (from *Illicium verum* J. D. Hook), geranium (from *Pelargonium graveolens*), and lemongrass (from *Cymbopogon citratus* DC). Essential oils were stored in air‑tight sealed glass bottles at 4 ±1°C.

### Chemical composition of essential oils

The relative composition of essential oils was determined, and the compounds were identified by gas chromatography with mass spectrometry (GC-MS). Essential oils were diluted in hexane to a concentration of l $\mu L.mL^{-1}$. Analyses were carried out using an Agilent 7890A GC coupled to an Agilent MSD5975C MS detector (Agilent Technologies, Palo Alto, CA, USA) with an HP-5MS column (30 m × 0.25 mm, 0.25 mm film thickness). One microliter of the sample was injected in split mode 1:12, at an injector temperature of 250°C and electron ionization energy of 70 eV. Analysis were measured in SCAN mode, the mass range was 40 – 400 m/z. Starting at 60 °C, the oven temperature was increased at a rate of 3 °C/min to a maximum of 231 °C, where it was kept constant for 10 min. The identification of constituents was based on a comparison of their mass spectra and relative retention indices (RI) against the National Institute of Standards and Technology Library (NIST, USA), as well as authentic analytical standards and data from the literature. Relatively proportion of EO constituents were assessed by Agilent 6890 GC-FID (Agilent Technologies, Palo Alto, CA, USA) with RTX5 column (Restek, Bellefonte, PA; 20 m × 0.18 mm, 0.2 µm film thickness). The same method for GC-MS was used. Relative proportions were calculated by dividing individual peak area by total area of all peaks. The response factor was not taken into account. Only compounds over 0.1% were included. The used standards are listed in Table 1.

### Antifungal activity of essential oils

The micro atmosphere method was used to study the effect of essential oils on the growth of strains of *Rhizopus* sp. The test was performed in sterile plastic Petri dishes (Ø 90 mm) containing 15 mL of potato dextrose agar (PDA, HIMEDIA India). Evaluation by filter paper was made by the method adapted from **Guynot et al. (2003)**. Essential oils were tested in concentration 0.625 $\mu L.cm^{-3}$ of air. A sterile filter paper (cca1.5 x 1.5 cm) was placed in the lid of the Petri dish and 50 µL of essential oil was pipetted by micropipette to the paper. Dishes were kept in an inverted position. Filter paper discs impregnated with sterilized distilled water were used as a control to confirm no solvent effect of bioactivity. Each isolate was inoculated on the center of Petri dishes with 5 µL of spore’s suspension ($10^5$ spores in 1 mL). Dishes were tightly sealed with parafilm and incubated for seven days at 25 ±1 °C (three replicates were used for each treatment). Diameters (Ø mm) of the growing colonies were measured on the 2nd, 4th, and 7th day with a digital caliper.

### Inhibition of mycelial growth

According to **Cakir et al. (2005)** and **Kordali et al. (2008)** growth inhibition of treated samples (*T*) against

control (*C*) was calculated by the percentage of growth inhibition using the following equation (1):

$$\% \text{ of inhibition } = \frac{C-T}{C} x\ 100 \qquad (1)$$

Where:
C is the mean of six replicates of hyphal extension (mm) of controls; T is the mean of six replicates of hyphal extension (mm) of plates treated with either essential oil.

**Minimum inhibitory doses (MIDs)**

Essential oils that completely inhibit the growth of all strains of *R. stolonifer* or strain of *R. lyococcus* were used to determine their minimum inhibitory doses (MIDs). EOs dissolved in dimethylsulfoxide (DMSO) were prepared at different concentrations (500, 250, 125, 63, 31.25, and 15.63 $\mu L.L^{-1}$ of air). For each fungal strain, a conidial spore suspension of $10^6$ spore in $mL^{-1}$ was prepared. The EVE™ Automatic cell counter (NanoEnTek, Korea) was used to determine the number of spores. Petri dishes (Ø 90 mm, three-sector, two replicates) containing 15 mL of PDA were inoculated by 5 µL spore suspension. Cultivation was carried out at 25± 1 °C and measured after 7 and 14 days. The MID (expressed as microliters of EOs per volume unit of the atmosphere above the organism growing on the agar surface) was defined as the lowest concentration of the oil which did not permit any visible growth after 7 or 14 days in comparison with control sets.

## Statistical Analysis

The size of colonies of isolates (mm) for each day of cultivation within treatment was evaluated. Also, the size of colonies of isolate for each treatment to the same isolate in the control group was evaluated too. The results were mathematically processed using the Microsoft Excel program and statistically evaluated by SAS/9.3 (2010). A used statistical model can be written in the following form:

$$yij = \mu + ISOLATEi /TREATMENTj/ + eij$$

Where:
yij = the measurements for size of colonies; µ = overall mean; ISOLATEi = the fixed effects of isolates (i = 1 to 6); TREATMENTj = the fixed effect of treatment (j = 1 to 5); eijk = random error, assuming eijkl ~ N(0, I σe2).

**Probit analyses**

The ability of strains to grow in the presence of EO was coded to a binomial scale (1 – growth observed, 0 – without growth). Such data were processed by probit analysis in Statgraphics Centurion XV (Statgraphics) software. Doses that inhibit the growth in 50% respectively 90% of cases (MID50 and MID90) were reversely predicted from the regression equation.

## RESULTS AND DISCUSSION

According to market data, there are about 400 species, from 67 plant families, which are cultivated on a large commercial scale for the production of essential oils **(Bhattacharya, 2016)**. In this research, we evaluated the antifungal properties of 8 essential oils from families *Oleaceae* (jasmine EO), *Rutaceae* (bergamot EO, bitter orange EO, grapefruit EO), *Acoraceae* (sweet flag EO), *Illiaceae* (geranium EO), *Lamiaceae* bergamot EO (*Mentha aquatica* L. var. *citrata* (Her.) Fresen), *Poaceae* (lemongrass EO). According to authors **(Ben Farhat et al., 2016; Méndez-Tovar et al., 2016; Dušková et al., 2016)**, the effect of the growing seasons, the different growth stage of plants, and climatic conditions of each year in terms of the essential oil content and composition were proven. Based on the above, we also focused on the composition of the essential oils we used. The identified compounds are listed in Table 1. The major components according to the concrete essential oil were: bergamot EO - (R)-(+)-Limonene (34.7%) and Geraniol (31.1%), bitter orange EO - (R)-(+)-Limonene (31.9%), grapefruit EO - 1,8-Cineole (92.2%), geranium - Citronellol (28.3%), lemongrass EO - β-Citral (28.3%), jasmine EO - (-)-Borneol (37.7%), Star anise EO - Trans-Anethole (87.1%), sweet flag EO - cis-Verbenol (87.6%).

Species of genus *Rhizopus* especially *R. stolonifer* are the most important postharvest pathogens for a great variety of fruits and vegetables. Several articles have been published on the possibilities of influencing the growth of these fungi by plant essential oils. Inhibitory effect of *Thymus vulgaris* EO on the *Rhizopus stolonifer* tested **Bhaskara Reddy et al. (1998)**, **Bosquez-Molina et al. (2010)**. Sage EO (*Salvia officinalis*), savory EO (Satureja horten-sis), and zataria or Shiraz thyme EO (*Zataria multiflora*) were tested by **Alizadeh-Salteh et al. (2010)** and **Alizadeh-Salteh et al. 2013)**. The essential oils from *Mentha piperita, Lavandula angustifolia, Foeniculum vulgare,* and *Cuminum cyminum* were tested by **Hadian et al. (2008)**.

Certain essential oils are highly effective in the vapor phase and could be used in the control of foodborne bacterial pathogens **(López et al., 2007; Nedorostova et al., 2009)**. **Tyagi and Malik (2011)** report significantly higher antimicrobial activity of some essential oils, which we observed in the vapor phase. In our research, we also used the vapor phase to test the effect of selected essential oils on *Rhizopus* growth. The antifungal activity of 7 essential oils against the *Rhizopus stolonifer* (4 strains) and *Rhizopus lyococcus* (1 strain) were determined, using the micro-atmosphere method (625 $\mu L.L^{-1}$ of air). The results are shown in Table 2 and Figure 1.

Two essential oils: lemongrass (*Cymbopogon citratus* DC) and geranium (*Pelargonium graveolens*) completely inhibited the growth of all strains. Lemongrass (*Cymbopogom citratus* DC) essential oil is known due to its broad-spectrum antimicrobial activity **(Leimann et al., 2009)**. According to **Abdulazeez, Abdullahi and James (2016)**, **Božik et al. (2017)**, **Císarová et al. (2020)** lemongrass oil has been also shown to be an effective fumigant for stored food commodities due to its bioactivity in the vapor phase. Lemongrass EO was found to significantly reduce colony development against key postharvest pathogens: *Colletotrichum coccodes*, *Botrytis cinerea*, *Cladosporium herbarum*, *Rhizopus stolonifer*, *A. flavus, A. parasiticus, A. ochraceus, A. westerdijkiae,* and *Aspergillus niger in vitro*. Lemongrass essential oil at concentrations of 0.5% and 1.0% was incorporated into 0.5% and 1.0% chitosan solution and evaluated as means of controlling anthracnose of bell pepper *in vitro* and *in vivo*.

**Table 1** Chemical composition of essential oils (in %) determined by by gas chromatography coupled with mass spectrometry (GC-MS).

| Compound | Essential oils | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **B** | **BO** | **GP** | **GR** | **L** | **J** | **AN** | **S** |
| **Asarone**[a] | | | | | | | | 5.4 |
| **Camphenea**[a] | | | | | 0.9 | 3.5 | | |
| **(+)-α-Pinene**[a] | 1.3 | 1.7 | 0.6 | | | 3.5 | 0.4 | |
| Estragol | | | | | | | 5.3 | |
| β-Phellandrene | 0.9 | 0.3 | 0.4 | | | 1.2 | | |
| **(-)-β-Pinene**[a] | 8.9 | 17.5 | | | 1.3 | | | |
| 6-Methylhept-5-en-2-ol | | | | | 1.2 | | | |
| β-Myrcene | 0.6 | 1.8 | 1.4 | | 6.0 | | | |
| α-Phellandrene | | | 0.2 | | | | | |
| **p-Cymene**[a] | 1.8 | | | | | 1.6 | | |
| **(R)-(+)-Limonene**[a] | 34.7 | 31.9 | | | 9.6 | 19.2 | | |
| **1.8-Cineole**[a] | | | 92.2 | | | 2.7 | | 1.1 |
| β-Ocimene | | 0.7 | | | | | | |
| **γ-Terpinene**[a] | 8.5 | | | | 0.9 | | | |
| **(-)-Linalool**[a] | 9.2 | 3.1 | | 4.3 | 1.5 | 4.3 | 1.0 | 4.1 |
| **(+)-Rose oxide**[a] | | | | 2.3 | | | | |
| Thujone | | | 0.3 | | | | | |
| cis-Limonene oxide | | | 1.4 | | | | | |
| **(1R)-(+)-Camphor**[a] | | | 0.6 | | | | | |
| **(±)-Citronellal**[a] | | 1.0 | | 2.8 | | | | 1.3 |
| **(-)-Borneol**[a] | | | | 5.2 | | 37.7 | | |
| **2-Undecanone**[a] | | | | | | 4.6 | | |
| Pseudo-limonene | | | | | | 3.6 | | |
| **5-Methylindole**[a] | | | | | | 0.6 | | |
| α-Terpineol | | 1.6 | | | | | | |
| cis-Verbenol | | | | | | | | 87.6 |
| **4-Carvomenthenol**[a] | | | 0.3 | | | | | |
| **Cinnamaldehyde**[a] | | | 0.4 | | | | | |
| Citronellol | | | | 28.3 | | | | |
| **β-Citronellol**[a] | | | 0.4 | | | | | |
| β-Citral | | 8.2 | | 0.7 | 28.3 | | | |
| **(−)-Carvone**[a] | | | 0.8 | | | | | |
| **Geraniol**[a] | 31.1 | 6.9 | | 11.8 | 5.0 | | 1.9 | |
| **Trans-Anethole**[a] | | | | | | | 87.1 | |
| Anisketone | | | | | | | 0.4 | |
| **α-Citral**[a] | 0.5 | 12.2 | | 1.4 | 35.2 | | | |
| Neryl acetate | | 1.8 | | | | | | |
| Benzyl benzoate | | | | | | 10.9 | | |
| Citronellyl formate | | | | 13.0 | | | | |
| **Carvacrol**[a] | | | | 3.1 | | | | |
| δ-Elemene | | | | 0.6 | | | | |
| α-Copaene | | | | 0.7 | | | | |
| **Geranyl acetate**[a] | | 6.7 | | 2.2 | 4.2 | | 0.5 | |
| **β-Caryophyllene**[a] | | 1.3 | | 0.8 | 2.3 | | | |
| **Farnesene**[a] | | | | | 2.0 | | | |
| **Citronellyl propionate**[a] | | | | 0.8 | | | | |
| Germacrene-D | | | | 4.3 | | | | |
| β-Bisabolene | | | | 0.7 | | | | |
| γ-Cadinene | | | | 0.5 | | | | |
| **Myristicin**[a] | | | | 1.4 | | | | |
| Acetyl eugenol | | | | 0.8 | | | | |
| (+)-Carvone acetate | | | | 0.6 | | | | |
| **Caryophyllene oxide**[a] | | | | 0.8 | | | | |
| **Cedrol**[a] | | | | 1.2 | | | | |
| γ-Eudesmol | | | | 5.7 | | | | |
| Tau-Cadinol | | | | 0.6 | | | | |
| β-Eudesmol | | | | 2.0 | | | | |
| α-Eudesmol | | | | 0.6 | | | | |
| **Total** | **97.5** | **96.7** | **99** | **97.3** | **98.4** | **98.4** | **96.6** | **99.5** |

Note: [a] – identification confirmed by co-injection of authentic standard, GS-MS – gas chromatography coupled with mass spectrometry, B – bergamot, BO – bitter orange, GP – grapefruit, GR – geranium, L – lemongrass, J – jasmine, AN – star anise, S – sweet flag.

Fungal growth was effectively controlled by 0.5% and 1.0% EO *in vitro* **(Ali, Noh and Mustafa, 2015)**. **Moore-Neibel et al. (2011)** described **t**he antimicrobial activity of lemongrass oil against *Salmonella* directly on the leafy greens, romaine, and iceberg lettuces, and both mature and baby spinach described**.**

As mentioned above, the geranium EO completely inhibited the growth of the *Rhizopus* strains used in this study, as well. This essential oil has the potential to be used in the food industry to prolong the shelf life of fresh and processed foods **(Verma, Chandra Padalia and Chauhan, 2016)**.

**Bouzenna and Krichen (2013)** tested the antifungal activity *Pelargonium graveolens* Eo against *Rhizoctonia solani,* and results showed that the essential oil was highly active at a dose of 12.5 µL.20 $mL^{-1}$ of PDA. **Naeini, Nazeri and Shokri (2011)** reported that *P. graveolens* EO has considerable anti-*Malassezia* activities.

Bitter orange (*Citrus aurantium* L.) essential oil completely inhibited the growth of *Rhizopus stolonifer* strains. But we recorded growth on day seven of cultivation at the *Rhizopus lyococcus* strain, in the presence of this essential oil. Bitter orange oil has been reported to possess various pharmacological properties. Properties of bitter orange oil for food preservation are discussed too **(Anwar et al., 2016)**. In contrast with bitter orange EO, grapefruit essential oil inhibited the growth of *R. lyococcus* completely but only weakly *Rhizopus stolonifer*. **Ng et al. (2016)** show that grapefruit oil (*Citrus paradisi*) exhibits an array of activities encompassing insecticidal and antimicrobial activities.

**Table 2** Effect of essential oils (treatment) in the vapor phase on the growth of *Rhizopus stolonifer* and *Rhizopus lyococcus* strains.

| Treatment essential oil | Strains of *Rhizopus stolonifer* | | | | Strain of *Rhizopus lyococcus* | std. Error |
|---|---|---|---|---|---|---|
| | 383 | 510 | 511 | 524 | 512 | |
| **Second day of cultivation - means** | | | | | | |
| Star anise | $0^{a}$ | $12.42^{b}$ | $7.11^{c}$ | $0.00^{a}$ | $0.00^{a}$ | 0.1548 |
| Bergamot | $7.63^{aA}$ | $9.14^{a}$ | $8.87^{a}$ | $7.67^{a}$ | $14.31^{b}$ | 0.1548 |
| Sweet flag | 90.00 | 90.00 | 90.00 | 90.00 | 90.00 | 0.1548 |
| Geranium | Completely inhibited growth of strains | | | | | |
| Grapefruit | $16.16^{aA}$ | $16.19^{a}$ | $28.81^{b}$ | $20.46^{d}$ | $0.00^{c}$ | 0.1548 |
| Jasmine | 90.00 | 90.00 | 90.00 | 90.00 | 90.00 | 0.1548 |
| Lemongrass | Completely inhibited growth of strains | | | | | |
| Bitter orange | Completely inhibited growth of strains | | | | | |
| Control | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | 0.1548 |
| **Fourth day of cultivation – means** | | | | | | |
| Star anise | $11.61^{aA}$ | $27.92^{bA}$ | $20.615^{cA}$ | $4.64^{dA}$ | $0.00^{eA}$ | 0.2232 |
| Bergamot | $9.24^{aA}$ | $14.48^{bA}$ | $13.66^{bcA}$ | $12.35^{bcA}$ | $90.00^{d}$ | 0.2232 |
| Sweet flag | 90.00 | 90.00 | 90.00 | 90.00 | 90.00 | 0.2232 |
| Geranium | Completely inhibited growth of strains | | | | | |
| Grapefruit | $27.75^{aA}$ | $32.63^{bA}$ | $90.00^{c}$ | $39.88^{dA}$ | $0.00^{eA}$ | 0.2232 |
| Jasmine | 90.00 | 90.00 | 90.00 | 90.00 | 90.00 | 0.2232 |
| Lemongrass | Completely inhibited growth of strains | | | | | |
| Bitter orange | Completely inhibited growth of strains | | | | | |
| Control | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | 0.2232 |
| **Seven day of cultivation – means** | | | | | | |
| Star anise | $22.43^{aA}$ | $90.00^{b}$ | $90.00^{b}$ | $34.47^{cA}$ | $9.77^{dA}$ | 0.2072 |
| Bergamot | $16.33^{A}$ | $18.55^{A}$ | $18.41^{A}$ | $21.51^{A}$ | 90,00 | 0.2072 |
| Sweet flag | 90.00 | 90.00 | 90.00 | 90.00 | 90.00 | 0.2072 |
| Geranium | Completely inhibited growth of strains | | | | | |
| Grapefruit | $36.12^{aA}$ | $90.00^{b}$ | $90.00^{b}$ | $61.61^{c}$ | $0.00^{dA}$ | 0.2072 |
| Jasmine | 90.00 | 90.00 | 90.00 | 90.00 | 90.00 | 0.2072 |
| Lemongrass | Completely inhibited growth of strains | | | | | |
| Bitter orange | 0.00 | 0.00. | 0.00 | 0.00 | $14.63^{bA}$ | 0.2072 |
| Control | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | $90.00^{B}$ | 0.2072 |

Note: a, b, c, d, e – different letters are significant within treatment at the level $p < 0.05$; A, B – significant difference ($p < 0.05$) of the same isolate within all treatments to the same isolate in control.

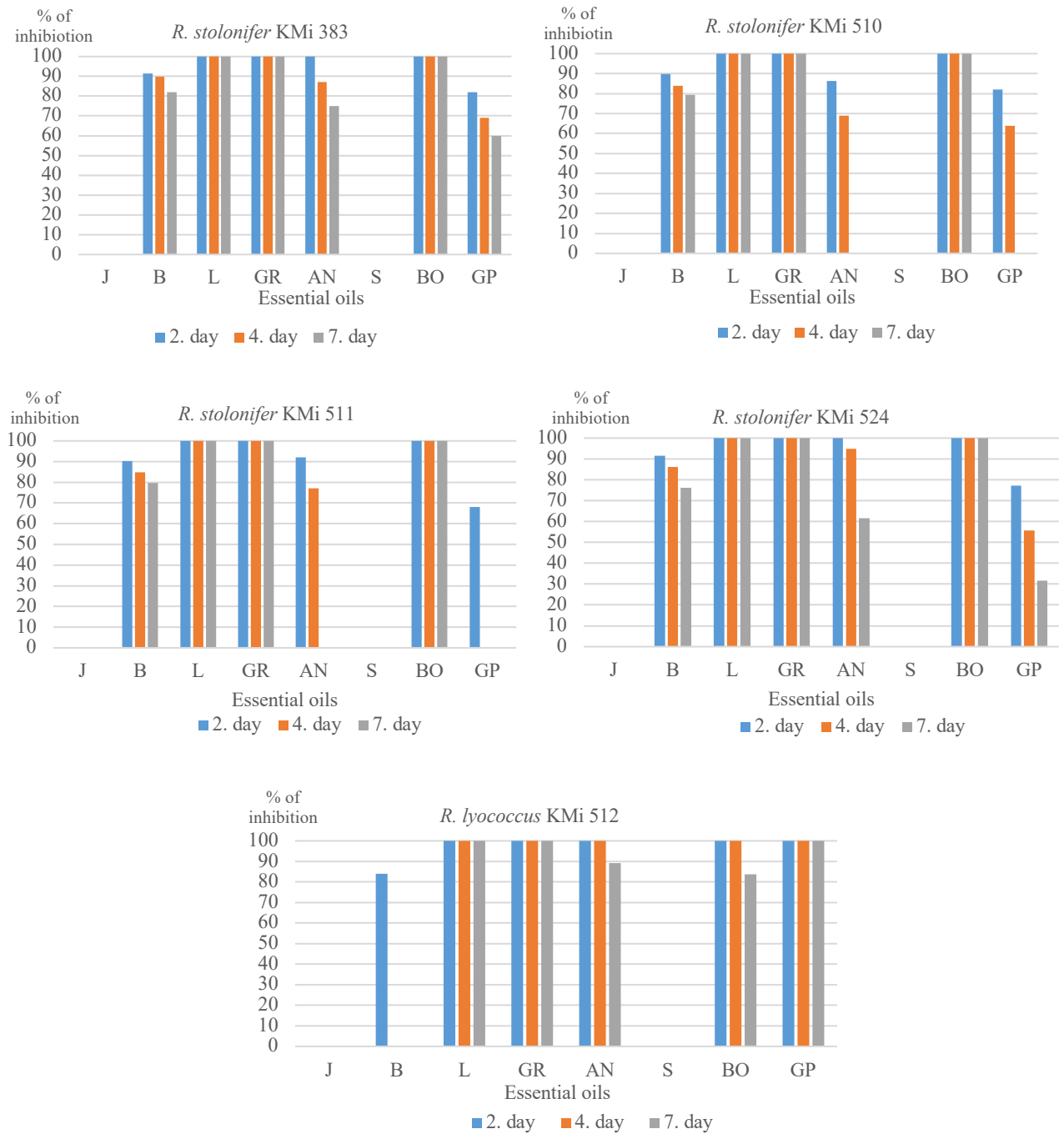


**Figure 1** Inhibition of *Rhizopus* spp. growth caused by tested essential oils in the vapor phase. Note: B – bergamot, BO – bitter orange, GP – grapefruit, GR – geranium, L – lemongrass, J – jasmine, AN – star anise, S – sweet flag, R. – *Rhizopus*.

**Viuda-Martos et al. (2008)** point out the good inhibitory activity of grapefruit EO on the moulds *Penicillium chrysogenum* and *Penicillium verrucosum* too.

The strains of the test species responded differently to the presence of bergamot EO. Significant antifungal activities were observed in all *Rhizopus stolonifer* strains. The strain of *Rhizopus lyococcus* began to grow intensively after two days of cultivation and no growth inhibition was found on the fourth day of cultivation. Some authors e.g. **Avila-Sosa et al. (2016)**, report that bergamot essential oil may be very helpful when applied to food preservation systems.

Jasmine (*Jasminum officinale* L.) and sweet flag (*Acorus calamus* L. var. *angustatus* Bes) essential oils did not inhibit the effect on the growth of tested strains genus *Rhizopus*. Apart from other uses of jasmine essential oil, it is active against various gram-negative, gram-positive bacteria and fungi. This property of jasmine oil allows it to be used in food preservation. It also possesses antioxidant activity **(Ahmed et al., 2016)**. The possibility of using sweet flag EO as a natural preservative in food was indicated by **Miao et al. (2016)** and other authors.

Star anise (*Illicium verum* J. D. Hook) essential oil completely inhibited the growth of two strain *R. stolonifer* (KMi 383 and KMi 524) to the second day of cultivation

and strain *R. lyococcus* to the fourth day of cultivation. The complete inhibition of the germination of spores of *Penicillium expansum* by star anise EO in the vapor phase is reported by **da Rocha Neto et al. (2019)**.

Essential oils that completely inhibited the growth of *R. stolonifer* or *R. lyococcus* isolates were used to determine the minimum inhibitory doses (MIDs). The results are shown in Table 3. High minimum inhibitory doses for *R. stolonifer* and *R. lyococcus* strains were determined. The best results (500 $\mu L.L^{-1}$ of air) for *R. stolonifer* KMi 383, KMi 510, Kmi 524, and *R. lyococcus* Kmi 512 on the 7th day of incubation showed lemongrass EO. MIDs for geranium EO were 625 $\mu L.L^{-1}$ of air for all strains *R. stolonifer* and strain *R. lyococcus* too. The same parameters were determined for geranium Eo for all strains of R. stolonifer and grapefruit Eo for the strain of *R. lyococcus*. On the 14th day of incubation, we found the ame MIDs, respectively higher. It was found that EOs have different effects on individual strains of *R. stolonifer*. **Tripathi and Shukla (2009)** showed absolute fungitoxic activity of geranium EO against Botryodiplodia theobromae (the cause an important postharvest fungal disease of mango), like we. These authors reported MIC 200 ppm for this EO.

Using probit analysis, predicted MIDs90 and MIDs50 were calculated. The results are shown in Table 4. The most effective tested essential oil was lemongrass, less effective bitter orange EO for *R. stolonifer* strains, and grapefruit EO for *R. lyococcus* strain.

The specific compounds isolated from the oils may be non-fungi toxic in nature. Different components of the oils as such may also check the development of races of fungi during their application due to more than one site of action. Fungi can easily develop resistant races against a single component due to its specific mode of action. The exploitation of the essential oils as such would be more economical than a single component as a fungitoxicant **(Tripathi and Shukla, 2009)**. The safe use of EO ingredients is also supported by their self-limiting properties as flavoring substances in food resulting in low levels of use; their rapid absorption, metabolic detoxication, and excretion in humans and other animals; the wide margins of safety between the conservative estimates of intake and the no observed-adverse effect levels determined from subchronic and chronic studies and the lack of significant genotoxic, developmental and teratology potentials **(Adams et al., 2011)**.

## CONCLUSION

In our research, we evaluated the antifungal properties of jasmine EO (extract from *Jasminum officinale* L.), bergamot EO (*Mentha aquatica* L. var. *citrata* (Her.) Fresen), bitter orange EO (from *Citrus aurantium* L.), grapefruit EO (*Citrus paradisi* Macfady), sweet flag EO (East Asian Calamus, from *Acorus calamus* L. var. *angustatus* Bes), star anise EO (from *Illicium verum* J.D.Hook), geranium EO (from *Pelargonium graveolens*), and lemongrass EO (from *Cymbopogon citratus* DC), two essential oils: geranium and lemongrass completely inhibited the growth of all isolates. Bitter orange essential oil inhibited the growth of all isolates of *Rhizopus stolonifer*, but isolate of *Rhizopus lyococcus* began to grow after four days of cultivation. In conclusion, certain essential oils are highly effective in the vapor phase. These could be used in further tests of their antifungal activity and could be used in the control of *Rhizopus* spp. or other fungal pathogens. In further research, we plan to test the effect of selected essential oils in *in vitro* (on selected fruits). In the next part we will test the impact of EOs on sensory quality of selected fruits.

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This article does not contain any studies that would require an ethical statement.

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# THE EFFECT OF SINGLE CLOVE BLACK GARLIC ON THE HEMOSTASIS STATUS AND LIPID PROFILE IN MALE SPRAGUE DAWLEY RATS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

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## ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) patients have alteration in hemostasis components. Thus, including excess expression of plasminogen activator inhibitor-1 (PAI-1), causing fibrinolysis disorders; the majority of these patients are hypercoagulable state prone to thrombosis. Some evidence suggests that garlic and garlic supplements have antithrombotic and anti-inflammatory properties. Besides, garlic stimulates fibrinolytic activity and normalizes plasma lipid imbalances. Black garlic is processed garlic that is produced through natural aging at a controlled temperature (70 °C) and high humidity (90%) for several days, without other additives. This study aimed to prove the effect of single clove black garlic (SCBG) (*Allium sativum Linn*) on PAI-1 levels and lipid profiles of NAFLD rats induced by a *high-fat fructose diet* (HFFD) containing 1.25% cholesterol and 0.5% cholic acid.The rats were then divided into healthy control group ($K_1^+$); NAFLD control group without treatment ($K_2^+$); 0.9 mg simvastatin treatment group ($K_1^-$); 45 mg metformin treatment group ($K_2^-$); SCBG 450 mg per 200g BW (X1); 900 mg per 200 g BW (X2); and 1350 mg per 200 g BW (X3). All treatments were administered for 4 weeks via oral gavage. As a result, significant differences in PAI-1 levels and lipid profiles between groups after the administration ($p$ = 0.001) were noted and also by simvastatin and metformin, respectively. There was a correlation between PAI-1 and lipid profile of SCBG treatment. In conclusion, the administration of SCBG (1350 mg per 200 g BB per day) for 4 weeks had a significant effect on PAI-1 levels, and the lipid profiles in *Sprague Dawley* rats modeled NAFLD ($p$ = 0.001). SCBG has provided benefits that can be useful in the management of NAFLD but it's not equivalent to medicine.

**Keywords:** single clove black garlic; PAI-1; lipid profile; NAFLD; cardiovascular disease

## INTRODUCTION

Several mechanisms have been described for the development of atherosclerosis in patients with NAFLD, including genetic predisposition, insulin resistance, and atherogenic dyslipidemia, oxidative stress, chronic inflammation, decreased adiponectin levels, and altered pro- and anticoagulant factor production, which are all present simultaneously **(Francque, van der Graaff and Kwanten, 2016)**. NAFLD, especially in its necro inflammatory form (NASH), can cause atherogenic dyslipidemia **(Kim et al., 2012)**. Also, an increase in procoagulant factors, such as fibrinogen, plasminogen activator inhibitor-1 (PAI-1), and tumor growth factor, all of which increase the risk of atherosclerosis, has been noted **(Alkhouri et al., 2010)**.

To manage NAFLD, lifestyle modifications are strongly suggested, especially dietary arrangements. However, patients usually fail to achieve these changes because they are unable or unwilling to change their dietary habits, they require safe and effective diet management for them. Garlic (*Allium sativum* L.) is a functional food containing an *allyl*-substituted sulfur compound that has been used for a long time both for seasoning and also for prevention and cure of diseases in many cultures **(Rivlin, 2001)**. Preclinical and clinical studies suggest a link between dietary habits and disease occurrence. Previous studies have shown that garlic consumption is inversely correlated to the incidence of hyperlipidemia, atherosclerosis, and thrombosis **(Agarwal, 1996)**. Garlic has many health benefits, but its raw form is not always consumed, because it has a strong odor and taste due to its organosulfur compounds, which makes some people uncomfortable. In recent years, various methods of processing raw garlic have been used, such as fermentation and heating for a long time, which eliminates pungent odors, imparts a sweet taste, and increases the beneficial effects **(Bae et al., 2014)**. Black garlic is processed garlic that is produced through natural aging at a controlled temperature (70 °C) and high humidity (90%) for several days, without other additives **(Toledano-Medina et al., 2019)**. Through

processing, the *S-allyl-L-cysteine* content in black garlic becomes up to five times higher **(Sasaki et al., 2007)**. Also, black garlic had a higher polyphenol content and SOD activity and scavenging activity against hydrogen peroxide were stronger than raw garlic **(Kim et al., 2012; Sato, Kohno and Niwano, 2006)**. The cardiovascular benefits of black garlic administration have been illustrated in various studies. However, so far, few studies have been done on the effect of SCBG administration on PAI-1 levels and lipid profiles in NAFLD. In this study, we evaluated the effective administration of SCBG, simvastatin, and metformin on the hemostasis status and lipid profiles of rats modeled NAFLD induced by a high-fat fructose diet (HFFD) containing 1.25% cholesterol and 0.5% colic acid.

## Scientific hypothesis

We investigate several hypotheses in our study:

1. The administration of SCBG at different doses (450 mg per 200 g BW per day; 900 mg per 200 g BW per day and 1350 mg per 200 g BW per day) for 4 weeks have a significant effect on PAI-1 levels, and lipid profiles of *Sprague Dawley* rats modeled NAFLD compared with simvastatin and metformin treatment groups.
2. There are correlations between PAI-1 and Lipid Profiles of *Sprague Dawley* rats modeled NAFLD.

## MATERIAL AND METHODOLOGY

This study was part of the research project entitled Study of Pre-clinical Benefit of Black Garlic (A*llium sativum Linn*) in Non-alcoholic Fatty Liver Disease (NAFLD).

**Samples**

This study used *Rattus norvegicus*, strain Sprague Dawley rats, weighing 180 – 200 g and aged 8 – 10 weeks old.

**Chemicals**

Plasma PAI-1 levels were determined using Rat PAI-1 ELISA kit, Finetest from Wuhan Fine Biotech Co. Ltd. Lipid profiles were determined using Cholesterol FS (10') 5 x 25 mL/1x 3 mL Standard, Triglycerides FS (10') 5 x 25 mL/1 x 3 mL Standard, HDL Precipitant 250 mL, LDL Precipitant 250 mL from DiaSys, Holzheim, Germany.

**Animals and Biological Material:**

The male Sprague Dawley rats (n = 42) were purchased from the Center of the Food and Nutrition Studies of Gadjah Mada University, Yogyakarta, Indonesia. An acclimatization period lasting seven days was scheduled before the beginning of the experiment. After the acclimatization period, rats were divided into seven groups (n = 6 per group): healthy rats given standard feed ($K_1^+$), NAFLD rats without treatment ($K_2^+$); 0.9 mg simvastatin treatment group ($K_1^-$); 45 mg metformin treatment group ($K_2^-$); single clove black garlic (SCBG) 450 mg per 200 g BW (X1); 900 mg per 200 g BW (X2); and 1350 mg per 200 g BW (X3). All animals, except the $K_1^+$ group, were fed a *high-fat fructose diet* (HFFD) containing cholesterol (1.25%) and cholic acid (0.5%) for 8 weeks.

The standard feed containing 15% crude protein, 3 – 7% crude fat, 12% moisture content, 6% crude fiber, 7% ash, 0.5% phosphorus, and 0.9 – 1.1% calcium, and vitamins. The composition of HFFD as follow was 177.5 $g.kg^{-1}$ pork oil, 172.8 $g.kg^{-1}$ fructose, 19.87 $kJ.g^{-1}$ energy, 20% protein, 35% carbohydrates, and 45% fat.

Black garlic used in this study was produced from the Biotechnology Agency for Technology Assessment and Application (BPPT), Tangerang, Indonesia, with the commercial name of Natural Black Garlic® Lanang, which has passed the test of acute toxicity by the Laboratory Toxicity and Safety Materials, School of Pharmacy Bandung Institute of Technology, Indonesia. The main raw material for Natural Black Garlic® Lanang is single clove garlic. It's processed garlic that is produced through natural aging at a controlled temperature (70 °C) and high humidity (90%) for 26 days without other additives (Figure 1).

**Instruments**

iMark™ microplate absorbance reader, Bio-Rad. Microprocessor Controlled Spectrophotometer, *OPTIMA SP-300*.

**Laboratory Methods**

PAI-1 levels were measured using the Enzyme-Linked Immunosorbent Assay (ELISA) method **(Crowther, 2009)**.

Total cholesterol and HDL-C levels were analyzed by Cholesterol Oxidase- Phenol Amino Phenazone (CHOD-PAP) and triglycerides were analyzed by Glycerol Phosphate Oxidase-Phenol Amino Phenazone (GPO-PAP) **(McPherson and Pincus, 2016)**. LDL-C levels were calculated with the Friedewald formula.

**Description of the Experiment**

Simvastatin, Metformin and Single Clove Black Garlic preparation as follows was 0.9 mg simvastatin and 45 mg metformin per 200g BW as comparative groups and SCBG dose 450 mg, 900 mg, and 1350 mg per 200 g BW, respectively, were mashed and diluted together with 3 mL of aqua bidestilata **(Hatton et al., 2015)**. All samples were administered by oral gavage **(Atcha et al., 2010)**.

**Sample preparation**: After 4 weeks of administration, all animals overnight fasted. Blood was collected from the *sinus orbital* **(Wolforth, 2000)** and centrifuged at 1,000 rpm for 15 minutes at 2 – 8 °C, then plasma was collected and stored at -80 °C for later analysis.

**Number of samples analyzed:** forty-two.

**Number of repeated analyses:** duplo

**Number of experiment replication:** There is no experiment replication in our study.

## Statistical Analysis

Bodyweight gain was recorded weekly, while examination data of PAI-1 levels and plasma lipid profiles were taken *post-test.* All data were analyzed statistically using IBM SPSS Statistics for Windows version 22.0. Shapiro-Wilk test was used to test the normality of data. The analyses used were paired *t*-test, one-way ANOVA, *post- hoc* LSD, Kruskal-Wallis, and Mann-Whitney. For the correlations between PAI-1 levels and lipid profiles used correlation Spearman test.

## RESULTS AND DISCUSSION

In this study, the HFFD containing cholesterol (1.25%) and cholic acid (0.5%) – supplied rats showed marked obesity (increases of body weight), hyperlipemia (increases of the plasma total cholesterol, triglyceride, and LDL-C levels with decreases of the HDL-C levels) and hemostasis status (increases of PAI-1 level). Overall, obesity, diabetes, and hyperlipemia were induced by 8 weeks of continuous HFFD supply, which is consistent with the findings of previous studies **(Abe et al., 2019; Jarukamjorn et al., 2016; Oligschlaeger and Shiri-Sverdlov, 2020)**. Administration of HFFD containing cholesterol (1.25%) and cholic acid (0.5%) aim to induced fatty liver in experimental animals. HFFD content is considered the same as a fast-food diet which contains high fat, high fructose, and high cholesterol (i.e. 2% w/w). Free fatty acids are known to trigger mitochondrial dysfunction, oxidative stress, and hepatic stellate cell activation. Fructose is known to be a major activator of lipotoxicity factors in the liver, leading to the development of NAFLD. Fructose consumption was found to be higher in patients with NAFLD compared to controls of the same age, and increased fructose consumption has been associated with the severity of fibrosis in patients with NAFLD **(Abe et al., 2019)**. Free cholesterol directly activates liver stellate cells, besides that high cholesterol in the composition of the fast-food diet also promotes the development of nonalcoholic steatohepatitis (NASH) **(Tomita et al., 2014)**. Thus, we conclude that excess amounts of fat, fructose, and cholesterol in HFFD exacerbate the lipotoxic environment and accelerate the progression of steatohepatitis and fibrosis in obese Sprague Dawley rats.

### Administration of HFFD against weight

After administration of HFFD containing cholesterol (1.25%) and cholic acid (0.5%) for 8 weeks, generally, the six groups of rats experienced an increase in body weight as well as the healthy group that was given standard feed. Weighing the rats was performed weekly starting from the acclimatization until the end administration of HFFD. Weighing the mice after acclimatization aimed to ensure that the experimental animal still within the inclusion criteria, namely, having a bodyweight of 150 – 200 g. Table 1 shows the changes in body weight of rats before and after conditioning. These changes probably due to the HFFD administration for 8 weeks during conditioning. HFFD containing high-fat (45%) and high-fructose (172.8 $g.kg^{-1}$ diet), contributing a lot of energy (19.87 $kJ.g^{-1}$) **(Jung et al., 2011)**. Fat also contains high energy compared to carbohydrates and protein. 1 g of fat is equivalent to 9 kcal of energy, while 1 g of protein and carbohydrates each is equivalent to 4 kcal of energy **(Hardinsyah and Supariasa, 2019)**.

In this study, the body weight of the animals significantly increased after HFFD administration in six group treatments ($p = 0.001$). except for the healthy group, which had an increase but not significant. The X2 group does not have significant weight differences before and after conditioning. In this study, the administration of HFFD attempted to apply the diet composition of NAFLD patients. The difference in food intake before and after HFFD administration between groups was related to the differences in the energy composition of the diets of different rats. The dietary intake of 15 g per rat per day was selected according to the nutritional needs of laboratory animals, which is considered the minimum amount needed by rats per day to ensure normal growth of them **(Fakhoury-Sayegh et al., 2015)**.

### Effect of single clove black garlic on body weight

In this study, the increases of weights were meaningfully and dose-dependently inhibited by treatment of SCBG and also by simvastatin and metformin, respectively. The X2 and X3 groups experienced a change in obesity status after the SCBG administration. The SCBG group with doses of 900 mg and 1350 mg per 200 g BW per day had a preventive effect on obesity, which had no statistical difference from the healthy group. Meanwhile, the group of SCBG 450 mg per 200 g BW per day only had a slightly reduced Lee index, so they were unable to change the obesity status of the rats to normal (Table 2).

HFFD feeding in this study aimed to induce a fatty liver in rats. The excess consumption of carbohydrates (fructose and sucrose) and fat (fatty acids and cholesterol) plays a key role in the development and advancement of obesity-related NAFLD through the activation of the lipid metabolism pathway modulated on a high-fat diet **(Jarukamjorn et al., 2016)**.

The obesity status of the X2 and X3 groups had changed after the SCBG administration. The treatment group of SCBG 900 mg per 200 g BW per day and 1350 mg per 200 g BW per day provided a preventive effect of obesity and had no statistical difference from the healthy group. Meanwhile, the group of rats given SCBG 450 mg per 200 g BW did not reduce the Lee index. Garlic is a rich source of polyphenol compounds and is highly potent in inhibiting lipid peroxidation. Polyphenols have the potential to prevent obesity by inhibiting enzymes related to lipid metabolism, for example, pancreatic lipase, lipoprotein lipase, and glycerophosphate dehydrogenase, or by regulating lipid homeostasis, for example, by regulating lipogenesis and fatty acid oxidation **(Chen et al., 2014)**. In conclusion that black garlic has an antiobesity effect **(Chang, Wu and Hsu, 2015; Chang et al., 2017; Chen et al., 2014; Lee et al., 2011; Kim et al., 2011)**.

### PAI-1 levels

PAI-1 level was measured once at the end of the study. The *post-hoc* LSD test showed significant differences in the PAI-1 levels in all groups of rats ($p = 0.001$). The PAI-1 levels were meaningfully and dose-dependently decrease by treatment of SCBG and also by simvastatin and metformin, respectively. It compared to the NAFLD group, without SCBG treated. However, when compared with the healthy group, the PAI-1 levels in the three treatment groups had not yet reached their PAI-1 levels (Table 3).

**Table 1** Effect of single clove black garlic on body weight of rats modeled NAFLD.

| Groups | Rats weight (g) | | | | | *P* |
|---|---|---|---|---|---|---|
| | Acclimatization | Conditioning | Administration | Δ1 | Δ2 | |
| **Controls** | | | | | | |
| Healthy Rats | 163.33 ±3.55 | 208.83 ±3.60$^{a}$ | 238.16 ±4.35$^{a}$ | 45.50 ±1.22$^{a}$ | 29.33 ±2.25 | 0.005 |
| NAFLD Rats | 164.83 ±2.56 | 242.50 ±2.26$^{a}$ | 305.16 ±2.56$^{a}$ | 77.67 ±0.81$^{a}$ | 62.66 ±1.63$^{a}$ | 0.004 |
| **Reference** | | | | | | |
| Simvastatin | 164.00 ±3.84 | 241.50 ±3.73$^{a}$ | 271.33 ±3.38$^{ab}$ | 77.50 ±1.04$^{a}$ | 31.70 ±0.98$^{b}$ | 0.002 |
| Metformin | 163.33 ±2.80 | 240.16 ±3.43$^{a}$ | 271.30 ±3.40$^{bc}$ | 76.83 ±0.98$^{a}$ | 31.16 ±1.98$^{b}$ | 0.001 |
| **SCBG treated** | | | | | | |
| X1 | 166.33 ±4.13 | 244.00 ±4.43$^{a}$ | 284.33 ±4.63$^{abcd}$ | 77.66 ±1.03$^{a}$ | 40.33 ±1.81$^{ac}$ | 0.001 |
| X2 | 165.17 ±2,32 | 242.33 ±2.58$^{a}$ | 272.66 ±3.14$^{abe}$ | 77.16 ±1.60$^{a}$ | 30.33 ±2.73$^{ab}$ | 0.061 |
| X3 | 163.00 ±2.83 | 240.33 ±2.42$^{a}$ | 270.66 ±3.66$^{abe}$ | 77.33 ±0.81$^{a}$ | 30.33 ±1.50$^{be}$ | 0.002 |
| ***p**** | **0.538** | **0.001** | **0.005**$^{*}$ | **0.001**$^{*}$ | **0.001**$^{*}$ | |

Note: Values are expressed as mean ± SD of six rats. Healthy, normal control group; NAFLD, Nonalcoholic fatty liver disease control group without treatment; X1, SCBG 450 mg per 200 g BW; X2, 900 mg per 200 g BW; X3, 1350 mg per 200 g BW. Simvastatin and metformin were administrated at dose levels of 0.9 mg and 45 mg per 200 g BW, respectively. ***p**** = paired t-test; ***p*** = one-way ANOVA test ; $^{abcdef}$ – different notation shows a significant difference with post-hoc LSD test.

**Table 2** Effect of single clove black garlic on obesity status of rats modeled NAFLD.

| Groups | Obesity Status (Lee index) | | | *p* |
|---|---|---|---|---|
| | Conditioning | Administration | Δ | |
| **Controls** | | | | |
| Healthy Rats | 286.32 ±2.82$^{a}$ | 287.82 ±3.40 | 1.50 ±1.55 | 0.028 |
| NAFLD Rats | 319.30 ±5.73$^{a}$ | 332.52 ±3.21$^{a}$ | 13.22 ±6.75$^{a}$ | 0.028 |
| **Reference** | | | | |
| Simvastatin | 323.80 ±5.62 | 290.55 ±3.27$^{b}$ | -33.24 ±2.51$^{a}$ | 0.028 |
| Metformin | 320.19 ±7.86$^{a}$ | 287.34 ±7.12$^{b}$ | -32.85 ±2.69$^{ab}$ | 0.028 |
| **SCBG treated** | | | | |
| X1 | 321.14 ±6.72$^{a}$ | 302.76 ±4.90$^{abcd}$ | -18.38 ±3.68$^{abcd}$ | 0.028 |
| X2 | 318.98 ±4.13$^{a}$ | 286.93 ±3.37$^{be}$ | -32.04 ±1.04$^{ab}$ | 0.028 |
| X3 | 318.67 ±8.94$^{a}$ | 286.93 ±3.37$^{be}$ | -34.21 ±4.11$^{abe}$ | 0.028 |
| ***p**** | **0.009*** | **0.001*** | **0.001*** | |

Note: Values are expressed as mean ± SD of six rats. Healthy, normal control group; NAFLD, Nonalcoholic fatty liver disease control group without treatment; X1, SCBG 450 mg per 200 g BW; X2, 900 mg per 200 g BW; X3, 1350 mg per 200 g BW. Simvastatin and metformin were administrated at dose levels of 0.9 mg and 45 mg per 200 g BW, respectively. ***p*** = wilcoxon test ; ***p**** = Kruskal-Wallis test; $^{abcdef}$ – different notation shows a significant difference with Mann-Whitney test.

**Table 3** Effect of single clove black garlic on the plasma biochemical parameters of rats modeled NAFLD.

| Groups | Biochemical parameters | | | | |
|---|---|---|---|---|---|
| | Hemostatis status (ng.ml$^{-1}$) | Lipid profile (mg.dL$^{-1}$) | | | |
| | PAI-1 | TC | HDL-C | LDL-C | TG |
| **Controls** | | | | | |
| Healthy Rats | 1.03 ±1.11 | 96.17 ±2.94 | 80.73 ±1.97 | 25.87 ±2.30 | 74.56 ±2.94 |
| NAFLD Rats | 30.86 ±1.26$^{a}$ | 219.93 ±4.80$^{a}$ | 24.47 ±2.32$^{a}$ | 81.83 ±2.17$^{a}$ | 142.44 ±4.75$^{a}$ |
| **Reference** | | | | | |
| Simvastatin | 4.02 ±3.32$^{ab}$ | 117.47 ±3.90$^{ab}$ | 64.54 ±2.98$^{ab}$ | 41.16 ±1.88$^{ab}$ | 95.45 ±2.12$^{ab}$ |
| Metformin | 2.98 ±1.68$^{abc}$ | 114.94 ±4.57$^{ab}$ | 66.78 ±3.09$^{ab}$ | 34.54 ±2.06$^{ab}$ | 94.78 ±2.92$^{ab}$ |
| **SCBG treated** | | | | | |
| X1 | 6.84 ±0.22$^{abcd}$ | 171.01 ±4.50$^{abcd}$ | 36.05 ±3.43$^{abcd}$ | 65.78 ±3.57$^{abcd}$ | 118.21 ±2.66$^{abcd}$ |
| X2 | 5.93 ±0.29$^{abcde}$ | 133.33 ±5.63$^{abcde}$ | 53.19 ±4.33$^{abcde}$ | 40.79 ±1.75$^{abde}$ | 106.56 ±3.32$^{abcde}$ |
| X3 | 4.86 ±0.24$^{abdef}$ | 121.07 ±2.78$^{abef}$ | 61.35 ±2.32$^{abdef}$ | 33.21 ±2.82$^{abcef}$ | 98.39 ±1.95$^{abef}$ |
| ***p*** | **0.001**$^{*}$ | **0.001** | **0.001** | **0.001** | **0.001** |

Note: Values are expressed as mean ± SD of six rats. Healthy, normal control group; NAFLD, Nonalcoholic fatty liver disease control group without treatment; X1, SCBG 450 mg per 200 g BW; X2, 900 mg per 200 g BW; X3, 1350 mg per 200 g BW. Simvastatin and metformin were administrated at dose levels of 0.9 mg and 45 mg per 200 g BW, respectively. ***p**** = One way ANOVA test; ***p*** = Kruskal-Wallis test; $^{abcdef}$ – different notation shows a significant difference with Mann-Whitney test.

Significant differences were noted in PAI-1 levels of all groups of rats ($p$ = 0.001). The PAI-1 levels in the healthy group were significantly different from those in the NAFLD, simvastatin, metformin, X1, X2, and X3 groups. The SCBG dose of 1350 mg per 200 g BW more effectively improved the hemostasis status in NAFLD rats than 450 and 900 mg per 200 g BW respectively.

The consumption of HFFD appears to have contributed to NAFLD development and increased the risk of progression to NASH **(Jarukamjorn et al., 2016)**, promote an increase in hemostasis status, namely, PAI-1 level. In this study, X1, X2, and X3 groups had lower levels of PAI-1 than the NAFLD group (HFFD without SCBG administration). In this study, the biological substances of garlic and its processes, which are involved in the antithrombotic function, are unknown. A*llicin*, one of the main components in garlic, is known to have some biological activity, but it does not seem to promote a t-PA activity or reduce PAI-1 levels. Black garlic contains less *allicin* in the production process due to *alliinase* inactivation, so it is odorless. However, whether the *alliin* in the diet was converted to *allicin* after administered in rats and whether SCBG in our study had a specific antithrombotic activity are uncertain. As is known, garlic contains many physiological functions; the composition and amount of each element are responsible for various functions, which may differ between the types and methods of manufacture. It should be noted that the physiological function of garlic is not homogeneous and depends on the number of active components and their combinations in garlic processing **(Fukao et al., 2007)**. Therefore, which components contribute to the antithrombotic activity in NAFLD rats is uncertain. In short, garlic greatly reduces the coagulation activity, which would prevent the formation of blood clots in the thrombus-modeled pathological rats. These results may explain the benefit of garlic and its preparations for blood and blood vessels.

In vitro and in vivo studies have shown that garlic appears to have antiplatelet, fibrinolytic, and antithrombotic effects, in addition to its anti-inflammatory properties **(Chang et al., 2005; Teranishi et al., 2003)**. Garlic also reduced PAI-1 levels in obese subjects compared to the placebo group **(Szulińska et al., 2018)**. Another study showed that administration of Methanolic Extract of Black Garlic (MEBG) reduced levels of PAI-1 ($p$ <0.05) in obese rats fed a high-fat diet **(Chen et al., 2014)**. Regarding the molecular regulation of decreased levels of PAI-1 and inhibition of adipogenesis in obese mice by black garlic supplementation through increased AMPK, forkhead box protein-01(FOXO1), Sirtuin-1(Sirt1), adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), perilipin, acyl-CoA-1 (ACO), carnitine palmitoyltransferase (CPT-1), and uncoupling protein-1(UCP1), thereby reducing lipogenesis, increasing lipolysis and fatty acid oxidation. The expression of cluster of differentiation-36 (CD36) levels decreased, resulting in less free fatty acid uptake in the adipose tissue. Also, black garlic increases adiponectin and downregulates PAI-1, resistin, tumor necrosis factor- α (TNF-α), and glucose transporter type-4 (GLUT4), thereby increasing insulin resistance in rats fed a high-fat diet **(Chen et al., 2014)**. Similarly, the downregulates PAI-1 mechanism of simvastatin and metformin **(Arruda de Faria et al., 2019; Mazza et al., 2012; Nascimbeni et al., 2016; Rodrigues, et al., 2019)**.

## Lipid profiles

In this study, the mean of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and triglycerides (TG) levels for all groups were significantly different ($p$ = 0.001). After administration SCBG, the X1, X2, and X3 groups have a significant decrease in triglyceride levels ($p$ = 0.001) compared to the NAFLD group (Table 3).

Consumption of high-fat diet results in the production of acetyl-CoA and malonyl CoA in the liver. Increased levels of malonyl CoA, as fuel for TG synthesis, will inhibit the expression of CPT-1, a key enzyme for lipolysis, thereby reducing fatty acid oxidation. As shown in this study, administration of HFFD and black garlic increases CPT-1 mRNA expression; thus, more TG is broken down (degradation) to supply free fatty acids as an energy source, lowering plasma cholesterol concentrations associated with decreased HMG-CoA reductase expression and ACAT. SREBP-1c levels in the liver also activate the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase and acyl-CoA cholesterol acyltransferase (ACAT) enzymes, which regulate cholesterol synthesis and catalyze cholesterol esterification **(Ha, Ying and Kim, 2015)**.

Calorie intake from fat and fructose promotes excess fat deposits in the adipose tissue. Excessive intake of fat from food will lead to an increase in lipogenesis activity and production of free fatty acids, resulting in the mobilization of free fatty acids from the fatty tissue to the liver and binding glycerol to form triglycerides. Giving a high-fat diet also increased levels of LDL and cholesterol in the circulation, triggering HDL to transport cholesterol from the liver to the circulation (*reverse cholesterol transport*). HDL is esterified into cholesterol esters, which can be taken directly to the liver then directly excreted or exchanged with triglycerides from VLDL and chylomicrons. When cholesterol ester excess, HDL-rich triglyceride (HDL low density) is breakdown by hepatic lipase resulting in lower circulating levels of HDL **(Dissard et al., 2013; Wong et al., 2016)**.

The results showed that the mean of total cholesterol, HDL, LDL, and triglycerides levels for all groups were significantly different ($p$ = 0.001). After SCBG administration, the X1, X2, and X3 groups had lower triglyceride levels ($p$ = 0.001) compared to the NAFLD group. These results supported the study of **Seo et al. (2009)** on db/db model rats given black garlic had lower triglycerides, cholesterol, fasting blood glucose, blood glycated hemoglobin levels, and higher HDL serum compared with rats with no black garlic intervention.

The lipid-lowering mechanism of black garlic in plasma and the liver of rats is described in Ha et al.'s study.
They have measured the expression of liver SREBP-1c mRNA transcription factors' key lipid metabolism and the decreased expression of SREBP-1c in rats fed extract black garlic, resulting in a decrease in liver sterol regulator element-binding protein-1c (SREBP-1c), Acetyl-CoA

**Table 4** Correlations between PAI-1 levels and lipid profiles.

| Lipid profiles | PAI-1 levels | |
|---|---|---|
| | *r* | *p* |
| **Total cholesterol** | 0.961 | 0.005 |
| **HDL** | -0.968 | 0.001 |
| **LDL** | 0.871 | 0.006 |
| **Triglycerides** | 0.961 | 0.004 |

Note: r = Spearman correlations.




**Figure 1** Natural Black Garlic® Lanang Premium.

carboxylase (ACC), fatty acid synthase (FAS), and G-6PDH mRNA levels, the target gene for SREBP-1c, indicating that the inhibition of the gene expression led to a decrease in the fat synthesis of the liver.

This is associated with a plasma TG concentration decrease. Similar results were reported: the methanolic extract Black Garlic (MEBG) administered in rats fed with a high-cholesterol diet inhibited lipid accumulation through *adenosine monophosphate-activated protein kinase* (AMPK) regulation and regulation of SREBP1c, ACC, and FAS **(Ha, Ying and Kim, 2015; Sobenin et al., 2019)**. Similarly, the lipid-lowering mechanism of simvastatin and metformin **(Arruda de Faria et al., 2019; Mazza et al., 2012; Nascimbeni et al., 2016; Rodrigues et al., 2019; Sobenin et al., 2019)**.

Black garlic and its constituent, *S-allyl-cysteine*, had antioxidant and hypolipidemic effects in rats with a high-fat diet **(Asdaq, 2015)**. Therefore, the high concentration of *S-allyl-cysteine* in black garlic extract induced blood lipid profile changes in *hepatic injury* rats **(Tran, Dam and Tram Le, 2018)**. These data support the results of this study in verifying the effect of single clove black garlic improving the lipid profile in NAFLD rats.

## Correlations between PAI-1 levels and lipid profiles

There were strong correlations between PAI-1 levels and lipid profiles, with *r* value = 0.76–0.99. The correlations were positive except for HDL-C levels ($p$ = 0.001; $r$ = -0.968), which means when HDL-C levels decrease, then the PAI-1 levels increase (Table 4).

Decreased plasma HDL cholesterol is the most common dyslipidemia in obese subjects and this condition is caused by hypertriglyceridemia, even if the levels are low or without elevated triglycerides. Hypertriglyceridemia is accompanied by a small increase in solid LDL, is highly atherogenic. The high concentration of non-esterified fatty acid (NEFA) released will be metabolized by the liver to increase the synthesis of liver triglycerides and very-low-density lipoprotein (VLDL), thereby triggering insulin resistance. High circulating levels of VLDL reduce HDL concentrations and increase small solid LDL. Dyslipidemia is also accompanied by hyperactive platelets, hypercoagulability with increased factor VII, and hypofibrinolysis with increased PAI-1. High plasma triglyceride levels increase oxidative stress by increasing superoxide production. Increased NEFA from adipocytes increases tissue factor (TF) and PAI-1 levels and increases platelet aggregation; this condition causes thrombosis. Lipoprotein (a) levels in obesity are increased, especially when associated with hyperglycemia. Lipoprotein (a) has LDL-like properties and is structurally similar to plasminogen. It, therefore, inhibits the binding of plasminogen to endothelial cells and interferes with the generation of plasmin, both of which are atherogenic and thrombogenic. Prothrombin levels and levels of vitamin K-dependent coagulation factor are increased with hyperlipidemia **(Darvall et al., 2007)**.

Significant correlations between PAI-1 levels and lipid profiles were noted in this study. Similarly, where a significant positive correlation was seen between PAI-1 and triglyceride levels; on the other hand, PAI-1 and

HDL-C levels showed a negative correlation in nondiabetic obese patients and the control group **(Festa et al., 1999; Somodi et al., 2018)**. Besides, another study showed a strong and significant positive correlation between the most atherogenic LDL subfraction and the densest concentration of the PAI-1 plasma levels was also noted. This is the initial finding to evaluate the correlation between the *small dense* LDL and PAI-1 plasma levels in obese nondiabetic patients **(Somodi et al., 2018; Väisänen et al., 1997)**. These data support the association between LDL-C and PAI-1 levels in accelerating atherogenesis and increasing the cardiovascular risk of obese patients; however, our hypothesis needs to be verified by further investigation. However, the correlation between HDL and plasma PAI-1 levels are not well understood. Also, there have been few studies on the correlations of PAI-1 with the functional and structural properties of different lipoprotein fractions especially on rats modeled NAFLD.

We were not able to evaluate the long-term effects of SCBG administration on the hemostasis status and lipid profiles in rats modeled NAFLD, as our intervention lasted 4 weeks. Given these limitations and our findings, we believe that additional studies with longer intervention periods are warranted to determine the long-term benefits of SCBG consumption on the hemostasis status and lipid profiles in rats modeled NAFLD. However, further studies are needed to elucidate the underlying mechanisms.

## CONCLUSION

The administration of Single Clove Black Garlic 450 mg; 900 mg, and 1350 mg per 200 gram BW for 4 weeks, respectively, affected the changes in body weight, PAI-1 levels, and lipid profile, compared to the healthy and NAFLD rats. The 1350 mg per 200 g BB of Single Clove Black Garlic is an effective dose that reduced PAI-1 levels and lipid profiles, which are equivalent to 15 grams per day when consumed by humans. More studies are needed in humans if Single Clove Black Garlic will be used as a food supplement in NAFLD.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

The use of animals in this research was approved by The Ethical Committee of Medical Research (KEPK), Faculty of Medicine, Diponegoro University, Indonesia (No. 11/EC/H/FK.UNDIP/III/2020, Date of approval: March 30th, 2020).

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# HYGIENE MEASURES IN SUPERMARKETS, RETAIL FOOD STORES, AND GROCERY SHOPS DURING THE COVID-19 PANDEMIC IN SLOVAKIA

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## ABSTRACT

The article presents the hygiene measures that are applied in the Slovak Republic in supermarkets, food stores, grocery stores as a result of the COVID-19 pandemic situation in Slovakia. These measures have been published by the Public Health Authority of the Slovak Republic in the relevant legal regulations and are based on the decisions of the Government of the Slovak Republic, which took into account the opinions of the experts of the Pandemic Commission of the Government of the Slovak Republic. In general, these measures are based on the mask-distance-hand principle. In public areas outside and inside, it was ordered to wear masks and later wear a respirator of FFP2 class in exterior and interior, gloves on hands or disinfection of customers' hands before entering the store, observance of 2 m distance of people standing in a row at the cash registers, maximum capacity of persons in stores was determined one person per 25 $m^2$ of sales area and later, this measure was tightened to 15 $m^2$ of sales area. Also, to perform regular ventilation of the premises and to have as many cash registers as possible so as not to create long lines of customers. All shops were closed at 8:00 PM. In the case of shopping centers, entry is prohibited for people with a body temperature higher than 37 °C, and disinfection of hands is mandatory, wearing a mask and later wear respirators of FFP2 class is necessary. In a stricter regime, during the peak of the pandemic, there was a restriction for persons to shop food only in the nearest retail/grocery or similar place from the place of residence to the extent necessary to procure the essential needs of life.

**Keywords:** hygiene measure; COVID-19; Slovakia; food supermarket; food store; grocery store; grocery shop; grocery

## INTRODUCTION

Every food business operator in European Union is required to follow the legal requirements for food safety. Supermarkets, grocery stores, and other businesses in the food retail sector are no exception in Slovakia. These legal requirements are given by EU General Food Law **(EU, 2002)**, EU Food Hygiene Low **(EU, 2004)** and the Slovak Food Law **(NR SR, 1995)** including several decrees based on this law, in which the sale of meat and meat products **(MPRV SR, 2012a)**, bakery products **(MPRV SR, 2014)**, perishable food **(MPRV SR, 2012b)** is described. The general principles of food safety require every business operator along the food chain to ensure that the safety of food is proactively managed and maintained.

During the COVID-19 pandemic, other relevant legal requirements were adopted by the Government of the Slovak Republic, Ministry of Health of the Slovak Republic, and The Public Health Authority of the Slovak Republic (PHA SR).

The PHA SR is a budgetary organization of the state with competence on the territory of the Slovak Republic with the registered office in Bratislava, linked with the financial relations to the budget of the Ministry of Health. The PHA SR is managed and its activity falls under the responsibility of the Chief Hygienist of the Slovak Republic Ján Mikas, PhDr., RNDr., MD, PhD. who is also the director of the office. The chief hygienist is appointed and dismissed upon the proposal of the Minister of Health of the Slovak Republic by the director of the office of the ministry. The Public Health Authority of the Slovak Republic is the supreme office for the regional public health authorities. It manages, controls, and coordinates the execution of state administration carried out by regional public health offices. More details on competencies and activities of the Authority in the field of protection, promotion, and development of public health are stated in the Act number 355/2007 Collection of Laws of the Slovak Republic (Act on the Protection, Promotion, and Development of Public Health and on Amendments to Certain Acts).

The development of the pandemic situation in Slovakia is presented in Figure 1 (Confirmed cases) and Figure 2 (Deaths). The Government of the Slovak Republic has set up a specialized website www.korona.gov.sk, where the public can find out basic information about the pandemic situation, including preventive hygiene measures, restrictions, and exceptions.

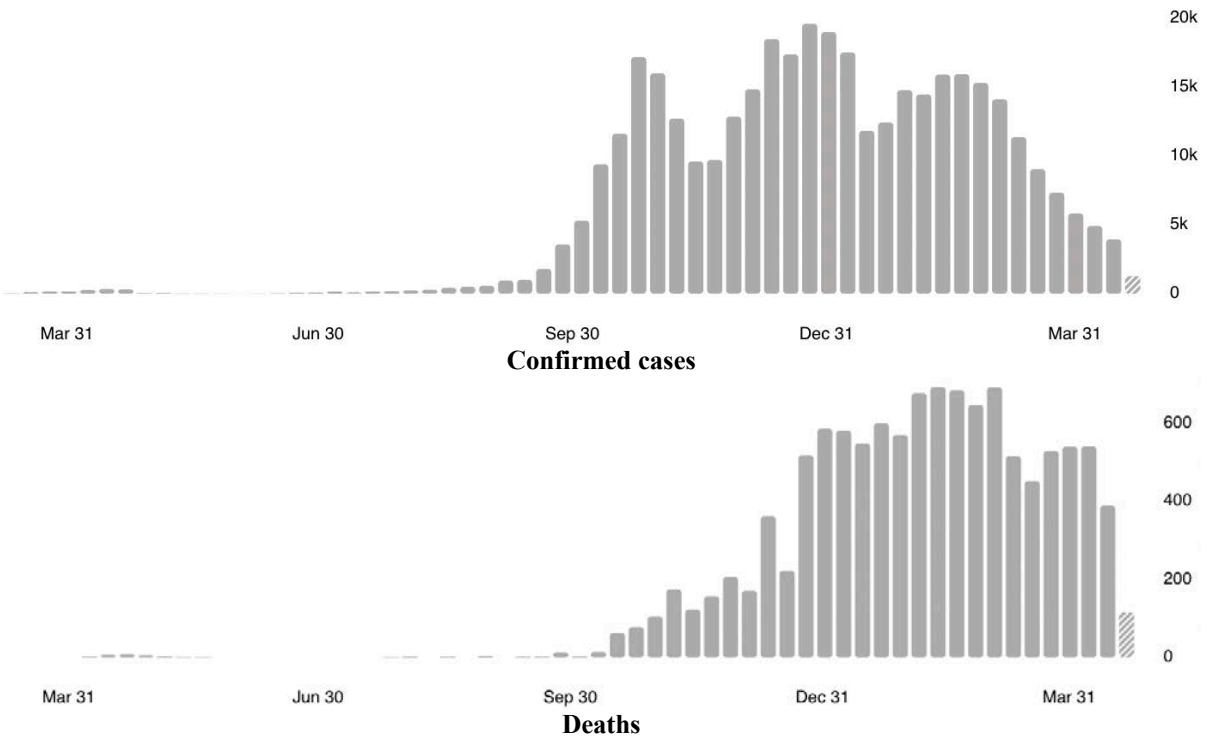



**Figure 1** Development of the epidemic situation in Slovakia 6.3. 2020. – 26.4.2021 (WHO, 2021).
Note: In Slovakia, from 3 January 2020 to 5:14pm CEST, 28 April 2021, there have been 381,180 confirmed cases of COVID-19 with 11,611 deaths, reported to WHO. As of 18 April 2021, a total of 1,310,241 vaccine doses have been administered.

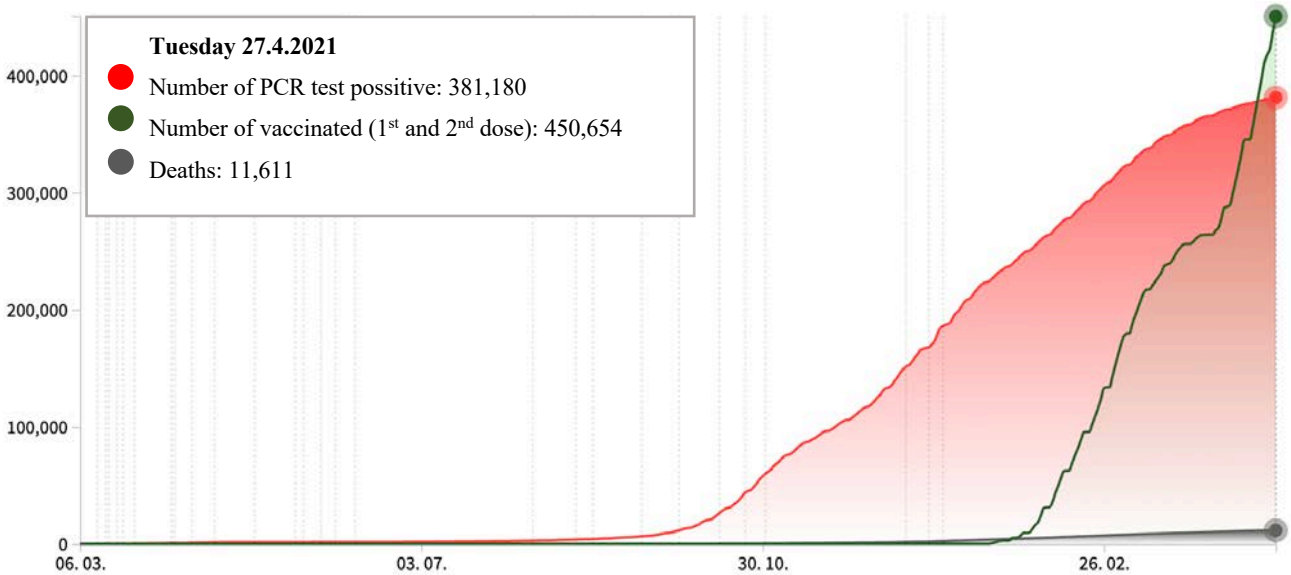



**Figure 2** Development of the epidemic situation in Slovakia 6.3.2020. – 27.4.2021 (MIRDI SR, 2021a).

**Covid Automat**

Covid Automat is the monitoring and signaling system of the Ministry of Health of the Slovak Republic. This system is providing early warning of uncontrolled spread of the disease so that the health system can take action the necessary preventive measures and the uncontrolled spread of the disease in advance, as well as during the ongoing critical situation, to contribute to its stabilization and gradual improvement. It enables the predictable set of measures – balancing economic activity and public health. This system aims to provide measures that are: simple, comprehensible, predictable, targeted, enforceable, secure, and consistent with the legislation. For more efficient and sustainable management of the pandemic, it is necessary to worsening of the epidemiological situation and preventing its deterioration by monitoring the cohort epidemiological criteria, the key ones being:

- number of cases (average number of cases in 7 days) = future workload,
- increase in the number of hospitalizations = current burden,
- Rt = dynamics.

The basis for quality and effective measures in pandemic management is compliance with 4 basic criteria that need to be taken into account when recruiting departmental traffic lights: Security, Insulation, Surveillance, and Protection **(MH SR, 2021)**. The covid automat shoving the epidemiological situation on the map (Figure 3).

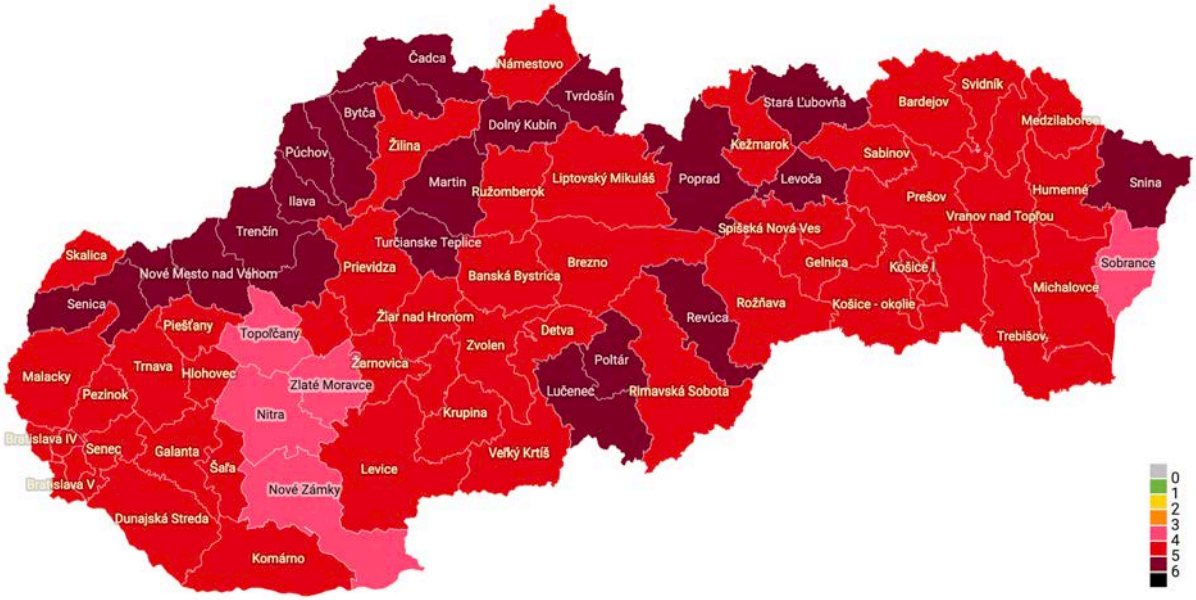



**Figure 3** Actual regional Covid Automat 26.4.2021 – 2.5.2021 (MIRDI SR, 2021b).

UNI Global Union has developed guidelines to protect food retailers from COVID-19 exposure. The recommendations were developed following a collection of best practices from UNI's affiliated trade unions around the world. They are designed to be implemented for all supermarket staff, despite different conditions in different regions. UNI encourages its sales offices to use guidelines when dealing with employers to ensure the protection and rights they deserve from food retailers. Christy Hoffman, UNI Secretary-General, said: "I was concerned and deeply saddened to see a growing number of grocery store workers who were infected with COVID-19 while bravely serving the public, despite the risk to their health and the health of their families." Too many have already died. It doesn't have to be that way. Supermarket workers and customers must be provided with the highest level of protection – as described in these guidelines." "These measures will not only ensure better safety for workers but will also help ensure the safety of supermarkets for all in the face of COVID-19," says Mathias Bolton, UNI store manager. "Healthy workers and safe shops are essential because supermarkets are the most visited places during this pandemic." Without proper safety and protection in shops, the risk of infection increases. That is why we say: protect workers, protect the public." UNI has launched a declaration obliging employers to work with uni and its affiliated departments to share and implement the highest hygiene and safety standards in stores across their operations. It has already been signed by the multinational retailer Carrefour, Auchan, and El Corte Inglés **(UNI Global Union, 2020)**.

Despite COVID-19 is a respiratory illness with a mode of invasion through the respiratory tract, not the gastrointestinal tract, an average food consumer is anxious and concerned about food safety **(Pressman, Satyanarayan and Roger, 2020)**.

Governments must take appropriate measures to prevent the spread of the disease. Examples of measures taken by governments include such as enforcing the proper lockdown among the people; ensure food security by providing the citizens with basic necessities or letting them buy food in the store from the retailers; enforcing and monitoring law and order during the whole pandemic and especially during lockdown period in public places, public transportation, markets, shop, and medical facilities; and identification and treatment of people suffering from coronavirus attack **(Hassen, El Bilali and Allahyari, 2020; Jain, Puri and Jain, 2021)**.

In addition to these measures, the government and the competent authorities should provide an information campaign for the population where consistently, simply, briefly explain to the population what is allowed and what is forbidden, what measures they must follow. Moreover, measures adopted by the competent authorities are then communicated by food retailers to customers at their premises. Individual retailers choose different ways of informing consumers. They should use various pictograms, posters, flyers, stickers, information banners, tv banners, websites, etc. to inform buyers.

The Public Health Authority of the Slovak Republic issued hygiene measures in the form of decrees and published them in the collection of laws of the Slovak Republic. The Government of the Slovak Republic and the Public Health Authority of the Slovak Republic regularly informed citizens and media at press conferences and in the national media Slovak Radio and Television. A special campaign was created, which was broadcast by the media at the time of the commercial broadcast between television programs and radio programs. Basic pictograms were published by the PHA SR to show the principle of Mask – Distance – Hands (Figure 4, a). On the other hand, the legislation did not make these pictograms mandatory. Supermarkets, retail food stores, and grocery shops start to improvise and invent information materials, pictograms especially in their corporate colors, and communicated them to the public (Figure 4, b, c, d). Size, contrast, resolution, limitations of people with color or visual impairments were unlikely to be taken into account. The blue color mostly indicating the recommendation and the red color signaling the restriction were not used consistently. Also, various corporate colors were used, yellow (Figure 7) green (Figure 12).




a) Note: Mask, distance, hands.




b) Note: Wear a mask, keep a distance, wear gloves or disinfectant.




c) Note: Safety measures COVID-19. Keep a distance of 2 m, put on your mask, disinfect your hands.




d) Note: Entry only with FFP2 respirator. Precautions COVID-19.

**Figure 4** Example of pictograms with information on measures to ensure protection against COVID-19 disease.

**Scientific Hypothesis**

Customers perceive anti-epidemiological measures applied in food stores as sufficient.

## MATERIAL AND METHODOLOGY

**Samples**

Our sample consisted of respondents from all over Slovakia. The structure of the respondents was:

- Total number of respondents: 1,000
- Man: 28%
- Woman: 72%

The age categories:

- 18 – 30: 31%
- 31 – 40: 26%
- 41 – 50: 18%
- 51 – 64: 15%
- 65 and >: 10%

Education:

- Basic (primary-school): 0%
- Secondary (high-school): 42%
- Higher education (university): 58%

**Description of the Experiment**

**Questionnaire preparation:** In creating the questionnaire, we relied on hygiene measures proposed by the Public Health Authority of the Slovak Republic and. We formulated some questions in such a way as to find out the overall perception of hygiene measures by the respondents. Respondents were allowed to answer the questions yes or no. There was a possibility to write the opinion regarding the questions. All questions were mandatory.

**Number of questions analyzed:** 14

1. Do you think that COVID measures in stores are sufficient? (Yes/No)
2. Was there a notice regarding the hygiene measures for customers in connection with COVID-19 before entering the store (picture, text)? (Yes/No)
3. Was there a disinfectant for customers before entering the store? (Yes/No)
4. Does the seller disinfect baskets and carts? (Yes/No)
5. Do customers have disposable gloves available for handling unpackaged products? (Yes/No)
6. Do you think that the frequency of disinfection of areas with which customers come into direct contact is sufficient (handles and handles on refrigerators/freezers, pastry tongs, belts on cash registers, etc.)? (Yes/No)
7. Was the overall cleanliness in the store satisfactory?
8. Is the observance of distances between customers in the store premises checked in the store? (Yes/No)
9. Is the observance of the spacing in the rows at the cash registers checked? (Yes/No)
10. Have you encountered a situation where a person of a younger age category was present in the store during the time set aside for the purchase of seniors from 9:00 to 11:00? (Yes/No)
11. Did you witness that the staff or customer did not have an FFP2 respirator in the store? (Yes/No)
12. Did you witness that the staff or customer did not have a properly fitted respirator in the store? (Yes/No)
13. Do you use the service of buying goods by delivery directly to your home? (Yes/No)
14. Do you only pay for your purchase with a payment card? (Yes/No)

**Conducting a questionnaire survey:** the online questionnaire survey was conducted via the social media Facebook and Instagram. The questionnaire was prepared by Google Form, firstly validated by our research team, and consequently shared with people on social media. Only one person from the research team had access to the collected raw data. We prevented any manipulation of these data. We complied with the requirements of the GDPR legislation of the European Union. Data collection was anonymous. Each participant could complete the questionnaire only once. The survey took place in the period: 15th of April 2021 – 25th April 2021.

**Number of answers:**

The total number of processed answers was14,0000.

**Creating a dataset:**

We processed the raw data. Each verbal answer was examined. Offensive and vulgar responses were deleted. We performed a grammatical correction of text answers. and prepared the final data set for further processing in Microsoft Excell (Office 365). The structure of the dataset was adapted to further statistical processing.

**Processing the answers:**

We evaluated all questions and visualized the consumers' opinions by figures.

We evaluated the individual text answers and formulated the most frequent opinions of consumers. These answers are presented in the discussion section of this article.

## Statistical Analysis

Multiple correspondence analysis was used to visualize the data obtained from the questionnaire survey. Statistical significance was determined based on the significance of the *p*-value. The statistical program R studio (vs. 1.3.959) was used for data processing. Multiple correspondence analysis (MCA) is an extension of simple correspondence analysis to summarize and visualize a data table containing more than two categorical variables. It can also be understood as a generalization of the analysis of the main components when the variables to be analyzed are qualitative instead of quantitative **(Abdi and Williams, 2010)**.

## RESULTS AND DISCUSSION

A COVID-19 pandemic is an unprecedented event in the past 100 years of human history. It has started at the end of the year 2019 when a novel coronavirus SARS-CoV-2 (named by the International Committee on Taxonomy of Viruses) was discovered in Wuhan, China **(Huang et al., 2020; Chan et al., 2020; Wu et al., 2020)**. The disease spreads rapidly to all provinces of China outside of Wuhan **(Li et al., 2020; Tan et al., 2020)**, since then COVID-19 has been spreading exponentially around the globe. Based on available data, the Centers for Disease Control and Prevention **(CDC, 2020a)** stated that COVID-19 appears to be disseminating primarily from individual to individual, resulting in respiratory disease, and deaths of elderly adults and individuals at any age who have severe medical problems **(Jribi et al., 2020)**. Dangerous for critically ill COVID-19 patients with pneumonia are symptoms on a large scale with no specific drug treatment. Severe inflammatory reaction and respiratory distress syndrome can lead to rapid progression of the disease and may cause death **(Wang et al. 2020a)**. An excessive release of inflammatory immune cytokines could increase inflammation of the lung and risk occurrence of acute respiratory distress syndrome (ARDS) **(Huppert, Matthay and Ware et al., 2019)**, this may lead to hypoxia and lung injury, causing a further release of inflammatory factors **(Butt, Kurdowska and Allen, 2016; Mokra and Kosutova, 2015; Paolone, 2017)**. The large area of infection and the inflammatory reaction causes many immunologic problems, such as cytokine release syndrome (CRS), which can rapidly lead to deterioration and death **(Zhao et al., 2019; Tanaka, Narazaki and Kishimoto, 2016)**. The Virus's health impact as well as the sharp effect on economic and business activities, mostly due to the lack of effective vaccines or treatments are well known today. Several countries, have preferred a shutdown approach to slow down the transmission of the novel virus, by reducing the number of social contacts, and stopping all unessential economic and business activities including shutting schools and universities, **Ferguson et al., 2020** proposed that lockdowns will need to be carried out on a fairly regular basis for more than a year. In many countries, government declarations of lockdown have led to a panic buying of food-like products **(Jribi et al., 2020)**, this kind of behavior is clearly described by the study of **Wang et al. (2020a)** who stated psychological effects, fear, anxiety, and stress caused by the pandemic. A partly positive report has been presented by the Centers for Disease Control and Prevention who stated that there is no proof that the novel coronavirus can be transmitted by interaction with food or food packaging **(CDC, 2020b)**. Besides this, it is always important to practice strict hygiene when handling food **(UNICEF, 2020)**. To reduce the transmission of the virus among the population, legislation has been adopted in Slovakia that also addresses the sale of food. An overview of specific measures in chronological order is given in the next chapter.

### The overview of the general COVID-19 pandemic measures in Slovakia

Slovakia was among the first countries in the world (and the second in Europe, after the Czech Republic) to make the wearing of masks mandatory in public **(URL 1, 2020)**.

The population of the Slovak Republic strictly adhered to the measures at the beginning of the pandemic when little information was available about the disease and people was scared. The government adopted the first measures in March 2020. The chronology of the most important measures was: 8/3/2020 secondary schools closed in Bratislava region; 9/3/2020 primary schools closed; 10/3/2020 events suspended; 12/3/2020 all event venues closed; 13/3/2020 compulsory 14-day quarantine upon returning from abroad; 15/3/2020 face masks compulsory in public transport and shops; 16/3/2020 non-essential stores closed; 25/3/2020 compulsory face masks in public; 6/4/2020 compulsory 14-day quarantine upon returning from abroad in a quarantine accommodation facility; 8-14/4/2020 curfew during the Easter seasion; 22/4/2020 allowed activities, shops under 300 $m^2$, long-term accommodation, outdoor non-contact sports, car selling, open-air markets; 6/5/2020 short-term accommodation without restaurants or eating in common areas, pedicures and manicures, outdoor tourism, taxi services, services and

weddings, massages, museums, galleries, libraries, exhibition halls; 20/5/2020 Concentration of people inside of stores increased from 1 person / 25 $m^2$ to 1 person / 15 $m^2$. Theatres and cinemas opened, events with less than 100 participants allowed. Shopping malls reopened. Swimming pools and gyms open for members of sports clubs. Restaurant interiors are now accessible. Face masks no longer mandatory when the distance between people is at least 5 meters. Schools and Kindergartens to be opened later on 1 June 2020. Residents of Slovakia can now pass the borders and not go into state quarantine as long as they return in under 24 hours; 1/10/2020 Face masks are again compulsory even in exterior spaces if separation of at least 2 meters between persons cannot be maintained. Mass events were restricted to less than 50 people. Restaurants and bars must close by 10 PM and shopping malls must maintain at least 10 meters squared and 2 meters separation per customer. Universities must switch to distanced learning; 15/10/2020 Mass events were banned, except for baptisms, funerals, and weddings, which must maintain at least 15 meters squared per person. Shops and shopping malls must also maintain at least 15 meters squared per customer and must check the customer's body temperature. 9 to 11 AM is reserved for senior citizens. Sports events must be without spectators. Restaurants must serve customers only in exterior spaces or as take-out. Fitness centers, wellness centers, aquaparks, pools, and saunas are closed. High schools must switch to distanced learning. Police may issue ticket up to €1,000 without warning. In October, a decision was taken to try to test every adult in the country. On 31 October and 1 November tests were undertaken in 5,000 test centers. 2.58 million people tested on Saturday with 25,850 tests being positive. By Sunday evening the testing campaign concluded with more than 3.62 million people tested during the weekend. 38,359 (or 1.06%) were positive. Testing was not compulsory but anyone not isolating without a negative test result in a curfew; 19/12/2020 a nationwide lockdown was instituted due to rising numbers of infected and deaths. Except for a few exceptions such as visiting family, going to/from work, buying basic essentials, visiting post offices, banks and doctors, people are required to stay home. The restrictions are planned to last until 10 January 2021; 1/1/2021, measures were toughened again. The government closed ski centers and churches, prohibited hotels from taking new guests, and visits between different households were prohibited. The restrictions will be in place until 24 January; 8-10/1/2021 decision was taken to try to test every adult in city Nitra. Testing was not compulsory but anyone not isolating without a negative test result in a harsher curfew in Nitra, In January, a decision was taken to try to test every adult in the country again. Testing was not compulsory but anyone not isolating without a negative test result in a harsher curfew; 8/2/2021 COVID automat was launched; 1/3/2021 measures were toughened again. Curfew was toughened between 8:00 PM and 1:00 AM. All shops were closed at 8:00 PM; 8/3/2021 FFP2 face masks in public transport and shops; 15/3/2021 Compulsory FFP2 face masks in interiors; 20/3/2021 Holidays were prohibited until 28 April; 25/3/2021 Movement in city and nature is only allowed between 5:00 AM and 8:00 PM; 29/4/2021 Movement in city and nature is only allowed between 5:00 AM and 9:00 PM **(MDSR, 2020; WIKIPEDIA, 2021; URL 2, 2021)**.

The first measures activated by the government at the beginning of the pandemic (March 2020) were very strict. The number of positive cases was minimal during this period and the population did not have direct experience with the disease. Positive cases were detected in the state quarantine in the accommodation facility, which had to be completed by residents returning from abroad and later in the home quarantine. The overall discipline to comply with the measures decreased continuously when the lift of the measures was adopted by the government (May 2020). People were more benevolent with the measures. Consequently, the borders were opened and people began to travel on vacation (summer 2020), they spent time in restaurants and pubs, summer terraces, and shopping centers, but the mass events were officially banned. People returning from and on vacation had to spend time in the home quarantine. The overall situation has deteriorated (autumn 2020) and the incidence of the virus in the population has increased. The number of infections and deaths increased (Figure 1) (December 2020 – March 2021). The overall discipline for the population to follow the measures was not optimal, probably, because the population did not have direct experience with deaths from this disease.

**The hygiene measures for employees**

By the Resolution of the Government of the Slovak Republic no. 77 (published in the Collection of Laws under number 49/2021) the state of emergency was repeatedly extended, declared by the Resolution of the Government of the Slovak Republic no. 587 of 30 September 2020, for 40 days with effect from 8 February 2021 in the entire territory of the Slovak Republic. At the same time, with effect from 8 February 2021, the government restricted freedom of movement and residence by a ban on going out from 8 February 2021 from 05.00. until 01.00 the following day, until further notice, but no later than the end of the state of emergency repeatedly extended by this resolution, ie after 19 March 2021, this restriction not applying to travel to and from employment in the case of a person who meets one of the exceptions: Travel to and from the employment, if this person proves a negative result by an RT-PCR test not older than 7 days or an antigen test certified in the territory of the European Union for COVID-19 not older than 7 days, or if the person has a history of COVID-19 and has proof of its survival for not more than three months, or if the person has been vaccinated against COVID-19 with a second dose of vaccine and at least 14 days have passed since that vaccination, or if his or her condition or a medical contraindication does not allow the COVID-19 test to be performed **(URL 3, 2021)**.

At the beginning of March, METRO adopted hygiene, disinfectant and other preventive measures, which are regularly updated. The stores are equipped with disinfection dispensers and stands for use by customers and employees at designated locations. Employees have been instructed to follow all hygiene measures defined by the Public Health Authority of the Slovak Republic or the World Health Organization (WHO). Replacement of air conditioning filters and disinfection of air conditioning was also carried out in all areas **(URL 4, 2020)**.

METRO employees have protective equipment at their disposal, such as a masks, gloves and disinfectants. At the same time, it demands that its customers respect the wearing of masks or other alternatives in all sales areas and in contact with METRO Distribution employees. "Metro thank all customers who behave responsibly during purchases in our stores, comply with current government regulations and thus protect not only themselves but also our employees," METRO also welcomes the recommendations on responsible purchasing issued by the Slovak Alliance of Modern Trade (SAMO) and encourages customers to continue to follow these rules **(URL 4, 2020)**. Hygiene recommendations have been implemented by all big food retail networks and small shops in the Slovak Republic.

**The overview of the Specific COVID-19 pandemic legislation requirements in Slovakia for supermarkets and grocery food stores**

Based on public decrees and measures of the Chief Hygienist of the Slovak Republic the following measures concerning the grocery stores are valid in Slovakia. We are providing them in chronological order:

**From 25th of March 2020**, it is ordered to close all grocery stores including bread, pastries and meat stores, fruit and vegetable stores, grocery stores for special nutritional purposes for infants and young children every Sunday of the month until further notice, and operators are required to disinfect the premises **(PHA SR, 2020a)**.

**From 30th of March 2020** according to § 48 par. 4 letter e) of Act no. 355/2007 Coll. of the Slovak Republic for clients and employees the following measures are ordered until the appeal: entry and stay in the store only with the covered upper respiratory tract, to apply a disinfectant on the hands, or to use disposable gloves. Provide 2 m distance in rows. One customer on 25 $m^2$ of sales area of store. Place the notice in a visible place for buyers at all entrances with the specified hygiene measures for the maximum number of customers at one time. Furthermore, it is ordered from Monday to Saturday, 9:00 to 12:00 to allow entry only to persons over 65 years **(PHA SR, 2020b)**.

**From 22nd of April 2020**, a complement to the previous measures: perform frequent ventilation in the premises and disinfect the contact surfaces, handles, shopping carts, and baskets regularly and wash the floors wet every day. From Monday to Friday from 9:00 am until 11:00 to allow entry only to persons over 65 years of age. The Public Health Authority of the Slovak Republic regulates the reserved purchase time for seniors over 65 years of age from 21/04/2020. The currently valid reserved shopping time for seniors over 65 is from 9:00 to 11:00 Monday to Friday **(PHA SR, 2020c)**.

**From 6th of May 2020**, a complement to the previous measures: the maximum number of customers in store is one customer per 25 $m^2$ of the sales area of the store. This condition does not apply to children **(PHA SR, 2020d)**.

**From 20th of May 2020**, change of previous measures: the number of customers in store at one time may not exceed the concentration of one customer per 15 $m^2$ of the sales area of the store, which does not apply to children. It is ordered to the operators to provide in the grocery store and drugstore from Monday to Friday from 9:00 to 11:00 entry only for persons over 65 years of age **(PHA SR, 2020e)**.

**From 27th of May 2020**, from 6:00, change of previous measures. the number of customers in store at any one time shall not exceed one concentration of one customer per 15 $m^2$ of the area of store intended for customers, this condition shall not apply to children; this condition also does not apply if the operator can ensure and maintain a distance between individual customers of at least two meters **(PHA SR, 2020f)**.

**From 3rd of June 2020** change of previous measures: in the grocery store there may be no more than one customer per 10 $m^2$ of the sales area of the store, this does not apply to children. The condition also does not apply if the operator allows ensuring a two-meter distance between customers **(PHA SR, 2020g)**.

**From 20th of June 2020**, complementing previous measures: grocery stores must also ensure the detailed implementation of regular cleaning and disinfection under the daily sanitation regime based on acceptable and established principles of good hygiene practice under Regulation (EC) No 1774/2002 of the European Parliament and the Council. 852/2004 on food hygiene **(PHA SR, 2020h)**.

**From 1st of October 2020**, change of previous measures: maintaining distances in the row of persons in the store of at least two meters, and this does not apply to persons living in the same household **(PHA SR, 2020i)**.

**From 15th of October 2020** change of previous measures: the concentration of one customer per 15 $m^2$ of sales area of the store cannot exceed, but this requirement does not apply to children from 14 years of age with an adult. It is ordered to the operators to provide from Monday to Friday from 9:00 to 11:00 entry only for persons over 65 years of age **(PHA SR, 2020j)**.

**From 19th of December 2020** change of previous measures: the concentration of one customer per 15 $m^2$ of sales area of the store cannot exceed, but this does not apply to children under 10 years accompanied by an adult, it is also prohibited for customers to consume food and beverages in indoors and outdoors parts of the store. On Mondays and Fridays from 9:00 to 11:00, entry is allowed only for persons over 65 years of age, except for 24.12. 2020 and 31.12.2020, when the valid shopping time for seniors is from 9:00 to 10:00 **(PHA SR, 2020k)**.

All facility operators are required to ensure that the number of customers in store at a time is one customer per 15 $m^2$ of sales area designated for customers, but this condition does not apply to children under 10 years accompanied by an adult when the area of store intended for customers does not reach 15 $m^2$. A maximum of one customer, except for children, may be in store at any one time. Operators of food stores should allow Monday to Friday from 9:00 to 11:00 entry is allowed only for persons over 65 years of age **(PHA SR, 2021a)**.

**From 2nd of February 2021** change of previous measures: according to the provisions of § 48 par. 4 letter c), e) and y) of Act no. 355/2007 of Coll. of the Slovak Republic as amended for the districts of Trnava, Hlohovec, and Piešťany, all facility operators are ordered to comply with the following measures: the number of customers in store at one time may not exceed the concentration of one customer per 25 $m^2$ from the sales area designated for customers, this does not apply to children up to 10 years accompanied by an adult. If the sales area of the store intended for customers

does not reach 25 $m^2$, then a maximum of one customer can be in store at a time (this condition does not apply to children under 10 years accompanied by an adult). Ensure the maximum possible use of available cash registers to minimize the number of customers waiting in front of cash registers. In the case of self-service cash registers, ensuring that the distances between persons in rows are maintained, including lateral distances of at least 2 m, does not apply to persons living in the same household. To ensure the maintenance of distances between persons in rows of at least 2 m in the interiors of the store, this does not apply to persons living in the same household **(PHA SR, 2021b)**.

**From 8th of March 2021** change of previous measures: according to the provisions of § 48 par. 4 letter c), e) and y) of Act no. 355/2007 of Coll. of the Slovak Republic, it is ordered to all facility operators to comply with the following measures: to ensure the maximum possible use of available cash registers to minimize the number of customers waiting in front of cash registers. In the case of self-service cash registers, ensuring that the distances between persons in rows are maintained, including lateral distances of at least 2 m, does not apply to persons living in the same household. In the interiors of the store to ensure the maintenance of distances between persons in rows of at least two meters, this does not apply to persons living in the same household. According to § 48 par. 4 letter e) of the Act, food business operators are ordered to allow from Monday to Friday from 9:00 am. to 11:00 am. entry only to persons over 65 years of age or persons with severe disabilities and their guides, based on a card of a person with severe disabilities or a card of a person with severe disabilities with a guide **(PHA SR, 2021c)**.

**From 15th of March 2021** change of previous measures: according to § 48 par. 4 letter r) of the Act no. 355/2007 of Coll. of the Slovak Republic to properly cover the upper respiratory tract (nose and mouth), all persons are ordered to use a preventive protective device, which is a respirator (not a mask) or similar device (always without exhalation valve) meeting at least all technical conditions and requirements for filter half mask with at least FFP2 classification according to the technical standard STN EN 149+A1. Wearing this kind of respirator is mandatory in the public in all commercial establishments **(PHA SR, 2021d)**.

**From 22nd of March, 2021**, change of previous measures: for the districts of Čadca and Kysucké Nové Mesto, all facility operators are ordered to comply with the following measures: the number of customers in store at one time may not exceed one customer per 25 $m^2$ children up to 10 years of age accompanied by an adult if the sales area of the store intended for customers does not reach 25 $m^2$. At most one customer can be in store at a time. This condition does not apply to children under 10 years accompanied by an adult. Ensure the maximum possible use of available cash registers to minimize the number of customers waiting in front of cash registers. In the case of self-service cash registers, to ensure that the distances between persons in rows are maintained, including lateral distances of at least 2 m, this does not apply to persons living in the same household. In the interiors of the store to ensure the maintenance of distances between persons in rows of at least 2 m, this does not apply to persons living in the same household **(PHA SR, 2021e)**.

In addition to the above requirements when the grocery shop is located in the shopping center: the shopping center should allow access to the premises only to a person with a body temperature of not more than 37 °C, each person entering this shopping center should disinfect hands. Only grocery stores, drugstores, and pharmacies can be open for the public in the department store. Other retail stores must be closed. Only the issue of goods purchased in advance in the e-shop is allowed **(PHA SR, 2020k)**.

In a stricter regime, during the peak of the pandemic, there was a restriction for persons to shop food only in the nearest retail/grocery or similar place from the place of residence to the extent necessary to procure the essential needs of life (in particular the purchase of food, medicine, medical devices, hygiene goods, drugstores goods, feed and other necessities for animals, provision of childcare, provision of care pets, refueling) **(PHA SR, 2021f)**.

**From 16th of April, 2021** a) Only persons with covered upper airways may enter and stay in the outdoor and indoor areas of the store; this obligation does not apply to the time required for the consumption of beverages and food, b) apply hand disinfection to entrants or provide disposable gloves at the entrance to the facility, c) visibly place a notice of the obligation to comply with the above hygiene measures on all entrances, to carry out frequent ventilation of the premises and to regularly disinfect contact surfaces, handles, shopping carts and baskets, used devices, tools and aids, to use disinfectants with viruidal effect for disinfection, e) to ensure that the distance between persons is at least 2 m; this does not apply to persons living in the same household or persons with severe disabilities and their assistants, f) the number of customers in store at one time may not exceed the concentration of one customer per 15 $m^2$ of the sales area of the establishment intended for customers; this does not apply to children under 10 years accompanied by an adult; if the sales area of the place intended for customers does not reach 15 $m^2$, there may be a maximum of 1 customer in the place at one time; this condition does not apply to children under 10 years of age accompanied by an adult, g) the obligation under letter f) is met if the area of store intended for customers does not reach 90 $m^2$.

All operators of department stores, where a department store is considered to be a defined enclosed space, permanent architectural object, which consists of a set of shops or premises intended for services and sales of goods, observe the following measures: a) equip the hygiene facilities of the store with liquid soap and paper towels, b) allow entry to the premises of the department store only to a person with a body temperature of 37 °C, c) the number of customers in the department store at one time must not exceed one customer per 15 $m^2$ of sales area of all operations in the department store intended for customers; this condition does not apply to children under 10 years of age accompanied by an adult, d) to disinfect sanitary facilities every hour, e) to ensure that public catering establishments serve food and drink only in the packaged state, f) it is forbidden to consume food and drinks in the premises of the department store, g) ensure the closure of seating sections and children's corners **(PHA SR, 2021g)**.

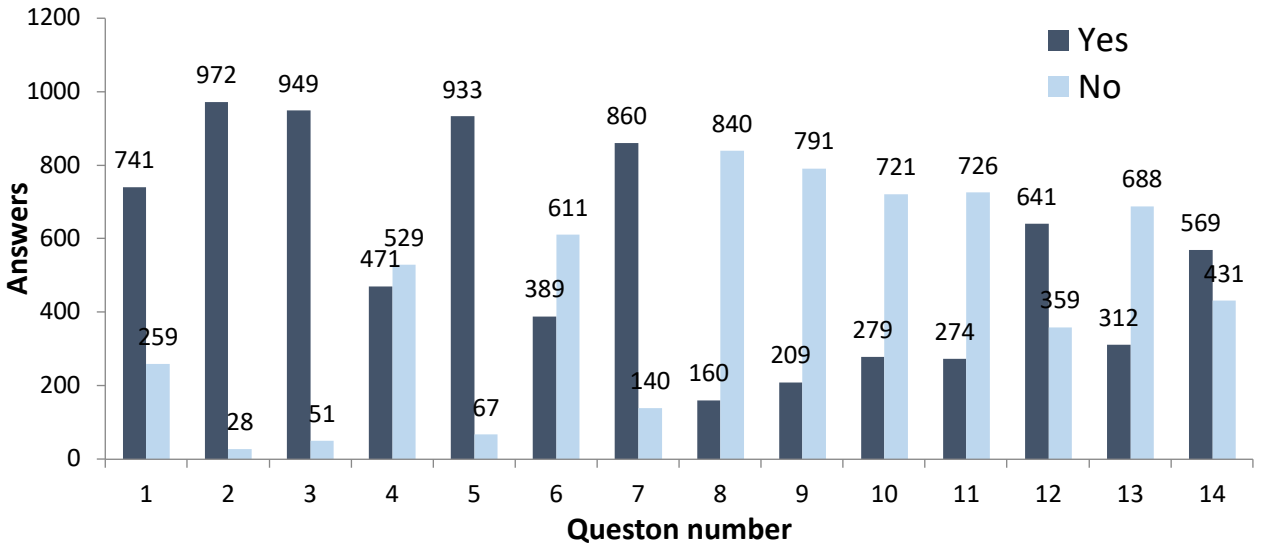



**Figure 5** Results of the questionairy survey (number of respondents = 1,000).

Gradually, in connection with the improvement of the pandemic situation in May 2021, the lift of the measures is expected. Other stores in shopping centers are opening again. The sale of food in markets and marketplaces is resumed.

**Results of the hygiene measures questionary survey**

The COVID-19 pandemic situation has changed the behavior of the population living in the Slovak Republic from many aspects of human life. It is well known, that the diverse site of the pandemic covers the loss of human lives, negative health impacts, negative economic aspects, etc. The shopping ability was driven by mentioned facts but also by several regulations that prohibit entrance to most of the shops and other typical services, with a few exceptions for groceries, drug, pharmacy or pet feed shops, with one general goal to minimize the close human contacts to slow down spreading of the virus. Remarkable was increased interest for food-like stuff, probably caused by the higher need for cooking activities caused by home office isolation, transferred to a higher frequency of grocery shops visiting. As such places are risky from the COVID-19 human-to-human share point of view for a long time, we have prepared the questionary with a package of fourteen questions focused on the most important anti-Covid regulations and routines to maintain a picture of real anti-pandemic customer protection in the Slovak market. One thousand respondents fill out the online questionnaire, on the principle of one choose answer per each question, based on Yes or No. Results of the hygiene measures survey are presented in Figure 5 and Figure 6.

**Questions:**

1. Do you think that COVID measures in stores are sufficient? **741 Yes, 259 No**
2. Was there a notice regarding the hygiene measures for customers in connection with COVID-19 before entering the store (picture, text)? **972 Yes, 28 No**
3. Was there a disinfectant for customers before entering the store? **949 Yes, 51 No**
4. Does the seller disinfect baskets and carts? **471 Yes, 529 No**
5. Do customers have disposable gloves available for handling unpackaged products? **933 Yes, 67 No**
6. Do you think that the frequency of disinfection of areas with which customers come into direct contact is sufficient (handles and handles on refrigerators/freezers, pastry tongs, belts on cash registers, etc.)? **389 Yes, 611 No**
7. Was the overall cleanliness in the store satisfactory? **860 Yes, 140 No**
8. Is the observance of distances between customers in the store premises checked in the store? **160 Yes, 840 No**
9. Is the observance of the spacing in the rows at the cash registers checked? **209 Yes, 791 No**
10. Have you encountered a situation where a person of a younger age category was present in the store during the time set aside for the purchase of seniors from 9:00 to 11:00? **279 Yes, 721 No**
11. Did you witness that the staff or customer did not have an FFP2 respirator in the store? **274 Yes, 726 No**
12. Did you witness that the staff or customer did not have a properly fitted respirator in the store? **641 Yes, 359 No**
13. Do you use the service of buying goods by delivery directly to your home? **312 Yes, 688 No**
14. Do you only pay for your purchase with a payment card? **569 Yes, 431 No**

We have performed the statistical analysis of the questionnaire survey. When using multiple correspondence analysis (MCA), we assume that we will plot a substantial part of the information from the obtained study data using two or more axes. Multiple correspondence analysis on the data obtained from the questionnaires extracted a total of 22 dimensions, which characterized the respondents' answers to 100%. The first dimension represented 12.75%, the second dimension 8.09%, and the third 7.62% of the total variation of the data 100%. The first two dimensions together characterized the respondents' answers to 20.84% (Figure 1). The graph shows age, gender, and education.

**Table 1** Results of the multiple correspondence analysis of the questionairy survey.

| Dimension 1 | | Dimension 2 | | Dimension 3 | |
|---|---|---|---|---|---|
| **Questions** | ***p*-value** | **Questions** | ***p*-value** | **Questions** | ***p*-value** |
| Q9 | 4.917E-138 | Age | 3.283E-79 | Age | 6.148E-121 |
| Q8 | 1.262E-135 | Q14 | 4.092E-68 | Q10 | 2.271E-91 |
| Q6 | 8.561E-106 | Q13 | 1.624E-51 | Gender | 1.449E-50 |
| Q12 | 2.600E-83 | Q4 | 1.301E-49 | Education | 4.773E-45 |
| Q7 | 1.620E-60 | Education | 7.466E-43 | Q1 | 2.787E-34 |
| Q1 | 4.424E-53 | Q7 | 4.552E-41 | Q12 | 3.760E-25 |
| Q11 | 1.984E-52 | Q12 | 5.809E-29 | Q11 | 5.357E-21 |
| Q4 | 3.221E-50 | Q1 | 4.099E-22 | Q4 | 6.544E-19 |
| Vek | 1.909E-47 | Q10 | 4.316E-21 | Q5 | 1.099E-16 |
| Q3 | 3.192E-10 | Q2 | 9.062E-15 | Q13 | 8.099E-04 |
| Q5 | 5.030E-07 | Q6 | 7.071E-12 | Q6 | 4.258E-03 |
| Education | 2.332E-05 | Q11 | 1.560E-10 | Q2 | 4.864E-03 |
| Q14 | 6.154E-05 | Q3 | 3.364E-03 | Q9 | 6.954E-03 |
| Q2 | 8.731E-05 | | | Q8 | 3.600E-02 |
| Q10 | 9.841E-03 | | | | |

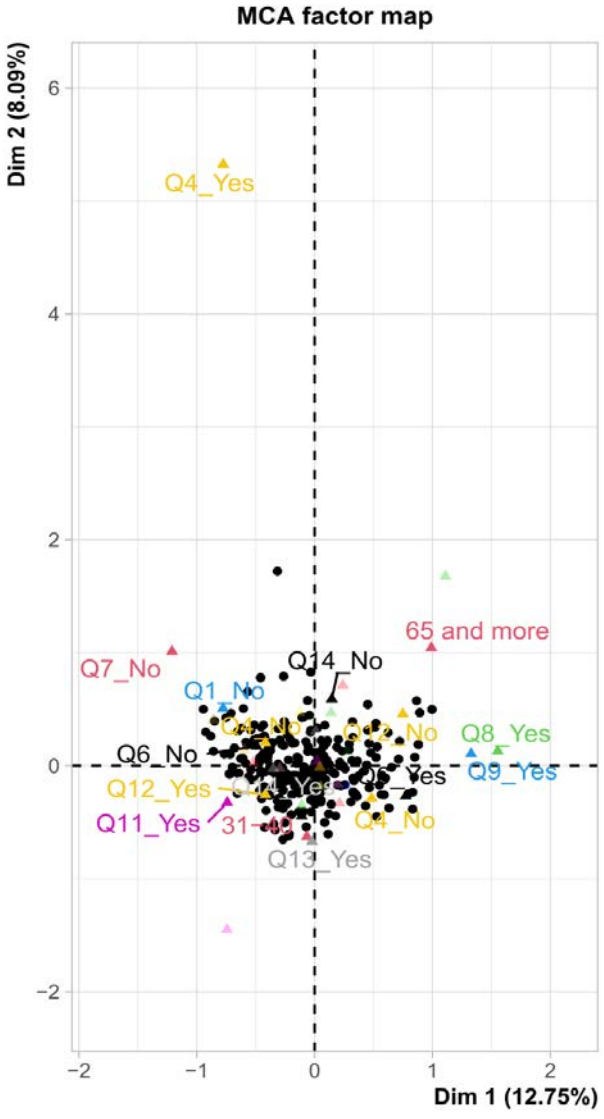



**Figure 6** Results of multiple correspondence analysis (MCA) of the first two dimensions.

The most statistically significant at the level of significance $p$ <0.05 (Table 1) was characterized by the following questions with the percentage of answers on the internal characteristic of the dimension: Q9 (Yes = 13.14%; No = 3.48%); Q8 (Yes = 13.78%; No = 2.63%); Q6 (Yes = 8.28%; No = 5.28%); Q12 (Yes = 4.00%; No = 7.16%); Q7 (Yes = 1.17%; No = 7.27%); Q1 (Yes = 1.94%; No = 5.56%); Q11 (Yes = 5.38 No = 2.03%); Q4 (Yes = 3.88%; No = 3.31%); Q3 (Yes = 0.07%; No = 1.32%); Q5 (Yes = 0.06%; No = 0.83%); Q14 (Yes = 0.25%; No = 0.32%); Q2 (Yes = 0.01%; No = 0.53%); Q10 (Yes = 0.17%; No = 0.07%). For second dimension: Q14 (Yes = 6.36%; No = 8.42%); Q13 (Yes = 7.91%; No = 3.59%); Q4 (Yes = 2.17%; No = 1.23%); Q7 (Yes = 1.29%; No = 8.00%); Q12 (Yes = 2.37%; No = 4.24%); Q1 (Yes = 1.30%; No = 3.73%); Q10 (Yes = 3.45%; No = 1.34%); Q2 (Yes = 0.09%; No = 3.20%); Q6 (Yes = 1.58%; No = 1.01%); Q11 (Yes = 1.64%; No = 0.62%) a Q3 (Yes = 0.02%; No = 0.46%). Dimension 3 is characterized by the following questions with a percentage of responses: Q10 (Yes = 14.53%; No = 5.63%); Q1 (Yes = 2.14%; No = 6.15%); Q12 (Yes = 2.18%; No = 3.91%); Q11 (Yes = 3.68%; No = 1.39%); Q4 (Yes = 0.02%; No = 0.13%); Q5 (Yes = 0.26%; No = 3.72%); Q13 (Yes =0.46%; No = 0.21%); Q6 (Yes = 0.30%; No = 0.19%); Q2 (Yes = 0.01%; No = 0.17%); Q9 (Yes = 0.34%; No = 0.09%) a Q8 (Yes = 0.22%; No = 0.04%).

Our questionnaire survey shows that most respondents think that the hygiene measures implemented in stores are sufficient in Slovakia. Almost one-third of the respondents believe that these measures are not sufficient, resp. could fail.

Almost all respondents stated that they were informed about the obligation to comply with hygiene measures in the form of a visual notice, a pictogram, before entering the store. However, some respondents stated that some notices were in a visually inaccessible place or were difficult to read.

Almost all respondents stated that they had a disinfectant available before entering the store. In some cases, the seller-provided a device for hand disinfection, but there was no disinfectant in this device, respectively this device was not refilled again.

More than half of respondents believe that the store does not disinfect shopping carts and baskets. Many respondents did not see any of the service staff to do baskets and shopping carts disinfection.

Therefore, many respondents used their disposable gloves and their disinfectant as a precaution or increased the dose of disinfectant when entering the store and with wet hands caught the handle of the basket or trolley.

Almost all respondents stated that the seller-provided disposable gloves for handling non packaged food such as bakery products. However, in some answers, they stated that disposable gloves were missing, resp. was not close to the food.

More than half of the respondents believe that the frequency of disinfection of surfaces with which they come into contact in the store and hand-held devices (handles and handles on refrigerators/freezers, pastry tongs, belts on cash registers, etc.) is insufficient.

Most respondents think that the overall level of cleanliness in stores is satisfactory.

Most respondents have experienced that there is no supervision of compliance with mandatory distances between customers.

Most respondents have experienced that there is no supervision of compliance with mandatory distances between customers waiting in line in front of the cash registers.

Many respondents have encountered a situation where a person of a younger age category was present in the store during the time set aside for the purchase of seniors from 9:00 to 11:00.

Many respondents have experienced that the staff or customer did not wear a respirator of FFP2 class. According to the comments, this problem mainly concerns customers.

Also, even more, respondents stated that the staff or customer did not have a properly fitted respirator in the store.

Almost two-thirds of respondents do not use the online service to buy food goods and their delivery directly to the home. Some consumers could not buy food online because their favorite retailer did not offer such an option. There were only two big food retailers who operated such a service in Slovakia for consumers.

More than half of customers prefer payment by credit card when making a purchase. However, many consumers also want to continue to pay in cash.

**Opinions of respondents**

The most frequent answers of the respondents included the following:

There should be a competent person (security man) at the entrance who will supervise the use of disinfectant because it has already been abandoned. Reduce the number of customers. Observe the spacing. Pensioners should shop only for limited hours and not go to the store whenever they want, they would be more protected. Gloves could also be mandatory. More frequent disinfection of trolleys, bag racks, etc.), limits the capacity of people (or control it). More frequent disinfection of surfaces with which customers come into contact. More frequent cleaning of shopping baskets. The disinfection of shopping carts and baskets is inconsistent. Disinfection of trolleys, gloves, distances is not observed! Observe the number of people per 1 $m^2$. More thorough control of hand disinfection. A more thorough inspection of properly fitted respirators/masks. Make sure everyone has the mask in place properly. Disinfect even more often and often check if people have anything to disinfect their hands with. Checking compliance with the measures. Checking antigen or PCR tests before entry, disinfection of the basket at entry. Disinfect baskets and trolleys, consistently release retirees only in the allotted time. I have a feeling that shopping carts are not disinfected at all. Fewer people at the store and more frequent disinfection of shopping baskets, often missing a disinfectant in the store. Temperature measurement in people before entering the store, disinfection of baskets after each use, disinfection of door handles at least once every 15-20 minutes. People manipulate with bare hands with unpackaged bread, vegetables, fruit and no one will warn them. Insufficient disinfection of areas such as cash registers, handles, baskets, pastry tongs. Insufficient hygiene measures for manipulation with pastry, fruit, and vegetables, insufficient hygiene for the cash register, for

handling goods and money. Negative tests are not checked, insufficient disinfection of baskets, door handles, the permitted number of people per square meter is not observed, poor disinfection of the hands of saleswomen at the cash register. I don't think that disinfection at the entrances is sufficient, I often find it diluted, people just apply it to their hands in a proforma so that it doesn't happen. Someone should stand at the entrance, they could encourage customers to better disinfect. I don't like that there is no limited number of customers in supermarkets, and so often, especially in the fruit and pastry section, there is a so-called head to head. The number of people per area is not observed. There are no controls, people do not use disinfectant, they rummage in pastries without gloves. The controls are simply not satisfactory, traders don't care, mainly to sell the goods. I've never seen saleswomen disinfect handles, baskets. Staff who control the number of people and hand cleaning and negative tests are missing, people have no respect, they do what they want. In my opinion, everyone should put on their gloves before entering the store and throw them away at the end. Mandatory gloves. The saleswomen have a mask under their noses and the temperature and the number of people are no longer checked, and people stand in a long line. Tighter regulation of the number of customers and control of valid measures. Stricter controls in stores. Who controls them at all? How is it possible that the measures are not complied with? People infected with the corona also have access to shops (and they know about it), it doesn't feel right to me. I don't even see the logic in the time reserved for retirees, so if they have to go to the shops in a reserved interval, then they should not go when others can go. Regulation of the number of people in the store. Weak control of a limited number of people per $m^2$. Monitoring of distances, no one checks it directly in the store. Stricter controls. Theoretically, they may be sufficient, but I rather see the problem in the customers themselves. In my experience, no one cared how many people entered the store. No one checked to see if people were disinfecting their hands. People did not keep a minimum distance of 2 m and many of them did not have the mask/respirator properly fitted. Crowds in the store at the cash register. People are also very close to pastries. The measures have already been relaxed, no one in the hypermarkets controls the numbers of people, does not clean the baskets, does not measure the temperature as in the beginning. There is disinfection in every store at the entrance, but few people use it, the same for masks and respirators, many people have not properly fitted them on their faces. In a few stores, someone oversees the number of people in the business or at least the proper wearing of a respirator, sometimes disinfection is not available at the entrance. First and foremost, regular disinfection should be added upon entering the store. A lot of people are buried, someone is still breathing down my neck, especially with vegetables in our store, or even with pastries or meat products. And sometimes I don't know if I have a disinfectant to squeeze my hands or feet, they have it differently everywhere. Lots of people in the same place. It would be good to cancel the hours for retirees. A large number of people in the store at the same time, young people between 9-11 can not go to the store but retirees can still (without insult). Get back to normal. No need to do such checks anymore, it is unnecessary. Few checks are performed. The employees are also in the front line and no one cares about them, and people still open their mouths when they reprimand them for their divorce. People get angry and frustrated at the saleswomen because they have to stick to the motto of our customer, our master. Closing operations is nonsense, it would be enough if people were waiting outside and there were only a few people in the store. Grouping of people on stock goods, no spacing 2 m apart if something is in action and few people are pushing each other. Increase the number of employees to check compliance with hygiene measures. One-way entry and exit are not provided by all retail chains in Slovakia.

The differences in the use of pictograms can be seen in the following figures: Billa supermarkets (Figure 7), Kaufland supermarkets (Figure 8), Tesco supermarkets (Figure 9), Tesco online shopping and delivery (Figure 10), Lidl supermarkets (Figure 11) Coop Jednota (Figure 12). Hygiene measures can be seen in the following figures: Coop Jednota and Tempo (Figure 13 and Figure 14), customers waiting in lines in front of stores (Figure 15), information notices in Tesco (Figure 16), measuring the temperature of people (Figure 17), hygiene measures in Lidl (Figure 18), Tesco (Figure 19), Tesco home delivery (Figure 20), restrictions on the purchase of non-foodstuffs (Figure 21). The Public Health Authority of the SR took over the official leaflet of the World Health Organization, translated it into Slovak, and made it available for download on its website (Figure 22). Several activities and inspections of compliance with regulations were performed by the police of the Slovak Republic and the municipal police in individual cities (Figure 23). Obligation to comply with measures in supermarkets Kaufland (Figure 24). Measures for customers in Metro (Figure 25). Problems with compliance with hygiene measures were regularly observed (Figure 26). Recommendation for one-way movent and disinfection (Figure 27).

The government took strong containment measures to prevent the spread of COVID-19 with restrictions on daily living such as social distancing and the closing of businesses and schools. While these measures are essential to stop the virus from spreading, several voices came to warn of their potentially disruptive impact on the agri-food system **(Hassen, El Bilali and Allahyari, 2020)**, supermarkets, retail food stores, and grocery.

Governments have various other issues to address, such as enforcing the proper lockdown among the people; providing the citizens with basic necessities like food, groceries, and vegetables, from the retailers; enforcing and monitoring law and order during the lockdown period pertaining to areas like transportation, markets, shops, public gatherings, and medical facilities; and identification and treatment of people suffering from coronavirus attack **(Jain, Puri and Jain, 2021)**. We agree that such measures must enable the population to buy food and necessities, including medicines. The measures themselves must therefore not be very restrictive, they must represent a healthy compromise between the needs of the people and the anti-pandemic commissions and public authorities.

Coronavirus disease 2019 (COVID-19) poses an occupational health risk to food system workers including farmers/producers, grocery store workers, emergency food system staff and volunteers (e.g., food pantry workers), and others.




**Figure 7**. Pictograms in Billa supermarkets, Slovakia **(Source: billa.sk)**.
Note: 1. Shopping list (Before you come to our store to shop, prepare a shopping list so that you do not stay in the store more than necessary). 2. Shop yourself (Go shopping yourself, in less frequent hours (usually between 1pm and 5pm)). 3. Protect yourself and others. Wear a mask, scarf or shawl, don't forget the gloves. 4. Keep a distance (Keep a distance of at least 2 meters from the others). 5. Wear gloves (Do not pick up fresh products with bare hands, use disposable gloves). 6. Think of loved ones (If possible, buy from others – parents, grandparents or neighbors). 7. We replenish the goods (We continuously replenish the goods for you, please buy in moderation). 8. Pay by card (Prefer payment by credit card). 9. Take care of hygiene (Wash your hands thoroughly with warm water and soap after purchase).
Note: Upon entering stores, we will not check customers for a negative test for COVID-19. Thank you for following these basic shopping rules.



**Figure 8**. Pictograms in Kaufland supermarkets, Slovakia **(Source: kaufland.sk)**.
Note: 1. Disinfection is very important in the fight against the virus. We also try to protect you, so we intensively clean and disinfect shopping carts and baskets. 2. When shopping, it is necessary to continue to wear a mask, ideally also gloves, and thus protect yourself and others. 3. You can disinfect the handle of the trolley or shopping cart yourself. Use our disinfection station, located at the entrance to the stores. This will reduce the risk of disease transmission. 4. Keep a safety distance of 2 meters between each other – In stores but also in private. This will prevent the spread of droplet infection. 5. Paying with a card means protecting yourself. Payment by smartphone is even more hygienic, as there is no need to enter a PIN code on the terminal. The fewer touches, the lower the risk of infection. 6. When coughing and sneezing, be sure to cover your mouth and nose thoroughly. Dispose of the handkerchief in a closed basket as soon as possible after use. 7. Especially the elderly and the sick need our help and support right now. If you have an older person in the immediate vicinity, you can possibly make a purchase. Observe precautionary measures in case of personal contact. 8. In addition to distributing leaflets to mailboxes, you will find the current offer in stores and still on our website, in the K-App or in the Newsletter. 9. Only through coherence and consideration can we prevent the spread of the virus together. That is why it is important to think not only about ourselves, but also about our fellow citizens. Valid: less "I", more "we"! 10. Let's be considerate and friendly to each other, despite the current situation. We try to do our best to cope with the difficult situation. We can do it together!




**Figure 9**. Pictograms in Tesco supermarkets, Slovakia **(Source: tesco.sk)**.

Note: 1. Respirator. Enter the store only with a respirator of class FFP2 and higher. It is necessary to have a covered nose and mouth with a respirator during the entire purchase. 2. Disinfect your hands. Thoroughly disinfect your hands when entering the store. For greater safety, we also recommend using protective gloves. 3. Keep safe distances. Keep a distance of at least 2 meters from other people in the store. 4. Wide alleys for greater safety. The alley in our stores are wide enough, thanks to which you can be sure that the safe zone and two-meter distances between all shoppers will be observed. 5. Cash registers protected by plexiglass. Protective plexiglass is installed in our stores to protect the health of all shoppers and our colleagues. Thanks to them, we can limit the contact of shoppers with our colleagues to the necessary minimum and you can safely complete your purchase. 6. Regularly disinfected baskets. We also don't forget to disinfect the store, which also includes shopping carts and baskets. We regularly disinfect them with a hygienic product that effectively protects against viruses and bacteria. 7. Contactless Scan & Shop service. If you want to shop fast and even safer, you can also use the Scan & Shop service with us and buy with a mobile phone or scanner. You can put the scanned products directly into your shopping bags, so you no longer have to unload your purchase at the checkout. 8. Convenient ONLINE shopping. If you want to shop from the comfort of your home, at Tesco we offer online delivery of food and other products thanks to the Tesco Online shopping service. We will deliver the food to your first door in compliance with the strictest safety measures. 9. One-way purchase. 10. Seniors and the severely disabled belong to the most vulnerable group. Priority shopping hours protected their health.

Note: Please keep in mind that our employees also have families at home that they care about. These measures protect not only you but also them. The current situation is not easy for any of us, but we assure you that we do everything in our power to bring you a safer purchase every day.



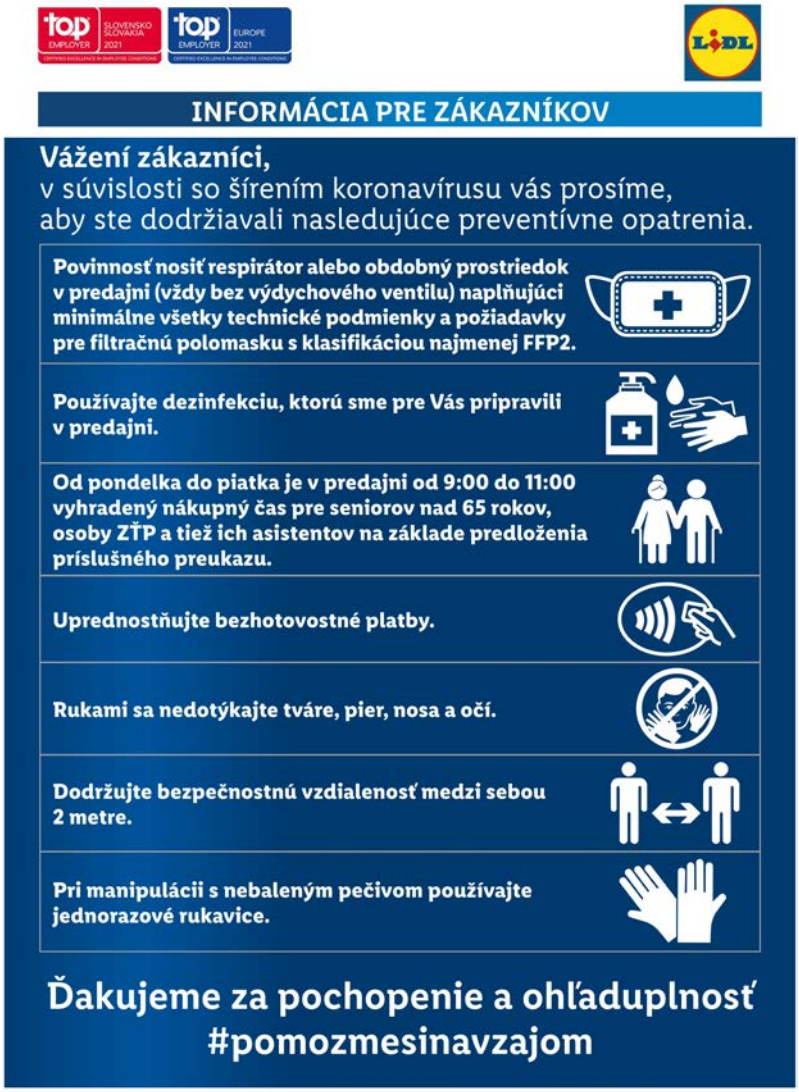

**Figure 10**. Pictograms: Tesco Online shopping and delivery, Slovakia **(Source: tesco.sk)**.

Note: Pictograms: 1. Wearing a protective mask (Please wear a protective mask on your face during the purchase). 2. Delivery only for the first lockable door (In order to maintain a safe distance, we now deliver the purchase only to the first lockable door). 3. Valid phone number (Please check if you have the current phone number in your Tesco account). 4. Please prefer online payment (If possible, please choose online payment to limit personal contact). 5. Maximum order size (Orders are limited to avoid excessive purchases. We'll notify you on the page when this limit is reached). 6. Delivery time reservation (We recommend that you book your delivery time in good time, you can complete your purchase later). 7. Online payment is convenient, simple, fast and more secure. 8. Payment for the purchase is charged to you only after receipt of the goods. 9. Contact with the driver is reduced, helping us to minimize the potential spread of the virus.

Note: Dear customer, we would like to inform you that from 3 March 2021 until further notice, the collection of food with the Click + Pick up service will only be possible until 20:00. We value your trust and thank you for respecting all measures that contribute to greater security of delivery and help protect you and our drivers. We do everything to ensure you the most convenient and secure online shopping. During the preparation and delivery of purchases, we observe the strictest hygiene measures – from the use of drapes and disinfection to maintaining a safe distance. For this reason, we now only deliver your purchases to the first lockable door, and we recommend that you prefer to pay online. Before delivery, the driver will contact you to agree with you on the delivery details according to your ideas. In order to best handle the current situation together, we would like to ask you to follow the instructions below. Please take a moment to find out how you can shop safer online these days.

Note: Why do we deliver all purchases in bags? We currently deliver each purchase in bags to meet hygiene standards. In the order, you can choose the delivery of goods in either plastic or paper bags for an additional fee. Please do not return plastic bags to our driver, as we are currently unable to accept them. Thank you for understanding. Why is it better to prefer online payment? We appreciate your choosing to pay for your orders online. It helps us reduce personal contact and keep your shopping as safe as possible.

Note: Important information for home quarantined customers. When taking over the purchase, it is important to strictly observe the above rules, which will prevent the spread of the disease. We want to deliver the goods to you as safely as possible, even if you are currently in quarantine. In order for us to succeed and at the same time be able to protect your health as well as the health of our drivers, we ask you to follow a few simple steps. In the order note, please inform us in advance that you are in home quarantine. We only accept online payments for your orders. We are very sorry, but if you are in quarantine and require payment at the door, your order may be canceled. If our driver contacts you to agree on a suitable method and place of delivery, please inform him that you are in quarantine. Please choose bagged delivery when ordering to minimize personal contact when picking it up. Thank you for following the rules that help us provide our service to as many customers as possible.




Translation of text:

INFORMATION FOR CUSTOMERS

Dear customers.
In connection with the spread of coronavirus, please follow the following precautions.

Obligation to wear a respirator or similar device in the store (always without an exhalation valve) meeting at least all technical conditions and requirements for a filter half mask with a classification of at least FFP2.

Use the disinfection that we have prepared for you in the store.

From Monday to Friday, shopping time is reserved in the store from 9:00 to 11:00 for seniors over 65, people with disabilities, and their assistants on the presentation of the relevant card.

Prefer cashless payments.

Do not touch your face, lips, nose, or eyes with your hands.

Keep a safe distance of 2 meters between each other.

Use disposable gloves when handling unwrapped pastries.

Thank you for your understanding and consideration
#let'shelpeachother

**Figure 11**. Pictograms in Lidl supermarkets, Slovakia **(Source: lidl.sk)**.




Shopping time for seniors only during the working week: Monday - Friday 9.00 - 11.00.

Use face protection when shopping and also everywhere away from home.

Keep a safe distance of at least 2 meters from other customers.

Use gloves or disinfectant during the purchase.

One shopper per 15 $m^2$ area.

Prefer cashless payment.

Thank you for being responsible.

**Figure 12**. Pictograms in Coop Jednota, Slovakia **(Source: Coop Jednota)**.
Note: Principles of safety, hygiene, and prevention when shopping. Uniformly and responsibly Coop Jednota. The best homemade food store.

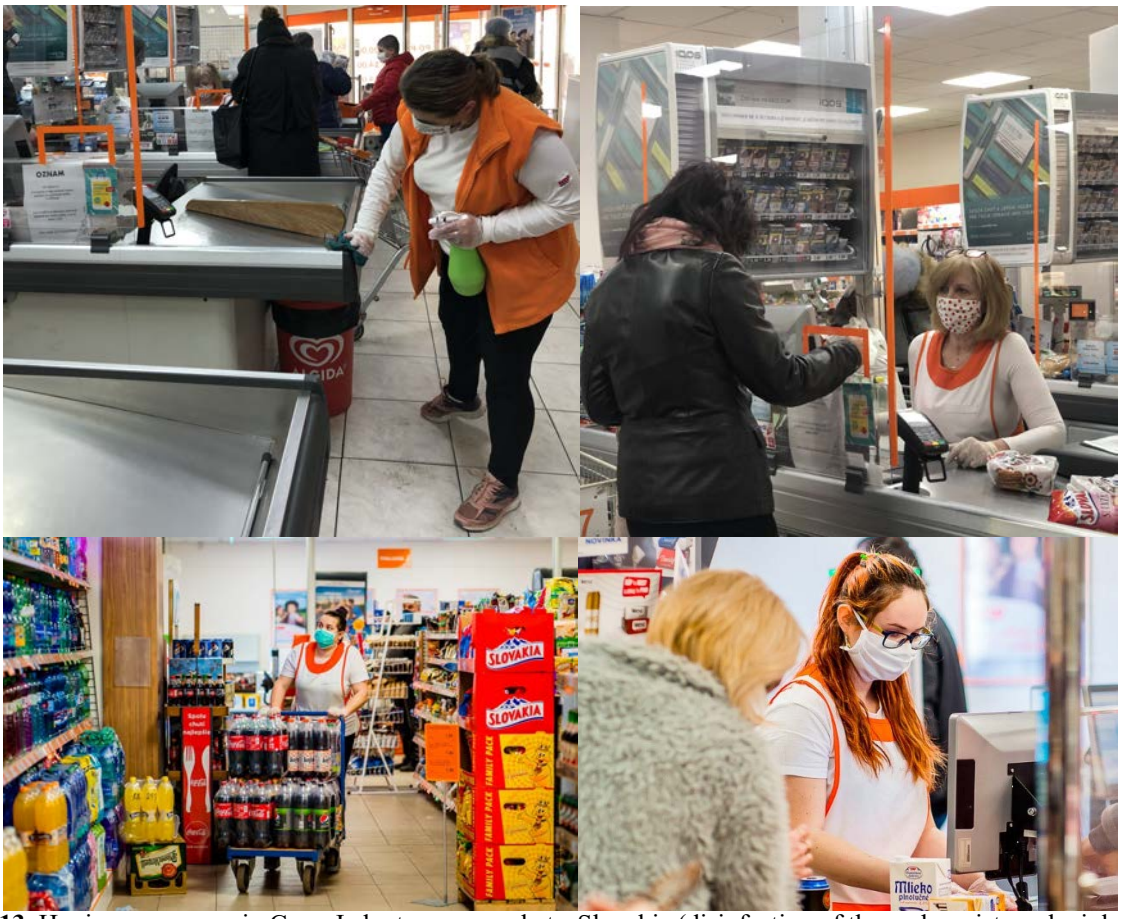



**Figure 13**. Hygiene measures in Coop Jednota supermarkets, Slovakia (disinfecting of the cash register, plexiglass separating the customer from the saleswoman, wearing masks) **(Source: Coop Jednota, Krupina, 2020)**.

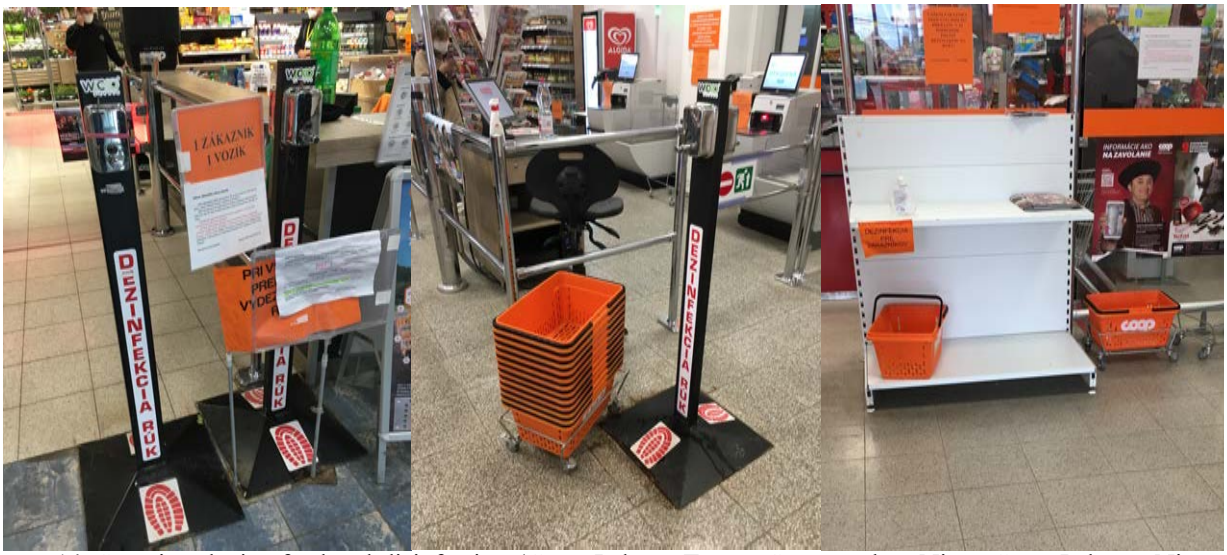



**Figure 14**. Stepping device for hand disinfection (Coop Jednota Tempo Supermarket, Nitra, Coop Jednota, Nitra and Coop Jednota, Nitrianske Hrnčiarovce, Slovakia).
Note: in Coop Jednota supermarkets and larger grocery stores of this retailer the hand disinfection is supervised by a security guard. In smaller grocery stores hygiene measures and hand disinfection are supervised directly by the saleswoman.



**Figure 15**. Customers waiting in lines in front of the stores **(Source: dennikn.sk – Tomáš Benedikovič; pravda.sk – Ivan Majerský)**.

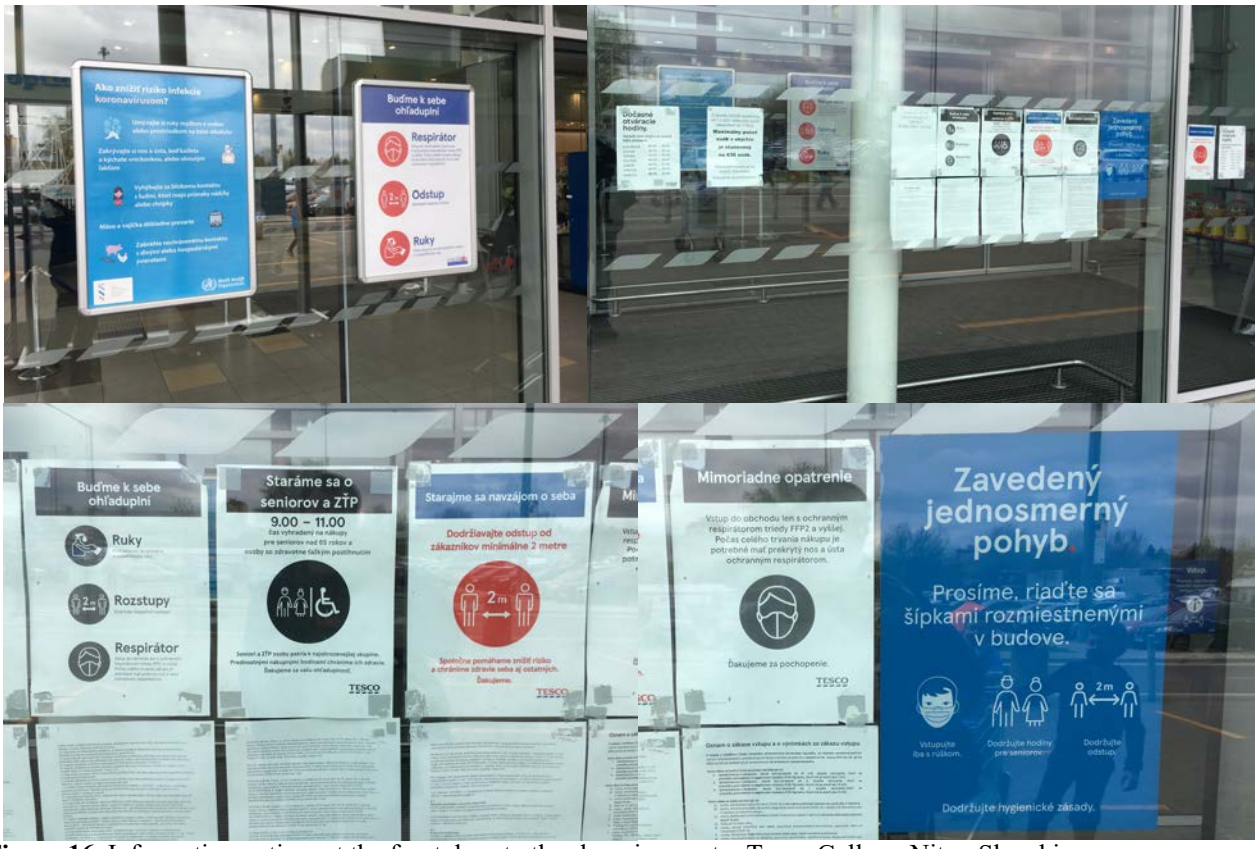



**Figure 16**. Information notices at the front door to the shopping center Tesco Gallery, Nitra, Slovakia.
Note: First picture: Official WHO and PHA SR poster: How to reduce the risk of coronavirus infection. Poster: Let's be careful with each other (Picograms: respirator, distance, hands with description). Second picture: Opening hours, The maximum number of people in the shopping center (Due to COVID measures, the number of customers is limited to 1/15 $m^2$ from 7.2.2021. The maximum number of people in the building is set at 635 people. We apologize for any inconvenience. Thank you for understanding). There are several other notices, which are shown in detail in the figures below. Third picture: Let's be careful with each other (Pictograms: hands, distance, respirator with description). We take care of seniors and the disabled (9.00 – 11.00. Let's take care of each other (time set aside for shopping for seniors over 65 and people with severe disabilities. The elderly and the severely disabled belong to the most endangered group. We protect their health with priority shopping hours. Thank you for your consideration), Let's take care of each other (Keep a distance of at least 2 m from customers. Pictogram 2 m distance. Together, we help reduce risk and protect the health of ourselves and others. We thank you), Fourth picture: Extraordinary measure (Enter the store only with a respirator of class FFP2 and higher. During the entire duration of the purchase, it is necessary to have a covered nose and mouth with a protective respirator. Thank you for understanding.), Introduced one-way movement (Please follow the arrows located in the building. Enter only with a mask. Observe the hours for seniors. Keep your distance. Follow hygienic principles). Below these pictures are the complete wording of the decree of the PHA SR and the notice of no entry and exceptions to the entry ban.

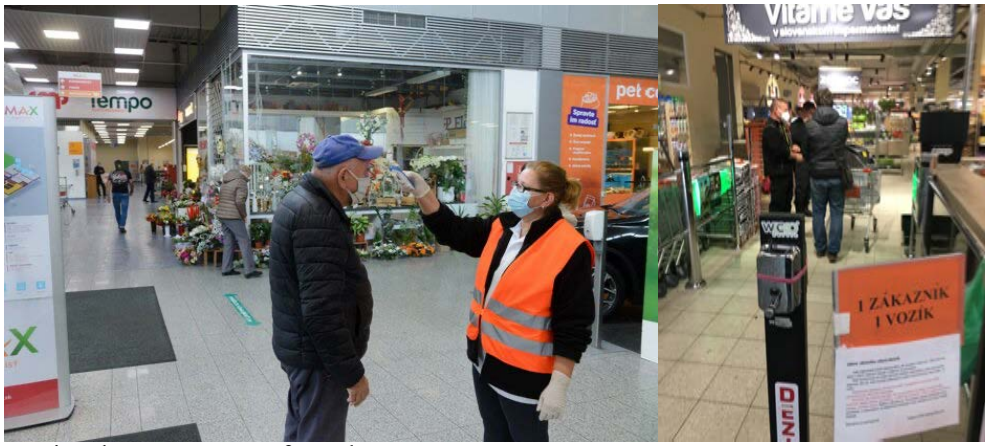



**Figure 17**. Measuring the temperature of people.
Note: Left: measuring the temperature of people entering the shopping center OC MAX in Nitra (**Source: TASR** and **sme.sk**). Right (measuring the temperature of people entering Tempo Jednota food supermarket, Nitra, Slovakia).

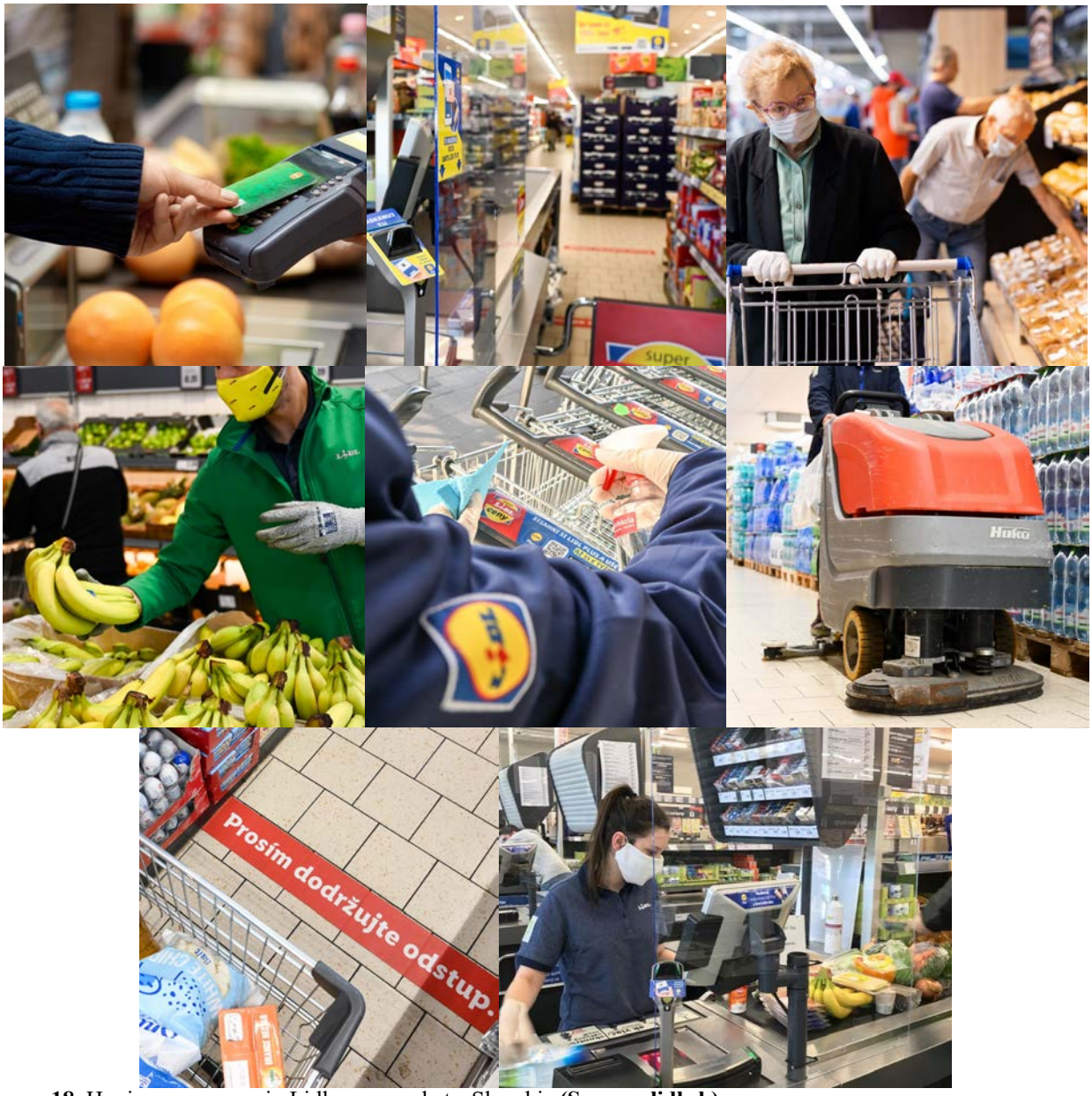



**Figure 18**. Hygiene measures in Lidl supermarkets, Slovakia **(Source: lidl.sk)**.
Note: to prefer payment with card, plexiglass is separating casher from customers, opening hours for seniors, wearing FFP2 respirators by employees, shopping cart disinfection, floor disinfection, keep a distance marks on the floor, blocking of goods behind plexiglass.




**Figure 19**. Hygiene notices in front of Tesco, Nitra, Slovakia.
Note: there are several large-scale posters informing consumers about hygiene requirements. Left: Let's be considerate of each other (Hand, Distane, Respirator. Disinfect your hands before entering the store. Keep a safe distance. Enter the store only with a respirator of class FFP2 and higher. It is necessary to have a covered nose and mouth with a respirator during the entire purchase). Right: Protect your health. Only a limited number of customers (453) can shop at the same time. We ask customers to enter the store only with protective gloves or after applying hand sanitizer. If you do not have protective equipment, please contact our colleagues. Thank you for respecting this regulation and following our colleague's instructions when entering the store. Blue pictogram: One-way purchase.




**Figure 20.** Home delivery service (tesco.sk).




**Figure 21.** Restrictions on the purchase of non – foodstuffs in Slovak food supermarkets.
Note: We ask you to comply with the decree of the Public Health Authority of the Slovak Republic, based on which the sale of goods in our stores was temporarily suspended, which does not serve to satisfy the necessary necessities of life. At Tesco, we continue to do everything we can to make a safer purchase. Thank you for understanding.




**Figure 22.** Official poster of the Public Health Authority of the Slovak Republic **(Source: uvzsr.sk)**.
Note: Left: Slovak version of the poster. Right: The original WHO English version of the poster.




**Figure 23.** Activities of the police of the Slovak Republic (Source: facebook.com/krpznr).
Note: Left: Preventive campaign of the police (Fraudsters continue to abuse the situation associated with the COVID - 19 pandemic for their enrichment. They do not slowdown in their activities and will do everything to gain the trust of seniors. Under various fictional stories, they often attract lifetime savings from them. We have therefore gone to shopping malls to repeatedly warn the most vulnerable citizens of the dangers they face if they are not still vigilant in the current pandemic. We warned citizens to be careful about strangers who, for example offer temperature measurements, the sale of protective masks or disinfectant gels by phone, so that they do not give money to people they do not know and do not let strangers into their homes under any circumstances. In order to always remember our advice and warnings at home, we handed over a magnet to the addressed citizens). Right: control of compliance with anti-pandemic measures in shops by citizens.







**Figure 24**. Hygiene measures in Kaufland, Slovakia.

Note: Left: Dear customers, concerning the measures of the Public Health Authority of the Slovak Republic with effect from 15.10.2020 until further notice, it is ordered not to exceed the concentration of one customer per 15 $m^2$ of sales area, to which we have adjusted the number of shopping carts. Max. number of customers in the store: 252. Middle: Dear customers, in connection with the COVID-19 pandemic, we would like to ask you to observe the following measures. Mandatory wearing of upper airway cover (respirator eg FFP2), Use hand disinfection, we have prepared it for you. Do not touch your face, lips, nose, or eyes with your hands. Distance in the rows of at least 2 m in the interior of the store. Use disposable gloves when handling unwrapped pastries. Opening hours: Mon-Fri: 9:00 - 11:00. Entry only for persons demonstrably over 65 years of age, persons with a disability card, and their assistants with a card. Prefer cashless payments. The permitted number of customers may not exceed the concentration of one customer per 15 $m^2$ of area. Keep a distance of at least 2 m in the ranks before entering the store. Opening hours: Mon-Sat: 7:00 - 20:00, Sun: 8:00 - 20:00. Right: hand disinfection equipment. Bottom: Dear customers, in connection with the COVID-19 pandemic, we would like to ask you to follow the following measures and recommendations. Mandatory wearing of upper airway cover (eg drape, scarf, scarf). Use hand disinfection, we have prepared it for you. Prefer cashless payments. Opening hours: Mon-Sat: 7:00 - 21:00, Sun: 8.00 - 20:00. Do not touch your face, lips, nose, or eyes with your hands. Use disposable gloves when handling unwrapped pastries. Distance in rows of at least 2 m. When coughing, be careful to cover your mouth and nose. Dispose of the handkerchief in a closable basket as soon as possible after use. The permitted number of customers may not exceed the concentration of one customer per 10 m2 of sales area. Thank you for your understanding and consideration. #wecandoittogether.




**Figure 25**. Notice for Metro customers **(Source: metro.sk)**.
Note: Left: Measure. Dear customers, for hygienic reasons we ask not to enter without a mask or alternative covering, e.g. scarf, scarf on the sales area. Thank you for understanding. Subway. Your success is our goal. Middle: Notice. Dear customers, on the basis of the measures taken in connection with COVID-19, it is obligatory to observe the following hygiene measures: Entry and stay in the store is possible only with an overlay of airways in the face. Respirator with filter class FFP2. Hand disinfectant must be applied at the entrance of the store. You will find the stand next to the card reader. Keep a minimum distance of 2 m in the rows and areas of the store. The number of shoppers in the operation will be regulated, only one buyer per 15 m2 of the sales area of the store. This concentration may not exceed. The number of customers per store is max. 565. Thank you for complying with the regulations. Metro. Your success is our goal. Right: Stand with hand disinfection.

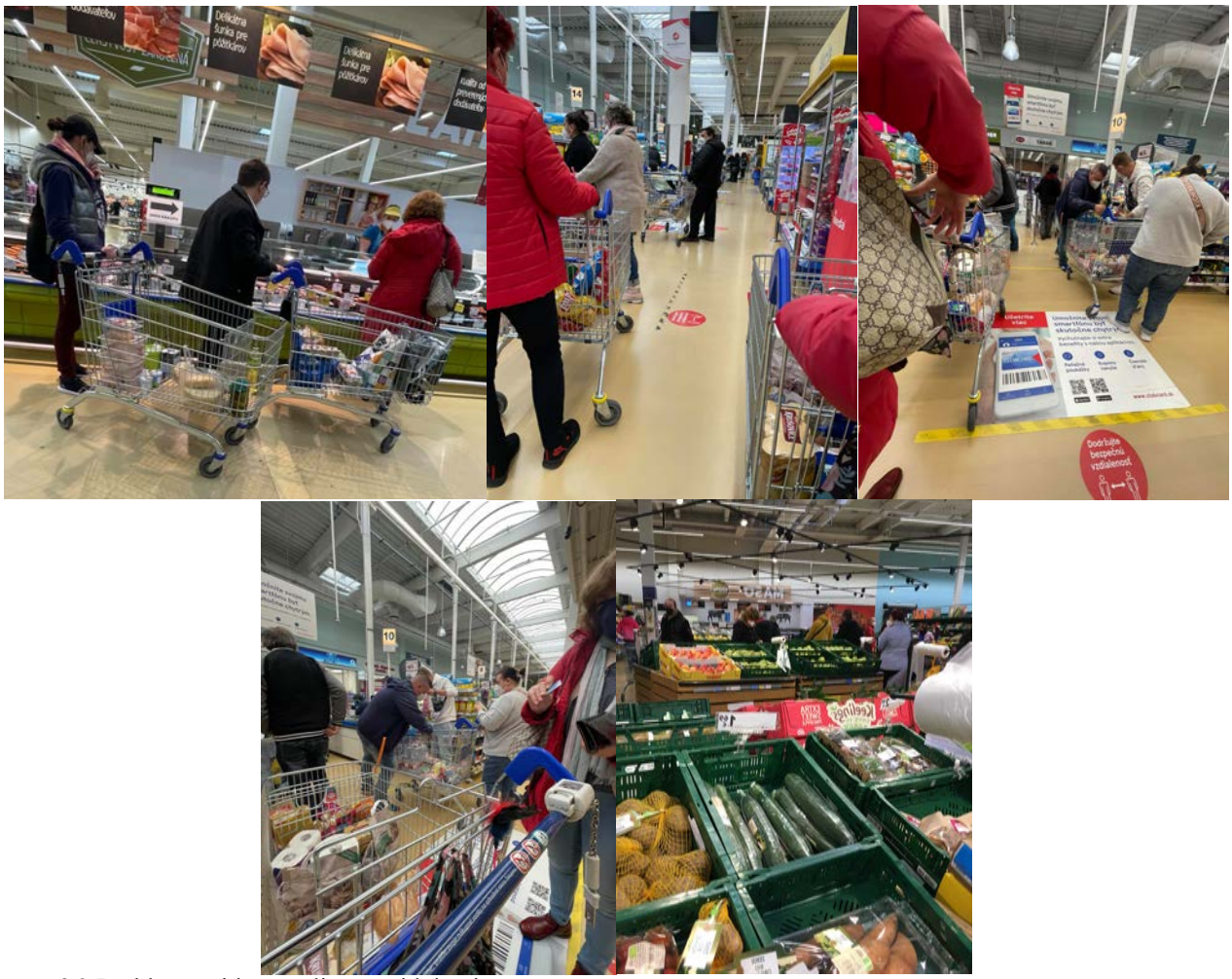


**Figure 26**. Problems with compliance with hygiene measures.
Note: Keeping the distance of 2 m is impossible during the purchase itself as well as while waiting for payment.

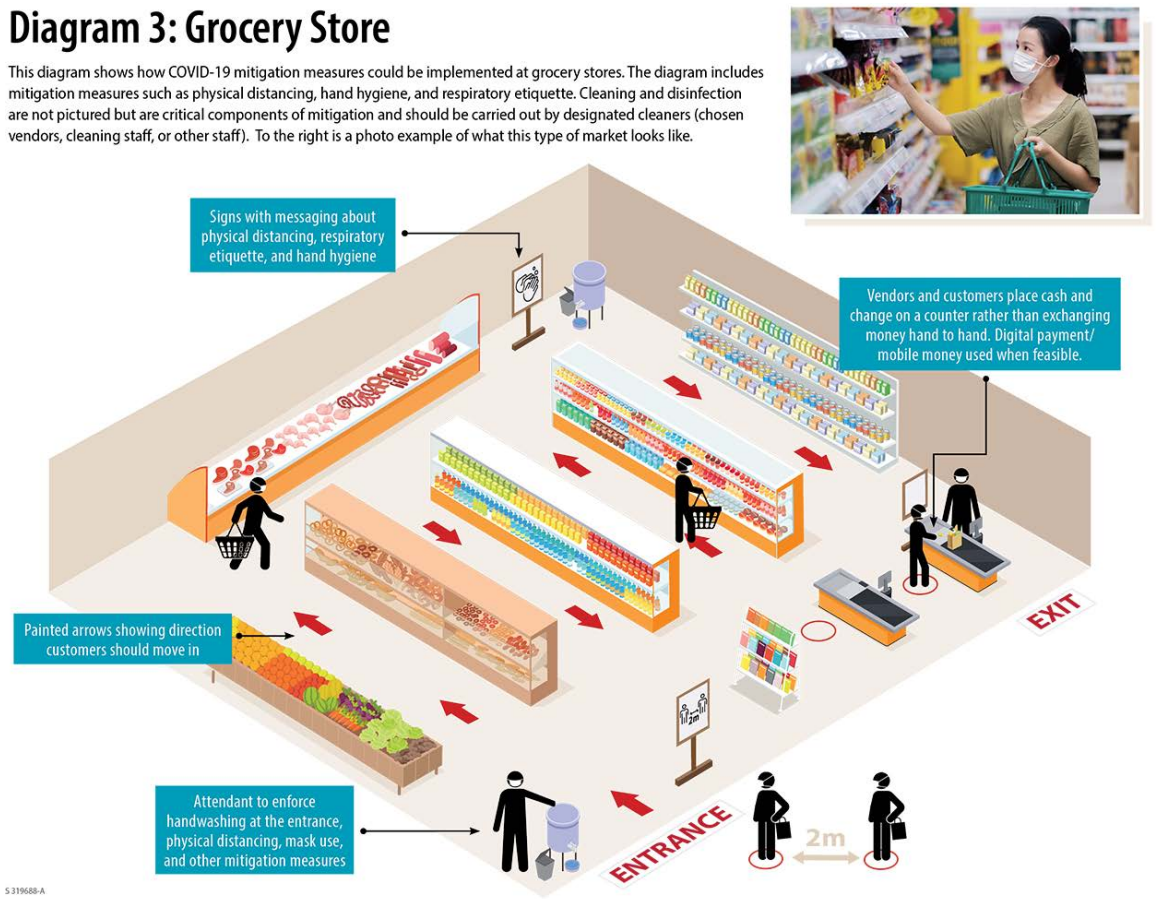



**Figure 27**. Examples of how the measurement should be implemented in grocery stores **(CDC, 2019)**.

These food system workers have been pushed to the front-line of this pandemic, providing essential services that support food consumption. Food system workers are some of the most economically vulnerable populations and are at risk of further financial disparities and contraction of COVID-19 during this pandemic. As we continue to grapple with the best strategies to support the food system and mitigate concerns around the spread of COVID-19, appropriate measures must be considered to better protect and support front-line food system workers that safeguard food access for all **(Parks et al., 2020)**.

The grocery retail industry is encountering unique challenges and opportunities during the 2019 Novel Coronavirus (COVID-19). The pandemic led to various transformations in the food retail industry, including changes in consumer perception and behavior. Although the pandemic has a situational nature, these transformations could have both temporary and long-lasting effects on reforms of the grocery retail industry **(Wang, et al., 2020b)**. Personal consumption expenditures have been significantly affected by the economic shocks in the COVID-19 era **(Dong et al., 2021)**. Panic buying during the novel Coronavirus (COVID-19) pandemic led to a temporary shortage of several staples and basic supplies in grocery stores, thus limiting the public's access to basic needs and exacerbating stress and anxiety **(Kassas and Nayga, 2021)**.We agree, many people have restricted shopping as such and are buying food in the nearest groceries, as ordered by the Public Health Authority of the Slovak Republic. However, we have also noticed that people are moving to the shopping center by car, even though most shops have to be closed. They justify this move by the need to buy their favorite foods, including pharmacy medicines and pet food at a specialist pet store.

As a result of the COVID-19 pandemic, the adoption of healthier food habits varied significantly with gender, age, and household income. There is a need to raise awareness among people on how to shop safely in grocery stores and that good hygiene practices should be followed in grocery stores to mitigate the risk of infection to consumers **(Shamim, Ahmad and Alam, 2021)**.

During the COVID-19 crisis food habits have changed among the population as food consumption is now carried out totally at home, and visits to groceries or food centers have decreased considerably **(Sinisterra-Loaiza, 2020)**, and in some countries, such as the Slovak Republic, there has been a complete decline in the hotel and catering sector. COVID-19 brought a high degree of disruption for society, firms, and consumers. Retail, grocery, and food services have been particularly affected as they were expected to maintain their operations while mitigating marketplace risks **(Aboelenien, Arsel and Cho, 2021)**.

In some countries like Slovakia where the restriction adopted by the authorities were too strict, many small food establishments went bankrupt. Supermarkets and grocery stores continue to operate, albeit in a restricted mode, supplying people with food and showing sales, and possibly making a profit.

COVID-19 continues to impose a series of unique challenges on the food retail and foodservice sectors in Canada. In May 2020, the expectation was that the public health crisis shutdowns of the restaurant sector would be

temporary. Grocery stores have solidified their changed realities through an increased focus on multiple channel retailing rather than a complete choice between either bricks and mortar or online.

Increased costs, resulting from the pandemic, are continuing to filter through the food system and we have a growing problem with food security for some Canadians given that employment in January 2021 was found to be at its lowest level since August 2020. Unemployment moves directly with lockdowns that are varied across the country **(Goddard, 2021)**. There is a sharp growth in demand for food deliveries and online grocery **(Gabriel, Darwish and Hassanien, 2021)**.

We agree, many respondents in our questionnaire survey stated that they use the home delivery service. Food delivery directly to the home in Slovakia was offered mainly by two retailers: Metro Cash & Carry and by Tesco.

Some municipalities ensured the purchase of food for the elderly and brought it directly to their house.

During the coronavirus disease-2019 pandemic, online grocery shopping experienced a never seen popularity in many countries. Amazon seems to follow its low-price strategy also in the grocery sector, even in times of high demand. However, during the lockdown phase, there were more price increases for certain highly demanded product groups such as frozen and prepared foods **(Hillen, 2021)**.

Online grocery shopping is a way of purchasing food products using a web-based shopping service. The current COVID-19 pandemic is determining a rethinking of purchase choice elements and consumers' behavior. The results of this work highlight that people having familiarity with buying food online, that have a higher educational level and consider food online channels easy to use, appear more satisfied with the food online shopping experience. These findings can be crucial for the future green global challenges as online shopping may help to reach competitive advantages for company sustainability **(Alaimo, Fiore and Galati, 2020)**. Online shopping for food products is quickly accelerating worldwide, particularly following the impact of the COVID-19 pandemic. Most e-grocers are multichannel supermarkets that have developed online channels. Variables defining retailer brand equity, i.e., retailer awareness, retailer associations, retailer perceived quality, and retailer loyalty contribute, together with perceived usefulness, to explain intentions to shop online at multichannel supermarkets **(Bandenhop and Frasquet, 2021)**.

## CONCLUSION

Hygiene measures against COVID-19 applied in food stores in the Slovak Republic are adequate and sufficient. It is very important to ensure that they are enforceable in practice. As commercial establishments are private, traders must ensure compliance with them themselves. The Public Health Authority of the Slovak Republic supervises their observance. The activities of the city police and the state police are necessary to ensure compliance with the measures in public places.

The most important hygiene measures in supermarkets, retail food stores, and grocery shops during the COVID-19 pandemic in Slovakia were: to wear masks and later wear a respirator of FFP2 class in public areas outside and inside, gloves on hands, or disinfection of customers' hands before entering the store, observance of 2 m distance of people standing in a row at the cash registers, maximum capacity one person per 25 $m^2$ of sales area and later one person per 15 $m^2$ of sales area, a limited number of shopping baskets and trolleys, specific purchase time only for seniors from 9:00 to 11:00 AM, to perform regular air ventilation of the premises and to have as many cash registers as possible so as not to create long lines of customers, regular disinfection of the shopping baskets and trolleys, regular disinfection of all surface in direct contact with customers like cash register belts, handles in refrigerators and freezers, pastry tongs, etc. In shopping centers, entry was prohibited for people with a body temperature higher than 37 °C. Shopping time allowed only to 8:00 PM.

During the peak of the pandemic, there was a restriction for persons to shop food only in the nearest retail/grocery or similar place from the place of residence to the extent necessary to procure the essential needs of life.

Most consumers think that the hygiene measures implemented in stores are sufficient. Almost one-third of the respondents believe that these measures are not sufficient or they are not properly performed by the seller.

It is very difficult to meet the measure of 2 m distance between buyers. The benevolent attitude of the population towards the measures taken and the indiscipline is a problem that the authorities must take into account when propose and adopt hygiene measures in legislation. The individual measures should be applied from a practical point of view, otherwise, they cause problems for all parties involved, official control bodies, sellers, and customers.

The grocery seller wants to sell groceries and not argue with the customer. It is annoying for the seller to constantly verbally warn customers to follow the specific COVID-19 hygiene measures. Such warnings may result in the loss of the customer and profit. On the other hand, in times of emergency, traders may be pleased that the government and public health authority have allowed them to operate their businesses and should therefore provide the measures required by the state. However, these measures must be based on sound reason. If the state wants to ensure food security and the necessary necessities of life in times of emergency measures for citizens, the help of the private sector and the participation of food retailers is necessary.

The information campaign on hygiene measures must be simple, clear and clearly communicated to citizens. We recommend unifying the visual appearance of pictograms, which should be used uniformly throughout the country and published in the legislation as mandatory. Ensure their contrast and good resolution, use blue color for notification or recommendation, and red color for prohibition. Uniform pictograms could then be incorporated by retailers into their corporate graphics.

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This article does not contain any studies that would require an ethical statement.

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# ASCORBIC ACID SUPPLEMENTATION SUPPRESSES CADMIUM-DERIVED ALTERATIONS IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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## ABSTRACT
Cadmium (Cd) a highly toxic environmental pollutant, that does not have any physiological function in the organism, represents a great concern for human health as it can be easily transported from its environmental sources to the food chain. Food, water, and air are the major sources of Cd exposure to the population. Cd-mediated impairments of the basic cellular properties largely depend on its ability to enhance the formation of reactive oxygen species (ROS) and thus triggers oxidative stress to the cell. With the use of fission yeast *Schizosaccharomyces pombe* (*S. pombe*) as the model organism, we have analyzed the impact of Cd on the cell growth intensity, as it represents the fundamental feature of all living organisms. Cells were incubated with different Cd concentrations for 3, 6, and 9 hours to investigate the effect of Cd on cell growth in a time and dose-dependent manner. Further possible Cd-derived alterations, as the peroxidation of membrane lipids or the functional impairment of the enzymatic antioxidant protection mechanisms, were investigated by determination of the MDA content and via catalase (CAT) activity detection. Moreover, ascorbic acid (AsA) pre-treatment was subjected to investigate the assumed positive effect of AsA against Cd toxicity. We show here on one hand that cells suffer under the influence of Cd, but on the other hand, they substantially profit from AsA supplementation. Because *S. pombe* is known to shares many molecular, and biochemical similarities with higher organisms, the effect of AsA in cadmium toxicity elimination might be expected to a similar extent also in other cell types.

**Keywords:** cell; cadmium; ascorbic acid; oxidative stress; contamination

## INTRODUCTION
As the problem of environmental contamination affects the entire ecosystem, it represents a global concern. Heavy metal contamination in soils and water may lead to severe human health alterations due to the metals' toxicity.

Although toxic ions naturally occur in the earth´s crust, anthropogenic activities including mining, industrialization, agricultural activities, waste disposal, etc. enhance their presence in the environment **(Okereafor et al., 2020)**. To the most threatening elements belongs cadmium (Cd) a non-essential toxic metal that is ranked at 7$^{th}$ position among the 20 highest toxins. Its natural occurrence in the earth's crust represents approximately 0.1 mg.kg$^{-1}$, but in sediments and marine phosphates its concentration may increase to 15 mg.kg$^{-1}$ **(Abedi and Mojiri, 2020; Hamid et al., 2019)**. Environmental contamination by Cd derives particularly from the use of phosphate fertilizers, recycling of electronic waste, smelting, and refining of metals, and combustion of fossil fuel **(Genchi et al., 2020)**. In such environments elevated Cd amounts deteriorate agricultural soils leading to Cd accumulation in crops ultimately reaching the human body. Scientists all around the globe evaluate food quality in terms of heavy metals contamination, including Cd. Rice is considered as the main Cd source worldwide, followed by seafood, vegetables, seaweed, grains, potatoes, and other types of food such as mushrooms, or raw cow milk **(Boudebbouz et al., 2021; Chiocchetti et al., 2020; Ćwieląg-Drabek et al., 2020; Huang et al., 2017; Jarosz-Krzemińska, Mikołajczyk and Adamiec, 2020; Kosečková et al., 2020; Liu et al., 2020; Satapathy, Panda and Jena, 2019; Suwatvitayakorn et al., 2019)**. Additionally, as Cd is naturally highly accumulated by the tobacco plant (*Nicotiana tabacum*), smokers incorporate a large amount of Cd from cigarettes **(Ganguly et al., 2018)**.

The adverse effects of Cd on human health are related to its long degradation period, unsuitability for decomposition, and its ability to accumulate in the kidneys, liver, and gut **(Fatima et al., 2019; Tinkov et al., 2018)**. Moreover, Cd is a proven (group I) human carcinogen according to International Agency for Research on Cancer classification. Cadmium exposure of either occupational or environmental origin can lead to apoptotic or necrotic events mediated through the cadmium-induced enhancement of reactive oxygen species (ROS) formation, excessive accumulation of $Ca^{2+}$ ions, and/or irregular expression of caspase 3, bcl2, and p53. Additionally,

cadmium was shown to interfere with the activity of antioxidant enzymes, such as catalase or superoxide dismutase **(Genchi et al., 2020)**. Cadmium-mediated cell damage due to oxidative stress induction suggests that antioxidants may be able to reduce cadmium toxicity. One of the prominent antioxidant supplements, ascorbic acid (vitamin C) has been shown to prevent oxidative impairments of the cell membrane induced by free radicals **(Erdogan et al., 2005)**. Recently **Darwish et al. (2019)** have shown that ascorbic acid supplementation helped HepG2 cells to recover from oxidative damage caused by food-relevant concentrations of Cd. In the presented study we investigated the effect of ascorbic acid (AsA) pre-treatment on Cd-mediated cellular impairments. A nonpathogenic yeast *Schizosaccharomyces pombe* was used as the model organism for the experimental analyses. *S. pombe* is a widely used model system for eukaryotic biology as it possesses great similarity of genome organization with mammals and shares many features with higher eukaryotes **(Awan, Manfredo and Pleiss, 2013; Fawcett et al., 2014)**. We assume that because of this high level of similarity the obtained results might predict the effect of AsA in the elimination of Cd toxicity in other cell types. Figure 1 represents the positive effect of AsA against Cd-induced toxicity.

## Scientific hypothesis

We predict that ascorbic acid due to its high antioxidant property will help cells to suppress the negative impact of Cd.

## MATERIAL AND METHODOLOGY

### Samples

### Biological Material

***Yeast strain***

For all analyses, the prototroph wild-type strain of the yeast *Schizosaccharomyces pombe* was used (JG 15459).

***Cultivation and growth conditions***

The overnight culture of the yeast cells was performed according to **Forsburg and Rhind (2006)** in the complete, liquid YES medium which contains 3% glucose, 0.5% yeast extract (YE), and supplements (S) containing 225 $mg.L^{-1}$ of amino acids: adenine, L-histidine, L-leucine, L-lysine, and uracil (AppliChem GmbH, Darmstadt, Germany). Incubation was performed at 30 °C under aerobic conditions ensured by stirring at 150 rpm.

### Chemicals

Ascorbic acid (AsA), Sigma-Aldrich, St. Louis, MO.

CdCl2, Sigma-Aldrich, St. Louis, MO.

Bovine serum albumin (BSA), Sigma-Aldrich, St. Louis, MO.

### Instruments

GloMax Multi Detection System Promega Corporation, Madison, WI, USA.

### Laboratory Methods

***Growth rate determination***

According to **Forsburg and Rhind (2006)**.

***Growth intensity ratio (GIR) assessment***

***Determination of catalase activity***

According to **Aebi (1984)**.

*Measurement of MDA content*

A slightly modified methodology of **Pekmez et al. (2008)** according to **Garre et al. (2010)** was used.

***Protein concentration***

According to Bradford assay **(Bradford, 1976)**.

### Description of the Experiment

***Growth rate determination***

For the experimental analyses, yeast cells from the overnight culture were either pre-treated with ASA (Sigma-Aldrich, St. Louis, MO) or left untreated. Cell cultivation was performed under stirring conditions at 150 rpm and 30 °C. The experimental group of yeast cells was pre-treated with 10 mM of AsA for 30 min under aerobic conditions at 30 °C. Cell concentration was determined as optical density measured at 600 nm by the use of the GloMax Multi Detection System (Promega Corporation, Madison, WI, USA). Cells were diluted to an $OD_{600}$ = 0.3 and subsequently transferred to 24-well plates where indicated final concentrations of Cd were added: 0, 1, 5, 10, 20, 40, 60, 100, 200, 300, and 400 μM of $CdCl_2$. Cells were incubated at 30 °C with shaking and after 3 hours of incubation, $OD_{600}$ was measured. The difference between the $OD_{600}$ value in the time point 3 h and the time point 0 h represents the growth intensity (GI) of the yeast cells after 3 hours of incubation. From the obtained data, the half critical concentration of Cd ($IC_{50}$) causing cell growth retardation to 50% in the presence or absence of AsA pre-treatment, was calculated. Freely available software Quest Graph™ IC50 Calculator was used to calculate the $IC_{50}$ value.

Growth intensity ratio (GIR) was calculated from the growth intensity (GI) values obtained after 3, 6, and 9 hours of incubation in the presence of different Cd concentrations with (+AsA) and without (-AsA) ascorbic acid treatment. Growth intensity of AsA pre-treated cells was expressed as a percent ratio from the AsA untreated cells according to the formula:

$$GIR = 100 \, . \, GI^{+AsA} / GI^{-AsA}$$

The GIR value above 100 represents more intense cell growth of the AsA treated cells compared to AsA untreated cells.

Growth rate determination at time points 3, 6, and 9 h after Cd treatment was selected because the fission yeast cell cycle takes, on average, 3 h. Hence, analyses of two and three live cycles of the yeast served as experimental proof of the long-term Cd impact on the cell growth ability. Moreover, the effect of AsA pre-treatment on Cd-induced impairments on this process was assessed.

***Analysis of oxidative stress and antioxidant defense***

The antioxidant activity of catalase (CAT), referring to $H_2O_2$ decomposition, was determined by the use of Agilent Cary 60 UV/VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) as the drop of absorbance at 240 nm for 90 s as described by Aebi **(Aebi, 1984)**. The analysis was initiated by the addition of 50 μL of 30 mM $H_2O_2$ to the reaction solution containing 100 μL of the sample reaching the final volume of 600 μL. The molar absorption coefficient (36 $mM^{-1}.cm^{-1}$) was used to define the specific catalase activity.

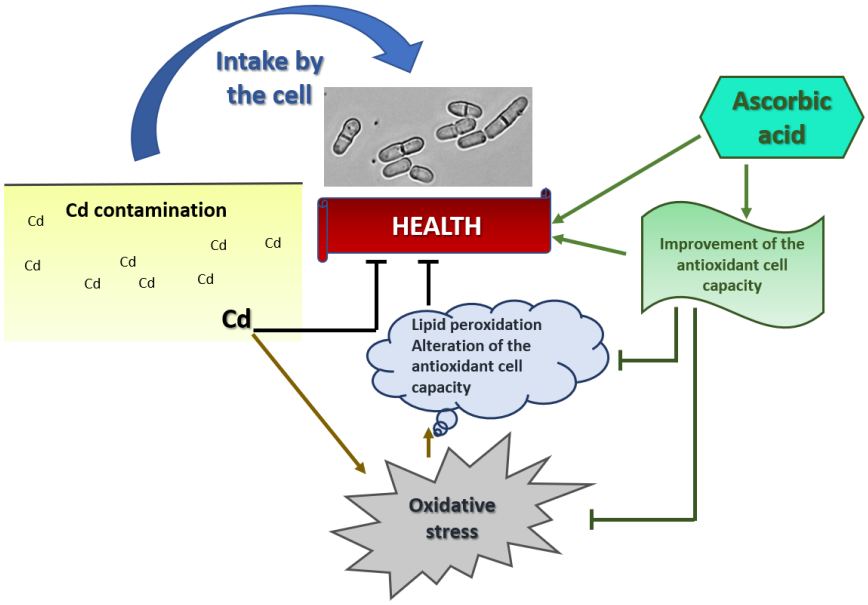


**Figure 1** Schematic draw represents the positive effect of AsA against Cd-induced toxicity.

Lipid peroxidation was estimated by measurement of malondialdehyde (MDA) content. Briefly, to 300 µL of supernatant from each sample, 600 µL of thiobarbituric acid (TBA) solution containing 15% trichloroacetate (TCA) supplemented by 0.375% (w/v) TBA, was added and boiled at 95 °C for 30 min. Subsequently, the sample was cooled on ice and centrifuged at 8500 rpm for 60 s. The light absorption of the supernatant at 532 and 600 nm was measured with the use of an Agilent Cary 60 UV/VIS spectrophotometer. For calculation of the MDA content, we used the molar absorption coefficient 153 $mM^{-1}.cm^{-1}$, results were expressed as nmol per µg protein. Determination of protein concentration with the bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard was performed at 600 nm according to Bradford assay **(Bradford, 1976)**.

***Sample preparation:*** Yeast cell material (sample) for each treatment were collected from the yeast culture as described in the cultivation methodology. For biochemical analysis, the samples were stored at -80 °C until analyses.

**Number of samples analyzed:** Each treatment was analyzed using seven individual samplex (n = 7).

**Number of repeated analyses:** Spectrophotometrical measurements were performed 3 times for each sub-sample.

**Number of experiment replication:** The number of repetitions of each experiment to determine one value was triple.

### Statistical Analysis

All data from the analyses are presented as the mean ± standard deviation (*SD*). Statistical analyses were performed using the Statistica 10 software (StatSoft Inc., Tulsa, OK, USA). The significance of the obtained differences was analyzed by ANOVA through Duncan and Fisher's LSD post hoc test.

Homogeneity and normality distribution of experimental data were evaluated by Lavene and Cochran's test. The statistical significance was set up to $p$ <0.05 *, 0.01 **, and 0.001 *** for the detected statistically significant differences.

## RESULTS AND DISCUSSION

### Ascorbic acid mitigates Cd-induced cell growth impairments

As it has been previously reported on various model systems, Cd induces alterations of cellular processes on different levels resulting in cell growth retardation **(Yamada et al., 2009; Sandalio et al., 2001; Xu et al., 2019)**. To evaluate the effect of Cd on the growth ability of *S. pombe*, cells were subjected to various Cd concentrations supplemented in the culture media. Increasing Cd concentration led to sequential abolishment of the cell growth (Figure 2 red symbols). The effect of ascorbic acid on Cd-mediated cellular impairments was investigated based on previous reports showing that ascorbic acid, as a strong antioxidant compound plays a significant role in alleviating various abiotic stresses **(Venkatesh and Park, 2014)**. Additionally, external compounds such as salicylic acid or selenium, through scavenging reactive oxygen species, can mitigate Cd toxicity **(Liu et al., 2016; Lin et al., 2012)**. Indeed, culture pre-treatment with 10 mM AsA largely improved the growth of cells exposed to Cd (Figure 2 green symbols). Figure 2 depicts the growth intensity of cells treated for 3 hours with various Cd concentrations and the effect of AsA supplementation. AsA markedly enhanced cell growth, additionally acknowledged by the $IC_{50}$ value. Cells pre-incubated with AsA display a 2.8 times higher $IC_{50}$ value meaning that in the presence of AsA cells require almost three times the amount of Cd to inhibit cell growth to 50% compared to AsA untreated cells.

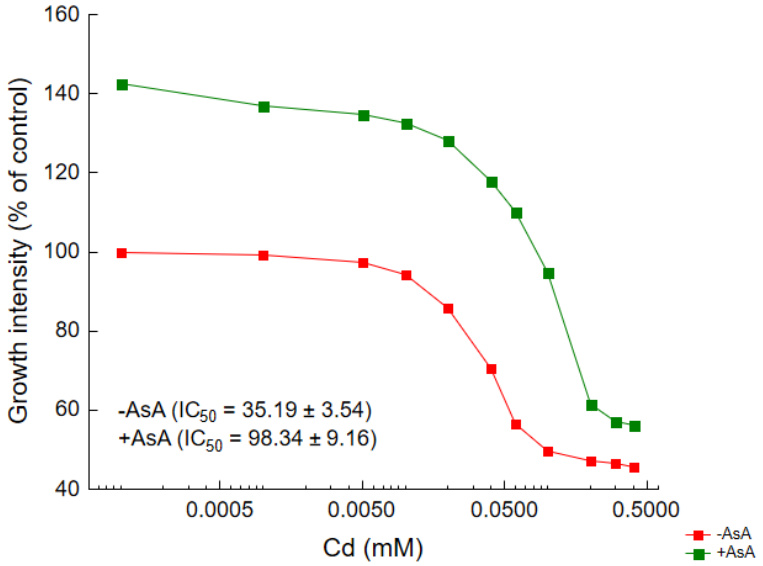


**Figure 2** AsA eliminates Cd-derived growth intensity alterations.
Note: Depicted curves represent growth intensity of cells treated (green symbols) and untreated (red symbols) with AsA upon increasing Cd concentrations after 3 h of incubation expressed as % of control. $IC_{50}$ values represent concentrations of Cd necessary for 50% cell growth inhibition without AsA supplementation (-AsA) or with AsA supplementation (+AsA).

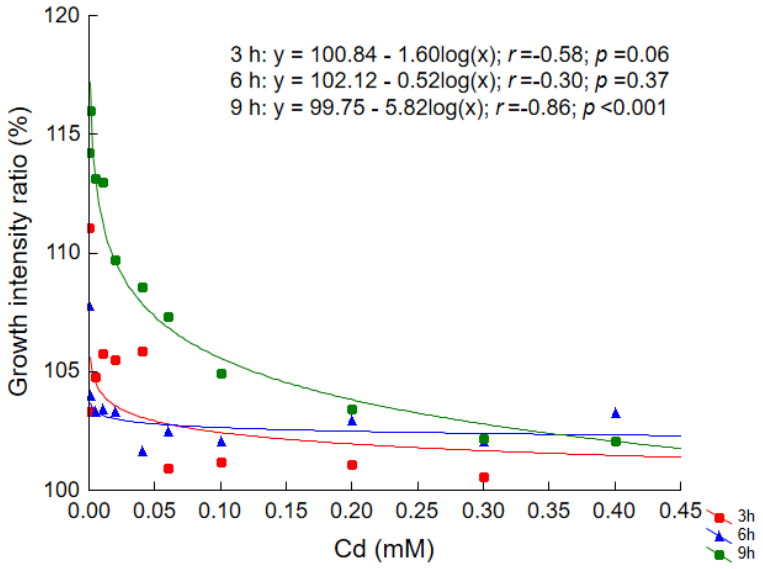


**Figure 3** Growth intensity ratio of AsA treated cells.
Note: Time dependent growth intensity ratio represents proportional increase in the cell density of AsA treated cells. Growth intensity ratio (GIR) is expressed in percent of AsA treated cells growth intensity to the growth intensity of AsA untreated cells. If GIR value equals 100 it would represent identical growth intensity for both, AsA treated and untreated cells. GIR value greater than 100 represents higher growth intensity of AsA treated compared to untreated cells. The red line and symbols represent GIR after 3 hours, blue line and symbols represent GIR after 6 hours, green line and symbols represent 9 hours of cells incubation with Cd (0, 0.001, 0.005, 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.3, 0.4 mM).

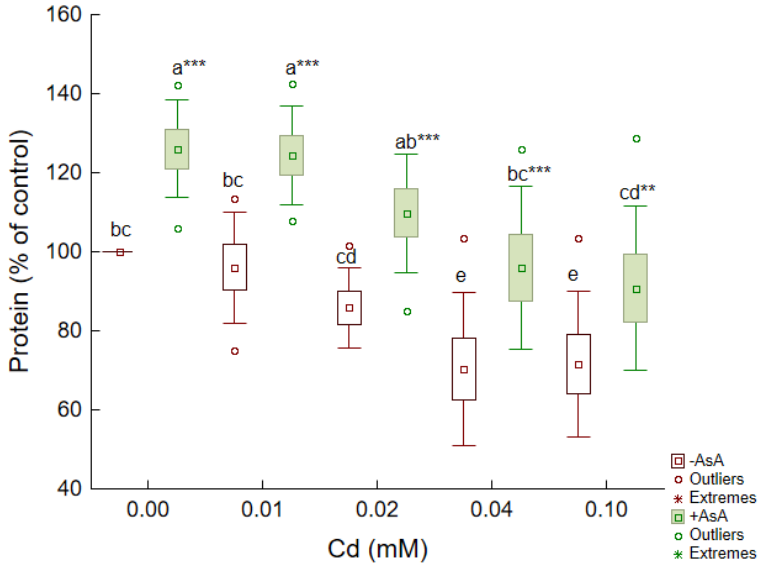


**Figure 4** Protein mass gain.
Note: The difference of the protein mass gain represents the difference between the cell density increase of AsA untreated (brown symbols) and AsA treated (10 mM) (green symbols) cells after three hours of incubation with different Cd concentrations (0, 0.01, 0.02, 0.04, 0.1 mM). Individual values are expressed as % of control, n = 7. Number of * indicate statistical significance ($p$ <0.05 *, 0.01 **, 0.001 ***), identical letters above bars denote that these values possess no significant difference.

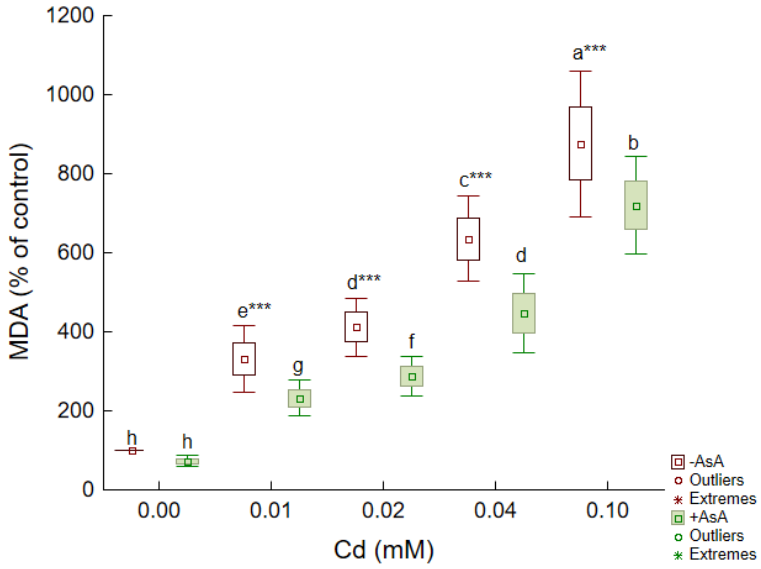


**Figure 5** AsA alleviates Cd-mediated lipid peroxidation.
Note: Measurement of the MDA content expressed as % of control represents the difference of the MDA production between AsA untreated (brown symbols) and AsA treated (10 mM) (green symbols) cells after 3 hours of incubation with different Cd concentrations (0, 0.01, 0.02, 0.04, 0.1 mM), n = 7. Number of * indicate statistical significance ($p$ <0.05 *, 0.01 **, 0.001 ***), identical letters above bars denote that these values possess no significant difference.

Moreover, growth curves of Cd-treated cells expressed as growth intensity ratio of the control show dose and time-dependent impact of cadmium and AsA on the cell proliferation (Figure 3).

Growth curves in Figure 3 show dose and time-dependent Cd-derived growth retardations and the significant positive impact of AsA pre-treatment. Hence, the data show that AsA pre-treatment mitigates the negative effect of Cd on cell growth. The statistical significance of the single and the mutual impact of Cd addition and AsA supplementation on the cell growth after 3, 6, and 9 hours of incubation was evaluated by the two-way ANOVA and for more accurate interpretation is depicted in Table 1 (After 3 h, After 6 h, After 9 h).

Another confirmation of the positive effect of AsA against Cd-mediated cell impairment is the protein content measurement that represents cell mass gain. As it is shown in Figure 4, AsA significantly enhanced the amount of protein in the presence of all Cd concentrations but also even in its absence. This implies that AsA widely improves cellular condition resulting in enhanced growth ability represented by elevated mass gain ability. Statistical evaluation by two-way ANOVA of the single and the mutual impact of Cd addition and AsA supplementation on the protein level is shown in Table 1 (Protein). Concomitant with our data a recently published study by **Zhang et al. (2019)** although on the different model system shows that exogenous application of AsA eliminates the negative effect of Cd on the growth of maize. Moreover, studies with guinea pigs also demonstrate that sufficient doses of ascorbic acid help to restore Cd-induced reduction of immune cells or eliminate nephrotoxicity **(Nagyova, Galbavy and Ginter, 1994; Kubová et al., 1993)**.

## Ascorbic acid protects cells against Cd-mediated oxidative stress

To further investigate Cd-mediated toxicity to our model system and its possible elimination by ascorbic acid, the Cd-mediated oxidative stress and antioxidant defense mechanisms have been analyzed. Numerous studies on various model organisms confirmed the cadmium-derived enhancement of ROS production. **Wang et al. (2019)**, **Singh et al. (2018)**, **and Li et al. (2019)** detected increased free radical (as $H_2O_2$, $O_2^-$) formation in the lettuce and tomato seedlings, respectively upon Cd treatment. Moreover, Cd-induced cytotoxicity by excessive ROS was demonstrated by **Zhuang et al. (2019)** in duck renal tubular epithelial cells, **Modareszadeh et al. (2021)** in *Arabidopsis*, and interestingly by **Khojastehfar et al. (2015)** also in MCF-7 human breast cancer cell line and by **Wang et al. (2015)** in PC-12 cells. Although Cd does not induce elevation of ROS generation directly **(Liu, Qu and Kadiiska, 2009)**, its impact on excessive free radical formation is unquestionable. Hence, we were interested if *S. pombe* cell exposure to Cd will cause oxidative stress to the yeast cells. As the end product of the membrane lipid peroxidation is malondialdehyde (MDA), its increasing content indicates an escalation of oxidative stress **(Requena et al., 1996)**. In our experimental conditions, increasing Cd concentration enhanced MDA production in a dose-dependent manner (Figure 5 brown symbols). AsA pre-treatment significantly reduced MDA content (Figure 5 green symbols) suggesting its protective role against Cd-mediated oxidative stress. Culture treatment with AsA did not significantly affect MDA content in the control cells without Cd supplementation, while the addition of all Cd concentrations led to the dramatic increase of MDA amount. However, cells pre-incubated with AsA were largely protected against Cd toxicity. More detailed statistical analyses by two-way ANOVA of the significance of the single and mutual impact of Cd addition and AsA supplementation on MDA content are shown in Table 1 (MDA). Our observation is supported by other studies, revealing that antioxidant additives such as *Senna alexandrina* extract or salicylic acid or components enhancing the antioxidant capacity of the cell such as selenium or silicon might serve as cells protectors against Cd-mediated oxidative stress **(Wang et al., 2020; Liu et al., 2016; Tang et al., 2015; Lin et al., 2012)**.

To confirm the protective role of AsA through its antioxidant property against Cd toxicity, the activity of catalase (CAT) the antioxidant enzyme responsible for $H_2O_2$ scavenging, was analyzed. Lower Cd concentration (0.01 mM) led immediately to the enhancement of CAT activity of cells without AsA treatment, while AsA pre-incubation was sufficient to protect cells against Cd-mediated oxidative stress without CAT support. Increasing Cd concentrations in AsA untreated cells at first further enhanced CAT activity, however high Cd concentrations (0.04, and 0.1 mM) consistently interrupted CAT activity, and thus subjected cells to oxidative stress. Strikingly, AsA pre-treatment promoted cellular CAT activity even under exposure to high Cd concentrations leading to extended cell protection (Figure 6). Statistical evaluation by two-way ANOVA of the significance of the single and mutual impact of Cd addition and AsA supplementation on the CAT activity is shown in Table 1 (CAT). Similarly, while CAT activity in Cd-treated roots of *Brassica napus* has dramatically increased, concomitant AsA supplementation brought CAT activity to a normal state hence triggering decreased sensitivity of the plant towards Cd **(Jung et al., 2020)**. Interestingly, similar to our observations, AsA supplementation to Cd-exposed rats largely improved their antioxidant defense status **(Chen et al., 2018)**.

A presented study reveals that the impact of Cd on the exposed organism, through oxidative stress induction, is highly conserved among species and is evident to a similar extent from yeast to higher organisms such as plants, animals, or humans. Contamination of food, water, and air represents the major pathway of human exposure to Cd including Cd exposure to children through breast milk leads to Cd-mediated adverse effects of human health through oxidative stress induction **(Wu et al., 2016; Kippler et al., 2012; Chunhabundit, 2016)**. Thus, we assume that according to our observation, people living in areas exposed to critical amounts of Cd in the environment that are at high risk of oxidative stress should consider supplementation of their nutrition with AsA.

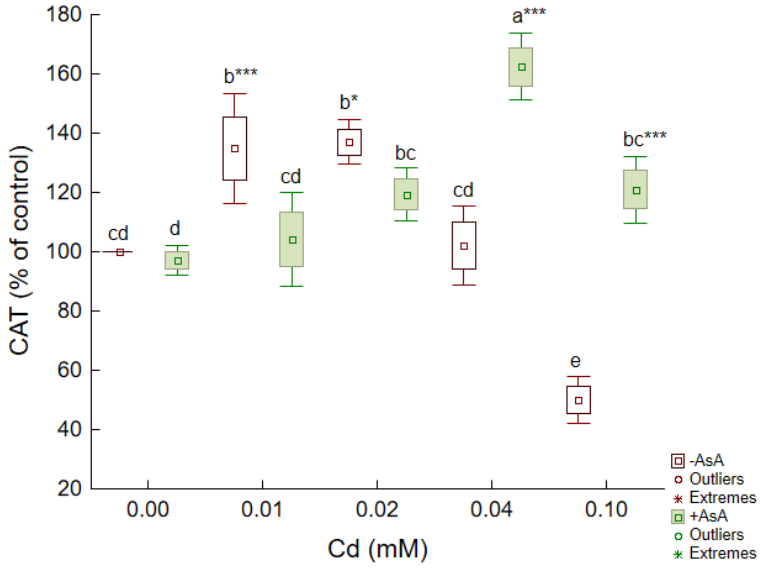


**Figure 6** Cadmium mediated impairments of the CAT activity are restored by AsA supplementation.
Note: CAT activity determination expressed as % of control depicts the differences of the CAT activity between AsA untreated (brown symbols) and AsA treated (10mM) (green symbols) cells after three hours of incubation with different Cd concentrations (0, 0.01, 0.02, 0.04, 0.1 mM), n = 7. Number of * indicate statistical significance ($p$ <0.05 *, 0.01 **, 0.001 ***), identical letters above bars denote that these values possess no significant difference.

**Table 1** Statistical analyses by two-way ANOVA for changes in the cell growth and biochemical parameters. AsA – AsA treatment, Cd – different Cd concentration, AsA×Cd – interaction of AsA and Cd effects.

| Trait | Effect | *SS* | *MS* | *F* | *p* |
|---|---|---|---|---|---|
| | | | *Growth intensity* | | |
| After 3 h | AsA | 0.0745 | 0.0745 | 38.0 | 0.000000 |
| | Cd | 3.2725 | 0.3273 | 167.0 | 0.000000 |
| | AsA×Cd | 0.0509 | 0.0051 | 2.6 | 0.009611 |
| After 6 h | AsA | 0.1269 | 0.1269 | 18.95 | 0.000045 |
| | Cd | 27.4592 | 2.7459 | 410.12 | 0.000000 |
| | AsA×Cd | 0.0964 | 0.0096 | 1.44 | 0.181151 |
| After 9 h | AsA | 0.0675 | 0.0675 | 5.95 | 0.017271 |
| | Cd | 62.0007 | 6.2001 | 546.18 | 0.000000 |
| | AsA×Cd | 0.0732 | 0.0013 | 7.02 | 0.000000 |
| | | | *Biochemistry* | | |
| Protein | AsA | 0.15156 | 0.15156 | 217.29 | 0.000000 |
| | Cd | 0.61470 | 0.15367 | 220.32 | 0.000000 |
| | AsA×Cd | 0.03920 | 0.00980 | 14.05 | 0.000001 |
| MDA | AsA | 2.9769 | 2.9769 | 204.522 | 0.000000 |
| | Cd | 49.1648 | 12.2912 | 844.446 | 0.000000 |
| | AsA×Cd | 0.6322 | 0.1580 | 10.858 | 0.000015 |
| CAT | AsA | 0.0116 | 0.0116 | 0.0187 | 0.892672 |
| | Cd | 10.8949 | 2.7237 | 4.3866 | 0.010434 |
| | AsA×Cd | 11.6358 | 2.9089 | 4.6849 | 0.007852 |

**Table 2** The effect of Cd and AsA supplementation on protein level, MDA content and CAT activity in absolute values.

| Cd (mM) | Protein (mg.mL$^{-1}$) | | MDA (mg.mL$^{-1}$) | | CAT (μM.mg$^{-1}$.min$^{-1}$) | |
|---|---|---|---|---|---|---|
| | -AsA | +AsA | -AsA | +AsA | -AsA | +AsA |
| 0 | 0.95 ±0.11 | 1.18 ±0.08$^{***}$ | 0.47 ±0.06 | 0.34 ±0.02$^{nd}$ | 2.98 ±0.85 | 2.89 ±0.12$^{nd}$ |
| 10 | 0.90 ±0.07 | 1.17 ±0.08$^{***}$ | 1.51 ±0.11 | 1.07 ±0.04$^{***}$ | 4.31 ±0.67 | 2.81 ±0.23$^{**}$ |
| 20 | 0.80 ±0.05 | 1.02 ±0.10$^{***}$ | 1.90 ±0.08 | 1.33 ±0.09$^{***}$ | 6.37 ±0.96 | 3.35 ±0.22$^{***}$ |
| 40 | 0.65 ±0.09 | 0.89 ±0.09$^{***}$ | 2.93 ±0.20 | 2.05 ±0.11$^{***}$ | 3.64 ±0.61 | 5.94 ±0.62$^{***}$ |
| 100 | 0.66 ±0.07 | 0.84 ±0.07$^{**}$ | 4.32 ±0.15 | 3.31 ±0.06$^{***}$ | 1.79 ±0.61 | 4.42 ±0.53$^{***}$ |

## CONCLUSION

A surrounding environment contaminated with increasing Cd concentration causes dose-dependent cell growth intensity alteration. Cell pretreatment with 10 mM AsA considerably improved the growth of cells resulting in a significantly increased gain of protein mass ($p$ <0.001). Cd-mediated oxidative stress led to lipid peroxidation resulting in the consistent increase of the MDA content ($p$ <0.001) upon rising Cd concentration. The strong antioxidant property of AsA caused that its pre-treatment markedly decreased MDA content ($p$ <0.001) suggesting cell membrane lipid preservation. Enzymatic protection against oxidative stress ensured by CAT was significantly altered by Cd ($p$ <0.05), while AsA pre-treatment restored CAT activity ($p$ <0.01) thereby protecting cells against Cd-induced oxidative stress (summarized in Table 2).

Taken together, our results demonstrate the positive effect of AsA against cellular impairments resulting from Cd toxicity upon its contamination. We show here that cells subjected to Cd contamination substantially profit from AsA supplementation. However, further studies are required to understand the exact protective nature of AsA on the overall cellular parameters under conditions of the contaminated environment.

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This article does not contain any studies that would require an ethical statement.

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# THE CHEMICAL COMPOSITION OF POLLEN, STAMINATE CATKINS, AND HONEY OF *CASTANEA SATIVA* MILL.

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## ABSTRACT

The chemical composition of pollen and honey primarily depends on the botanical and geographical origin of the species, as well as other factors – climatic conditions, soil type, plant species, etc. The present study was to knowledge the biochemical profile of pollen, staminate catkins, and honey samples of *Castanea sativa* Mill. which were examined under conditions of Ukraine. Proteins are the major components of pollen and staminate catkins (169.0 ±1.60 g.kg$^{-1}$ and 69.8 ±1.67 g.kg$^{-1}$, respectively), while saccharides are predominant in honey samples (38.0 ±1.32 g.kg$^{-1}$ fructose, 32.5 ±0.68 glucose g.kg$^{-1}$ and 6.1 ±0.06 g.kg$^{-1}$ sucrose). Glutamic acid (13.30 g.kg$^{-1}$), aspartic acid (13.05 g.kg$^{-1}$), and proline (12.45 g.kg$^{-1}$) were predominant nonessential amino acids in the chestnut pollen. The content of macro and microelements was found in the pollen and staminate catkins much higher than in the honey. All *Castanea sativa* samples are a very valuable source of potassium as the main mineral element contained in pollen (7400 mg.kg$^{-1}$), staminate catkins (7760 mg.kg$^{-1}$), and honey (981 mg.kg$^{-1}$). Microelements such as manganese and iron prevailed in pollen (478 mg.kg$^{-1}$ Mn and 461 mg.kg$^{-1}$ Fe), and staminate catkins (247 mg.kg$^{-1}$ Mn and 109 mg.kg$^{-1}$ Fe), and heavy metals (Hg, Sr, Sn, Sb, Li) are present only in the pollen samples with the most abundant Sr (12.8 mg.kg$^{-1}$) and Sn (1.9 mg.kg$^{-1}$) content and can be used as indicator suggesting the environmental pollution status in the region. Regarding the vitamin content, vitamin C was the most represented in all samples. Obtained results indicate that chestnut is species with important constituents such as amino acids and vitamins, with low content of heavy metals and high content of biogenic elements that may be used in phytotherapy and phytopharmacology.

**Keywords:** *Castanea sativa*; staminate catkins; pollen; honey; amino acid

## INTRODUCTION

*Castanea* Mill. (Chestnut), belonging to the family *Fagaceae* is distributed in over ten species in temperate climate regions, including Asia, Europe, and North America. Among chestnut trees, *C. sativa, C. crenata, C. dentata,* and *C. mollissima* are important species consumed as a crop. Chestnut trees are valuable not only as food but also as a honey source plant, are already classified as major honey plants in many countries. The inflorescence is composed either of male or female flowers, or staminate and pistillate catkins occur together. Only the male flowers produce nectar that is secreted on the surface of the dislocated at the base of filaments in staminate catkins, from which is possible to produce 27.2 kg of honey per 100 trees **(Farkas and Zajácz, 2007)**. Chestnut pollen, bee pollen, and bee bread were also identified to contain functional and specific substances **(Ivanišová et al., 2015; Hudz et al., 2017a; Hudz et al., 2017b; Nikolaieva et al., 2017)**, mineral elements **(Motyleva et al., 2018b)** which show anti-inflammatory, antimicrobial **(Avşar et al., 2016)**, antioxidant activity **(Feas et al., 2012; Alves et al., 2013; Avşar et al., 2016; Kızılpınar Temizer et al., 2018; Nikolaieva et al., 2019)**, and hepatoprotective effect **(Yıldız et al., 2013)** was high in *C. sativa* honey **(Turski et al., 2016)**.

Major honey components are carbohydrates (mainly monosaccharides, fructose, glucose, and sucrose) and water. In small amounts, honey also contains minerals, proteins, organic acids, volatile components, phenolics, vitamins, and pigments. Although present in small amounts, these components are very important for honey characterization and nutritive properties **(White, 1975)**. A much better parameter for botanical and geographical origin identification was the analysis of free amino acids in honey, which was reported by several authors **(Hermosín, Chicón and Cabezudo, 2003; Cotte et al., 2004; Nozal et al., 2004; González Paramás et al., 2006)**. Pollen

contains an average of 1500 mg.100g$^{-1}$ of amino acids, which means approximately ten times more than the average concentration in honey **(Davies, 1975)**. The major amino acid in honey is proline (80 – 90% of the total amino acids) except lavender, thyme, and rosemary kinds of honey where the major amino acid is phenylalanine **(Hermosín, Chicón and Cabezudo, 2003; Cotte et al., 2004)**. The presence of proline is of animal origin and the quantity of proline depends on the time spent by the nectar in the bee's crop **(Petrov, 1974)**. The proline content was proposed by the International Honey Commission as a honey ripeness and possible sugar adulteration indicator, with a minimum value of 180 mg.kg$^{-1}$ for fresh, genuine honey **(Bogdanov et al., 1999)**. Besides proline, 26 amino acids (both essential and nonessential) were also identified in honey **(Hermosín, Chicón and Cabezudo, 2003)**.

Due to ecological-geographical conditions very important are not only differences in some compounds (biogenic elements, heavy metals), which can increase/decrease the quality of pollen or honey but also evaluation of pollen sizes **(Grygorieva et al., 2013; Grygorieva et al., 2017; Motyleva, Gruner and Semenova, 2018a, Horčinová Sedláčková et al., 2018, Horčinová Sedláčková et al., 2020)**.

The chemical composition of honey primarily depends on the botanical origin of species or cultivars. However, climatic conditions and/or geographical origin can also affect the chemical composition even within the same honey type **(Anklam, 1998)**.

The aim of the present study was to knowledge the biochemical profile of pollen, staminate catkins, and honey samples of *Castanea sativa* cultivar introduced under conditions of Ukraine, such as determination of saccharides, carotenoids, vitamins, amino acids, minerals (macro- and microelements) and heavy metals were examined. Comparative data of the mineral and amino acid composition of pollen and staminate catkins from different ecological-geographical zones little reflected in the literature.

**Scientific hypothesis**

*Castanea sativa* is a major honey plant in many countries is a potential source of phytochemical compounds used in food and health products against less-researched and less-used staminate catkins and chestnut pollen. The scientific hypothesis of this study was to examine the pollen, staminate catkins, and honey samples of *Castanea sativa* species and compared them due to their phytochemical, amino acid, and mineral profiles as appropriate substances of phytotherapeutic and phytopharmacology preparations.

## MATERIAL AND METHODOLOGY

**Biological Material**

We examined the biological material of *C. sativa* cultivar (staminate catkins, chestnut dried pollen, and monofloral chestnut honey) introduced in M. M. Gryshko National Botanical Garden of Ukraine in Kyiv (Figure 1 and Figure 2). Staminate catkins and pollen were harvested in June 2020 in dry conditions and with no precipitation. Samples were obtained from one cultivar of three trees.

**Chemicals**

Ethanol (Centralchem s.r.o., Bratislava, Slovakia, p.a.).

Acetonitrile (Fisher Chemical, Loughborough, UK, HPLC grade).

Petroleum ether (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany, Sigma Grade, ≥99%).

Ninhydrin (Ingos, Czech Republic), nitric acid (Analytika Praha Ltd, Czech Republic).

Hydrochloric acid (Analytika Praha Ltd, Czech Republic).

Methyl cellosolve (Ingos, Czech Republic).

Filter with 0.45 μm pore size (Labicom, Czech Republic).

Tin chloride ($SnCl_2$) (Centralchem s.r.o., Bratislava, Slovakia, p.a.).

**Instruments**

HPLC system with an ELSD detector (Agilent Technologies 1260 Infinity, Santa Clara, CA, USA).

HPLC system with ninhydrin and a VIS detector (Model AAA-400 amino acid analyzer, Ingos, Czech Republic).

UV-VIS spectrophotometer (UV Jenway Model 6405, UV/VIS, England).

ICP-OES system (Ultima 2, Horiba Scientific, France).

ES column (Zorbax SB-C18, 4.6x25.0 mm, 5 μm particle size, Agilent, Santa Clara, CA, USA).

Microwave oven (Milestone 1200, Milestone, Italy).

Vertical shake table (GFL, Germany).

Centrifuge (EBA 21, Hettich, Germany). Cation exchanger (LG ANB sodium cycle, Laboratory of Spolchemie).

**Laboratory Methods**

**Phytochemical analyses**

***Determination of dry matter, ash, and protein content***

Total dry matter, ash, and protein content were determined according to the EN method **(ČSN EN 12145, 1997)**. Total lipid content was determined according to methods specified in the ISO method **(ISO 659, 1998)**.

***Determination of saccharides***

For the determination of saccharides, 1 g of sample was extracted with 10 mL of extraction solution (ultrapure water and ethanol mixed in ration 4:1) in a 50 mL centrifugation tube placed on a vertical shake table (GFL, Germany). After 1 h of extraction, samples were centrifuged for 4 min at 6000 rpm in a centrifuge (EBA 21, Hettich, Germany); the supernatant was filtered using a filter with 0.45 μm pore size (Labicom, Czech Republic) and filled up to 50 mL in a volumetric flask with ultrapure water. An Agilent Infinity 1260 liquid chromatography (Agilent Technologies, USA) equipped with an ELSD detector was used for the determination of saccharides. A Prevail Carbohydrates ES column (250/4.6 mm) was used as a stationary phase and acetonitrile (VWR) mixed with water in a 75:25 volume ratio was used as the mobile phase.

***Determination of carotenoid***

Total carotenoid content expressed as beta-carotene was analyzed at a wavelength of 445 nm spectrophotometrically (VIS spectrophotometer UV Jenway Model 6405 UV/VIS). Sample (1 g) was disrupted with sea sand and extracted with acetone until complete discoloration. Petroleum-ether was added and then water, in purpose to the separation of phases. After the separation, the petroleum ether-carotenoid phase was obtained and the absorbance was measured **(ČSN 560053, 1986)**.



**Figure 1** Middle phase of blooming *Castanea sativa* Mill. cultivar.



**Figure 2** Inflorescences of staminate catkins *Castanea sativa* Mill.

***Determination of mineral contents***

Sample for elemental analysis was prepared using the wet ashing method in a microwave oven (Milestone 1200, Milestone, Italy). A total of 0.25 g sample matrix was decomposed in a mixture of nitric acid (6 mL) (Analytika Praha Ltd, Czech Republic) and hydrochloric acid (2 mL) (Analytika Praha Ltd, Czech Republic).

After the decomposition sample was filtered using a filter with 0.45 μm pore size and filled up to 25 mL in a volumetric flask with ultrapure water. Elemental analysis was performed using ICP-OES (Ultima 2, Horiba Scientific, France) according to the procedure described by **Divis et al. (2015)**.

***Determination of amino acids***

Amino acids were determined by ion-exchange liquid chromatography (Model AAA-400 amino acid analyzer, Ingos, Czech Republic) using post-column derivatization with ninhydrin and a VIS detector. A glass column (inner diameter 3.7 mm, length 350 mm) was filled manually with a strong cation exchanger in the LG ANB sodium cycle (Laboratory of Spolchemie) with average particles size 12 μM and 8% porosity. The column was tempered within the range of 35 to 95 °C. The elution of the studied

amino acids took place at a column temperature set to 74 °C. A double-channel VIS detector with the inner cell volume of 5 µL was set to two wavelengths: 440 and 570 nm. A solution of ninhydrin (Ingos, Czech Republic) was prepared in 75% v/v methyl cellosolve (Ingos, Czech Republic) and in 2% v/v 4 M acetic buffer (pH 5.5). Tin chloride ($SnCl_2$) was used as a reducing agent. The prepared solution of ninhydrin was stored in an inert atmosphere ($N_2$) in darkness at 4 °C. The flow rate was 0.25 ($mL.min^{-1}$) and the reactor temperature was 120 °C.

**Determination of vitamins**

***Determination of Vitamin C concentration***

Vitamin C concentration was determined according to the method described by **Vikram, Ramesh and Prapulla (2005)**. Precisely 1 g of the sample was mixed with meta-phosphoric acid and centrifuged at 10,000 rpm for 10 min. at 4 °C in a refrigerated centrifuge (Model H-2000C). The supernatant was sieved through Whatman No 4 filter paper. The filtrate was diluted with 1 mL of 0.8% meta-phosphoric acid and filtered with a 0.450 Millipore filter and 20 µL of the sample injected into the HPLC (Shimadzu 20 A-Series, with column -NH2 -LUNA-100A (250X4.6mm), Diameter – 5uLand Refractive Index Detector.

***Determination of Vitamin A content***

The method described by **Okwu (2004)** was used to determine vitamin A. Sample (1 g) was weighed into a beaker and macerated with a 10 mL mixture of acetone and n-hexane (1:1) and filtered. Thereafter, 10 mL of 50% $(NH_4)_2SO_4$ solution was added, vigorously shaken, and allowed to settle. The upper layer was collected, and the absorbance read in Spectrophotometer (Spectro21D, Pec Medicals, USA) at 450 nm against hexane as blank beta-carotene served as standard. Retinol equivalent was obtained by the conversion factor: 1 mg retinol equal to 12 mg of dietary beta-carotene.

***Determination of Vitamin A content***

Analysis Tocopherols and tocotrienols were analyzed by normal-phase HPLC using a Merck-Hitachi system (Merck, Darmstadt, Germany) with fluorescence detection (excitation: 292 nm, emission: 330 nm). Chromatographic separation was achieved within 45 min at 35 ±10 °C using a Eurospher-100 DIOL column (250 × 4.0 mm, 7 µm), preceded by a Eurospher-100 DIOL guard column (5×4.0 mm, 7 µm) (both from Knauer, Berlin, Germany). An isocratic mobile phase of n-hexane/MTBE (98:2, v/m) was used at a flow rate of 1.5 $mL.min^{-1}$. Individual tocochromanols were identified by comparing their retention times with those of external standards and quantified by 5-point calibration curve of externalstandards.

***Determination of Vitamin B1, B2, and B6***

**Reagents**

All reagents and standards were of analytical grade. Vitamins standards were from Sigma-Aldrich with a purity ≥99%. Potassium hydroxide (KOH), concentrated sulfuric acid ($H_2SO_4$), and acetic anhydride was 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).

**Standard**

Preparation 1 $mg.mL^{-1}$ of working standards i. e. thiamine chloride hydrochloride, riboflavin, pyridoxine hydrochloride, and folic acid were dissolved in methanol. Solutions were further serially diluted to the concentration of 5 – 10 $\mu g.mL^{-1}$ for thiamine chloride hydrochloride, 1.5 – 2 $\mu g.mL^{-1}$ for riboflavin, 5 – 10 $\mu g.mL^{-1}$ for pyridoxine hydrochloride, and 1 – 2 $\mu g.mL^{-1}$ for folic acid. 50 µl of each solution was used for analysis.

**Sample**

Preparation. Each sample was weighed and triturated into fine powder. The powdered sample was suspended in 50 mL of methanol, sonicated for 15 min, and dilute to 100 mL with methanol. 50 µL of each was for HPLC analysis.

Chromatographic Conditions Shimadzu LC system (LC 20A pump) equipped with UV/Visible detector was used for analysis. A single wavelength of 254 nm was used for simultaneous detection. A Promosil HPLC column C18 was used at 35 ºC. Methanol – 0.05 M sodium acid phosphate (80 + 20, v/v) at pH 7.0 was used as mobile phase. The flow rate was adjusted to 0.8 $mL.min^{-1}$. 50 µL of sample and standard solutions were injected in a single injection.

**Description of experiment**

***Sample preparation***

Freshly flowers (male catkins) were collected randomly from the three genotypes at the ballon stage (June 2020). Pollen samples released from male flowers (catkins) were further dried under laboratory conditions at 20 – 25 °C and prepared for analysis.

***Honey samples***

A total of 5 honey samples were collected from Ukrainian beekeepers. The samples were stored at room temperature in the dark and analyzed within the month after extraction. To confirm the beekeeper's denomination, the samples were subjected to preliminary analyses. Uniflorality of samples was confirmed according to electrical conductivity.

**Number of samples analyzed:** 18
**Number of repeated analyses:** 3
**Number of experiment replication:** 1

**Statistical Analysis**

Basic statistical analyses were performed using PAST 2.17. Data were presented as mean ±standard error of the mean (SEM) and differences between means were considered at $p < 0.05$. The variability of all these parameters was evaluated using descriptive statistics.

## RESULTS AND DISCUSSION

Bee pollen contains high amounts of carbohydrates, amino acids, fatty acids, minerals, and vitamins. The contents of these ingredients depend on the botanical origin of the pollen. The presence of these components shows that bee pollen can be used for human nutrition **(Campos et al., 2008)**.

In honey, the content of proteins, including the enzymes, is relatively low and has a minor nutritive significance. On the other hand, the proteins, including the enzymes, are usually used as honey quality evaluation parameters. This is because protein content and enzyme activities vary regarding the botanical and geographical origin of the honey **(Rodriguez-Otero et al., 1994; Flanjak et al.,**

**2016; Anklam, 1998; Won et al., 2008)**. **Roulston, Cane, and Buchmann (2000)** present that pollen contains from 2.5 to 61% of protein.

The phytochemical compounds in samples of pollen, staminate catkins, and honey of *Castanea sativa* are shown in Table 1. Our results show that the pollen and staminate catkins have the highest content of cellulose (954.6 $g.kg^{-1}$ and 190.6 $g.kg^{-1}$, respectively) and proteins (169.0 $g.kg^{-1}$ and 911.3 $g.kg^{-1}$). In the dried pollen from different plant species, the protein content varied from 142.1 – 314.0 $g.kg^{-1}$ **(Human and Nicolson, 2006)**, 150.4 – 276.9 $g.kg^{-1}$ DW **(Carpes et al., 2009)**, 242.3 – 341.8 $g.kg^{-1}$ DW **(Estevinho et al., 2012)**, 191.0 – 271.0 $g.kg^{-1}$ DW **(Feas et al., 2012)**, 125.0 – 251.5 $g.kg^{-1}$ DW **(Nogueira et al., 2012)**, 142.4 – 289.5 $g.kg^{-1}$ DW **(Yang et al., 2013)**, 160.3 – 323.4 $g.kg^{-1}$ **(Lilek et al., 2015)**. **Flanjak et al. (2016)** compared the protein content of various species and their study presented that chestnut honey had the highest protein content (the mean was 94.8 $mg.100g^{-1}$) followed by sage (the mean was 79.2 $mg.100g^{-1}$), honeydew honey (the mean was 59.4 $mg.100g^{-1}$). Black locust honey had the lowest protein content (the mean was 30.4 $mg.100g^{-1}$). These differences are probably due to the different plant species and different environmental conditions. The protein content may be a bioactive indicator that contributes to the pharmacological activities of honey **(Tonks et al., 2003; Yusof et al., 2007; Chua, Lee and Chan, 2015)**.

Three free sugars, namely, fructose, glucose, and sucrose, were detected in all samples, while the honey samples have the highest content of carbohydrates (fructose 38 $g.kg^{-1}$, glucose 32.5 $g.kg^{-1}$, and sucrose 6.1 $g.kg^{-1}$). Saccharides represent the main components of honey, and many papers have been published for using sugars as an indication of adulteration but cannot be used to identify the floral or geographical origins of honey **(Sivakesava and Irudayaraj, 2002)**.

**Kim et al. (2020)** presented free sugar content and sugar content per catkin in floral nectar of various *Castanea* spp. Fructose was present in the interval from 61.5 (*C. mollissima*) to 74.8% (*C. crenata*), glucose in the range of 33.7 (*C. dentata*) to 20.5% (*C. crenata*), sucrose ranged from 3.2 (*C. sativa*) to 13.4% (*C. dentata*), free sugar content varied from 22.6 (*C. crenata*) to 82.5 $\mu g.\mu L^{-1}$ (*C. mollissima*), sugar content per catkin was determined in the range from 0.31 (*C. sativa*) to 1.35 $mg.catkin^{-1}$ (*C. crenata*) and nectar volume per catkin in the interval from 8.7 (*C. dentata*) – 42.7 $\mu L.catkin^{-1}$ (*C. crenata*) **(Kim et al., 2020)**.

**Carocho et al. (2014)** presented results of saccharides of *C. sativa* staminate catkins: fructose – decoctions (152.08 –160.41 $mg.g^{-1}$), infusions (123.58 – 148.94 $mg.g^{-1}$); glucose – decoctions (149.09 – 191.91 $mg.g^{-1}$), infusions (145.71 – 164.07 $mg.g^{-1}$); sucrose – decoctions (25.69 – 27.01 $mg.g^{-1}$), infusions (26.67 – 35.68 $mg.g^{-1}$).

Regarding the vitamin content, vitamin C was the most represented in all samples in the following amounts in pollen 95 $mg.kg^{-1}$, staminate catkins 71 $mg.kg^{-1}$, and honey 55 $mg.kg^{-1}$.

The fat content predominates in pollen and staminate catkins against chestnut honey in amounts 55.4, 30.0, and 0.3 $g.kg^{-1}$, respectively.

The fat content of the polyfloral bee pollen from different plant species varied in the intervals 10.0 to 130.0 $g \cdot kg^{-1}$ DW **(Campos et al., 2008)**, 23.3 – 33.2 $g.kg^{-1}$ **(Estevinho et al., 2012)**, 43.0 – 63.0 $g.kg^{-1}$ DW **(Feas et al., 2012)**, 23.5 – 33.3 $g.kg^{-1}$ DW **(Nogueira et al., 2012)**, 37.0 – 65.0 $g.kg^{-1}$ DW **(Soares de Arruda et al., 2013)**, 6.6 – 107.9 $g.kg^{-1}$ DW **(Yang et al., 2013)**, 60.7 $g.kg^{-1}$ – 157.9 $g.kg^{-1}$ **(Lilek et al., 2015)**.

**Table 1** Contents of some phytochemical compounds in pollen, staminate catkins and honey of *Castanea sativa* Mill.

| Components | Pollen (*mean ±SD*) | Staminate catkins (*mean ±SD*) | Honey (*mean ±SD*) |
|---|---|---|---|
| **Cellulose ($g.kg^{-1}$)** | 954.6 ±29.32 | 190.6 ±3.10 | <1 |
| **Dry matter ($g.kg^{-1}$)** | 138.2 ±2.86 | 911.3 ±19.66 | – |
| **Water (%)** | – | – | 14.43 ±0.11 |
| **Proteins ($g.kg^{-1}$)** | 169.0 ±1.60 | 69.8 ±1.67 | 6.5 ±0.16 |
| **Fats ($g.kg^{-1}$)** | 55.4 ±0.15 | 30.0 ±0.11 | 0.3 ±0.01 |
| **Beta Carotene ($mg.kg^{-1}$)** | 3.0 ±0.04 | 2.4 ±0.009 | 2.0 ±0.01 |
| **Vitamin A ($mg.kg^{-1}$)** | <0.1 | <0.1 | <0.1 |
| **Vitamin E ($mg.kg^{-1}$)** | <0.1 | 24.0 ±0.13 | <0.1 |
| **Vitamin B1 ($mg.kg^{-1}$)** | 1.5 ±0.04 | 5.8 ±0.03 | 2.1 ±0.04 |
| **Vitamin B2 ($mg.kg^{-1}$)** | <0.5 | 1.3 ±0.01 | 0.5 ±0.03 |
| **Vitamin B6 ($mg.kg^{-1}$)** | <0.5 | 3.2 ±0.01 | 1.1 ±0.009 |
| **Vitamin C ($mg.kg^{-1}$)** | 95.0 ±2.10 | 71.0 ±2.17 | 55.0 ±0.96 |
| **Folic acid ($mg.kg^{-1}$)** | 0.7 ±0.02 | 1.8 ±0.02 | 0.6 ±0.01 |
| **Nicotinamide ($mg.kg^{-1}$)** | <0.5 | 5.8 ±0.05 | 10.9 ±1.21 |
| **Fructose ($g.kg^{-1}$)** | 3.0 ±0.01 | 4.1 ±0.05 | 38.0 ±1.32 |
| **Glucose ($g.kg^{-1}$)** | 1.8 ±0.04 | 2.2 ±0.02 | 32.5 ±0.68 |
| **Sucrose ($g.kg^{-1}$)** | 4.6 ±0.05 | 2.1 ±0.04 | 6.1 ±0.06 |

**Note:** *mean* – arithmetic mean; *SD* – standard error of the mean.

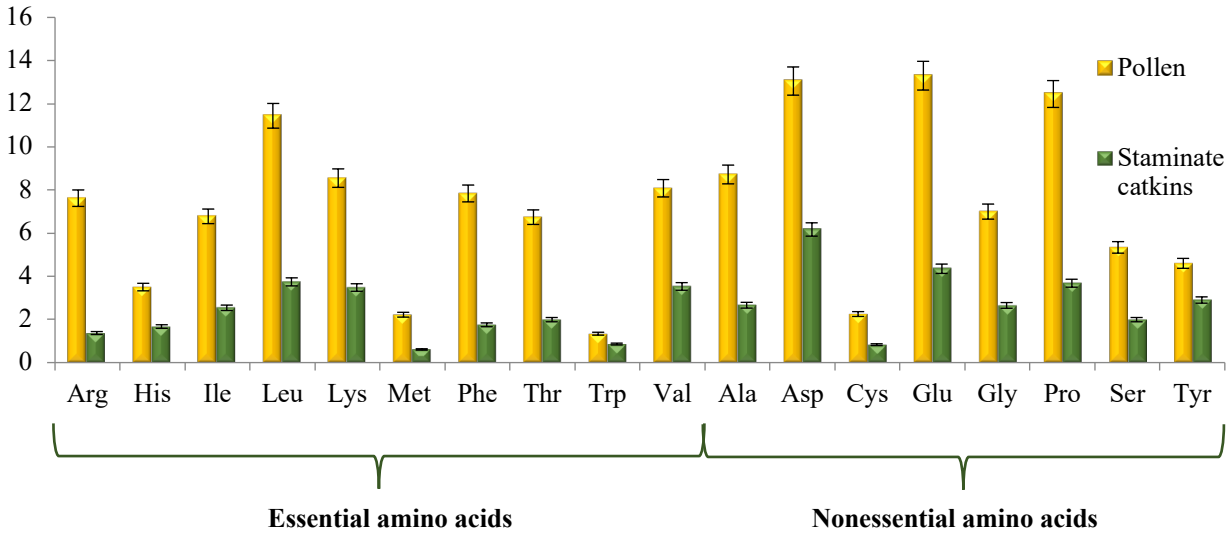


**Figure 3** Amino acid composition from pollen and staminate catkins of *Castanea sativa* Mill. (g.kg$^{-1}$).

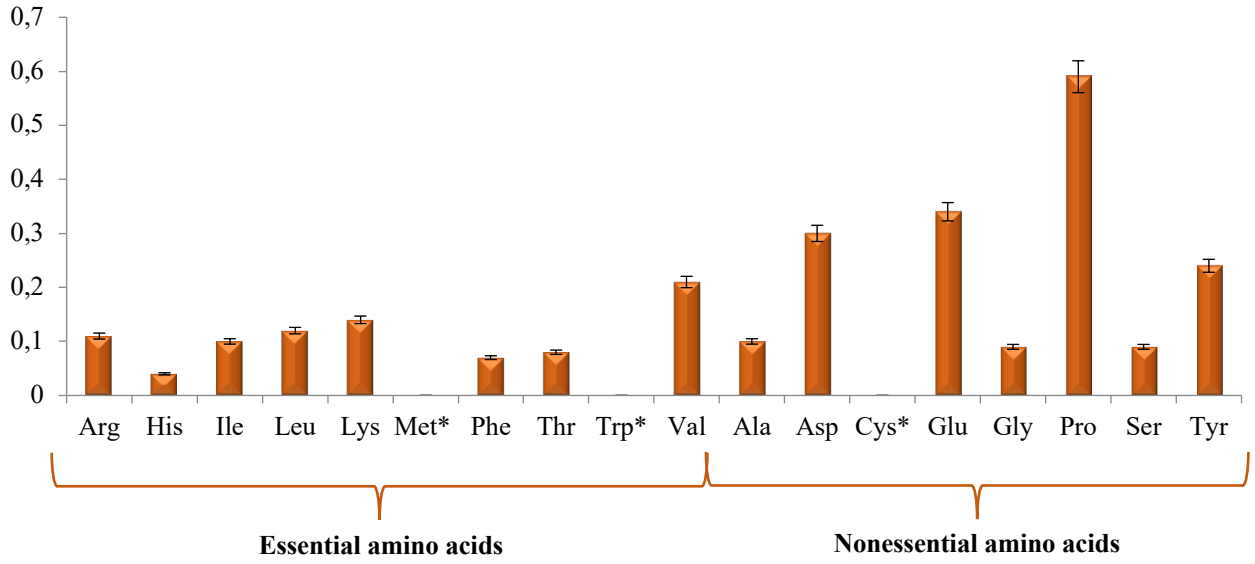


**Figure 4** Amino acid composition from the honey of *Castanea sativa* Mill. (g.kg$^{-1}$).
Note: * – methionine, tryptophan and cysteine determined at the trace level (<0.01).

Data presented in Figure 3 showed that eighteen (10 essential and 8 non-essential) amino acids were found in pollen and staminate catkins samples. Pollen had the greatest content of amino acids in comparison with staminate catkins and honey. Glutamic acid (13.30 g.kg$^{-1}$), aspartic acid (13.05 g.kg$^{-1}$), and proline (12.45 g.kg$^{-1}$) were the predominant nonessential amino acids in pollen of *C. sativa*. Their content represents 38.41% of total amino acids. High amounts of leucine (11.44 g.kg$^{-1}$) are also found in the pollen of *C. sativa* where its content is almost comparable to proline one.

According to many authors in bee pollen predominate aspartic acid, glutamic acid, glycine, and leucine **(Lilek et al., 2015; Taha, Al-Kahtani and Taha, 2019)**. **De Simone et al. (1980)** in their studies noted that proline almost always appears as the dominant amino acid in pollens of all examined species. According to them in *Pinus pinea* and *Pinus nigra*, proline represents more than 81% of the total amino acids. **Lilek et al. (2015)** demonstrate that in fresh pollen *C. sativa* predominates proline (2.96 g.100g$^{-1}$), slightly less glutamic acid (1.91 g.100g$^{-1}$) and aspartic acid (1.77 g.100g$^{-1}$).

The most abundant amino acid in staminate catkins was found to be aspartic acid (6.16 g.kg$^{-1}$), slightly below glutamic acid (4.35 g.kg$^{-1}$), which is 13.19 and 9.30% of the sum of total amino acid.

**Kim et al. (2020)** studied the nectar of *Castanea* spp. male flowers and results showed that the predominant amino acids as asparagine, proline, glutamic acid were detected in amounts of 12.9% (*C. crenata*) – 41.0% (*C. sativa*) of asparagine, 7.3% (*C. mollissima*) – 29.6% (*C. crenata*) of proline and 7.8% (*C. sativa*) – 13.9% (*C. crenata*) of glutamic acid of the total amino acids.

**Table 2** The concentration of the determined macro- and microelements and heavy metals in the pollen, staminate catkins and honey samples of *Castanea sativa* Mill.

| Components (mg.kg$^{-1}$) | Pollen (*mean ±SD*) | Staminate catkins (*mean ±SD*) | Honey (*mean ±SD*) |
|---|---|---|---|
| **K** | 7400 ±117 | 7760 ±219 | 981 ±77 |
| **P** | 3845 ±87 | 1819 ±109 | 96 ±1.6 |
| **Ca** | 0.27 ±0.010 | 5940 ±227 | 170 ±1.7 |
| **Mg** | 1730 ±66 | 2040 ±169 | 69 ±1.4 |
| **Na** | 98 ±1.8 | 31.7 ±0.07 | 25.1 ±0.5 |
| **Fe** | 461 ±56 | 109 ±13 | 13.6 ±0.6 |
| **Mn** | 478 ±43 | 247 ±34 | 3.7 ±0.3 |
| **Zn** | 126 ±19 | 30.1 ±0.8 | 2.1 ±0.05 |
| **Cu** | 16.7 ±0.9 | 10.4 ±0.3 | <1 |
| **Ni** | 41.5 ±1.1 | –* | –* |
| **Sr** | 12.8 ±0.5 | –* | –* |
| **Sn** | 1.9 ±0.03 | –* | –* |
| **Hg** | 0.027 ±0.001 | –* | –* |
| **Se** | <0.05 | –* | –* |
| **Sb** | <0.25 | –* | –* |
| **Li** | <0.5 | –* | –* |

Note: mean – arithmetic mean; *SD* – standard error of the mean; * – non-sampled.

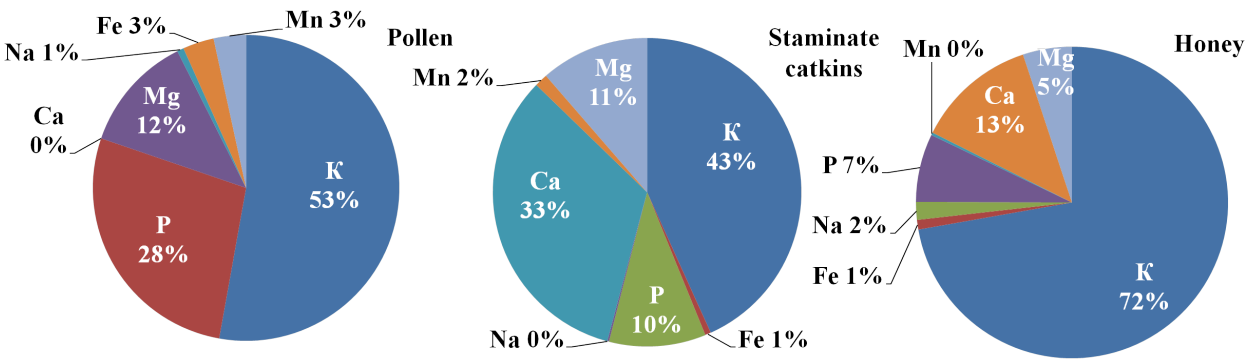


**Figure 5** Macro- and microelement percentage composition in the pollen, staminate catkins and honey samples of *Castanea sativa* Mill.

The main amino acids found for honey were proline (0.59 g.kg$^{-1}$), which is 22.52% of the sum of total amino acids. Lower but also important amounts of glutamic acid (0.34 g.kg$^{-1}$), aspartic acid (0.30 g.kg$^{-1}$), tyrosine (0.24 g.kg$^{-1}$) and valine (0.21 g.kg$^{-1}$) were present (Figure 4). Among the component methionine, tryptophan and cysteine are found to be present at the trace level (<0.01).

Honey contains approximately 0 – 1.5% amino acids. The proline is the main amino acid in pollen **(Ball, 2007; Wang and Li, 2011)** accounting for 50 – 85% of total amino acids **(Anklam, 1998; Czipa, Borbély and Győri, 2012)**. Lower values indicated immaturity or falsification of honey with sugar **(Von der Ohe, Dustmann and Von der Ohe, 1991; Bogdanov et al., 1999; Hermosín, Chicón and Cabezudo, 2003)**. Furthermore, amino acid content can serve as a useful indicator for estimating the floral origin of honey **(Iglesias et al., 2004; Senyuva et al., 2009)**. Proline originates from the salivary secretions of honey bees during nectar conversion **(da Silva et al., 2016)**. The amount of proline is important not only as a criterion in determining the quality value and maturity of the honey but also in determining the detection of its adulteration with sugar. **Bayram and Demir (2018)** reported that they found proline in *C. sativa* honey amount 503.46 to 692.88 mg.kg$^{-1}$. **Yılmaz and Küfrevioğlu (2001)** found proline values in the range of 300 – 860 mg.kg$^{-1}$ in their studies on honey (from different plant species) collected from the Eastern and South-eastern Anatolia Regions. **Oddo and Piro (2004)** reported that they found an average proline amount of 468 in 721 mg.kg$^{-1}$ honeydew honey samples. **Manzanares et al. (2011)** found proline values in the range of 664 – 1689 mg.kg$^{-1}$ in pine honey and 310 – 1057 mg.kg$^{-1}$ in flower honey. **Flanjak et al. (2016)** in a comparative study presented that the lowest proline content had the black locust honey with a mean of 157.0 mg.1000g$^{-1}$. Chestnut honey had the highest proline content with a mean of 699.0 mg.1000g$^{-1}$ **(Flanjak et al., 2016)**. **Czipa, Borbély and Győri (2012)** found that the proline value of 143 honey samples of different plant origin obtained from countries such as Hungary, Tasmania, New Zealand, Malaysia, Thailand, South Africa, Finland from 242 to 2283 mg.kg$^{-1}$. The honey proline content was in the range 39 – 632 mg.g$^{-1}$ (acacia honey), 99 – 617 mg.g$^{-1}$ (lime honey), and 84 – 450 µg.g$^{-1}$ (rape honey) **(Kečkeš et al.,**

**2013)**. The average proline content in the analyzed honey varied from 140.14 to 389.66 μg.g$^{-1}$ **(Kowalski et al., 2017)**. **Chen et al. (2017)** discovered higher proline content (an average of about 317 μg.g$^{-1}$ and a maximum above 600 μg.g$^{-1}$) in rape honey. It was determined that the proline content of honey samples from different plant species in the North-Eastern Anatolia Region of Turkey (Erzurum, Narman, Oltu, Tortum) was between 443 – 715 mg.kg$^{-1}$ **(Cengiz, Tosun and Topal, 2018)**.

The present study, bioaccumulation, and biosorption of minerals and heavy metal concentration (K, P, Ca, Mg, Na, Fe, Mn, Zn, Cu, Ni, Sr, Sn, Hg, Se, Sb, Li) were observed in pollen, staminate catkins and chestnut honey (Table 2 and Figure 5).

Macro-elements (Na, K, Ca, Mg, P) are the most represented group, dominated by potassium contained in pollen (7400 mg.kg$^{-1}$), staminate catkins (7760 mg.kg$^{-1}$) and honey (981 mg.kg$^{-1}$). Potassium is the main mineral element with an average of one-third of the total. Some research has suggested that the trace element content in honey is mainly based on the botanical origin of honey, for instance, light blossom honey having a lower content than dark honey such as honeydew, chestnut, and heather **(Sevlimli, Bayulgen and Varinlioglu, 1992; Gonzalez-Miret et al., 2005)**. The content of mineral substances in honey samples ranges from 0.2 to 10.3 g.kg$^{-1}$ **(White, 1975)**. **Rodriguez-Otero et al. (1994)** determined the macro- and microelements content of 91 original honey samples. Potassium was the most abundant of the elements with an average content of 1500 mg.kg$^{-1}$.

Calcium is the 2$^{nd}$ most abundant element in the staminate catkins (5940 mg.kg$^{-1}$) and honey (170 mg.kg$^{-1}$), while in pollen it is only in a small amount (0.27 mg.kg$^{-1}$). Calcium was the prominent element in the honey sample in the amount of 170 mg.kg$^{-1}$. According to the data, the Ca contents of the honey sample in the present study was higher than the ones established in Serbia (20.1 mg.kg$^{-1}$) **(Jevtić et al., 2012)**, Portugal (40.3 mg.kg$^{-1}$) **(Alves et al., 2013)**, Hungary (47.9 mg.kg$^{-1}$) **(Czipa, Andrási and Kovács, 2015)**, but lower than in Italy (356 mg.kg$^{-1}$) **(Pisani, Protano and Riccobono, 2008)**.

We can compare the percentages of individual components, which show that potassium was the most represented element in the honey (72%), pollen (53%), and staminate catkins (43%). Phosphorus was the second most abundant element in pollen (28%) of the total minerals, while calcium represented in staminate catkins 33% and the honey samples 13% of the total minerals.

Microelements (Mn, Zn, Cu, Fe, Se, and Ni) are the second represented group of biogenic elements, where the content of manganese and iron prevailed in all samples. The content of manganese and iron predominate in pollen (478 mg.kg$^{-1}$ Mn and 461 mg.kg$^{-1}$ Fe) and staminate catkins (247 mg.kg$^{-1}$ Mn and 109 mg.kg$^{-1}$ Fe) than in the honey samples (13.6 mg.kg$^{-1}$ Fe and 3.7 mg.kg$^{-1}$ Mn).

**Kızılpınar Temizer et al. (2018)** determined heavy metal content in three unifloral honey samples of *Castanea sativa* from the Black Sea region in Turkey. Fe contents in honey samples were found in amounts from 6.280 mg.kg$^{-1}$ to 20.515 mg.kg$^{-1}$ and showed similarity to the other study in Turkey **(Yücel and Sultanoğlu, 2013)**, and higher than performed in Portugal (0.70 – 7.06 mg.kg$^{-1}$) **(Alves et al., 2013)**, in Serbia (0.58 – 4.21 mg.kg$^{-1}$) **(Jevtić et al., 2012)**, and New Zealand (0.67 – 3.39 mg.kg$^{-1}$) **(Vanhanen, Emmertz and Savage, 2011)**. **Kızılpınar Temizer et al. (2018)** determined Mn contents in amounts from 21.58 to 26.52 mg.kg$^{-1}$, which are higher compared to our honey sample. The mineral content in honey is also related to the geographical origin, therefore it is possible to be used to differentiate between honey of different geographical origins **(Davies, 1975; Wang and Li, 2011)**. **Fernandez-Torres et al. (2005)** reported that the concentrations of Zn, Mn, Mg, and Na in eucalyptus, heather, orange blossom, and rosemary honey from Spain were strongly dependent on their botanical origin.

Heavy metals (Hg, Sr, Sn, Sb, Li) are present only in the pollen samples with the most abundant of Sr (12.8 mg.kg$^{-1}$), Sn (1.9 mg.kg$^{-1}$), Hg (0.0027 mg.kg$^{-1}$) content and other in the trace level (<0.05 mg.kg$^{-1}$).

Heavy metals such as Cd, Pb, Cr, and Ni in honey can be used as indicators suggesting the environmental pollution status in the region **(Porrini et al., 2003; Wang and Li, 2011)**. It has been reported that Pb contamination of honey in polluted and nonpolluted areas was not significantly different because of the high variability of the data, whereas the highest values were often found in polluted areas **(Bogdanov, 2006)**.

## CONCLUSION

The chemical composition, amino acid content, and minerals determined in pollen, staminate catkins, and honey obtained from the *Castanea sativa* cultivar were analyzed due to evaluate the representation of individual components in selected chestnut parts and the chestnut product. Total amino acids predominate in the pollen than in the staminate catkins and honey. Glutamic acid, aspartic acid, and proline were the most abundant. The fat content predominates in pollen and staminate catkins. The content of macro and microelements was found in the pollen and staminate catkins much higher than in the honey. All *Castanea sativa* samples are a very valuable source of potassium, which is necessary for the water and electrolyte balance. Vitamin analyses showed the presence of vitamin C in predominant quantity. Heavy metals present only in the pollen samples can be used as indicators suggesting the environmental pollution status in the region. These results showed significant amounts of biogenic substances in dried pollen, staminate catkins, and honey samples, which indicated that various plant parts are appropriate for phytotherapy and phytopharmacology.

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The authors declare no conflict of interest .

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This article does not contain any studies that would require an ethical statement.

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# BASIL – A COMPARISON OF THE TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY IN SELECTED CULTIVARS

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## ABSTRACT

During the lifetime, the human body forms a considerable amount of free radicals damaging DNA, cell membranes, and their components. A wider application of basil (*Ocimum basilicum*), an aromatic plant and one of the common gastronomic commodities in the human diet could help to prevent the formation of free radicals and to remove them from the human body. Therefore, determination of antioxidant activity and total content of phenolic substances in selected cultivars of basil (Ohře, Sweet green, Salad leaf, Purple opal, Thai) in a fresh and frozen state and the mixture with another plant (garlic, mint, rocket, spinach) in the fresh and refrigerated state was performed. The total content of phenolic substances in basil was established by the spectrophotometric Folin-Ciocalteu method. DPPH spectrophotometric method was used to analyze the antioxidant activity. The results have shown statistically significant differences between basil cultivars under the same growing conditions. The values of antioxidant activity in frozen samples ranged from 5.1 ±0.4 mg.g$^{-1}$ AA FW to 11.71 ±0.18 mg.g$^{-1}$ AA FW and the total phenolic content varied between 2.77 ±0.16 mg.g$^{-1}$ GAE FW in TH and 8.93 ±0.13 mg.g$^{-1}$ GAE FW. A statistically significant difference between fresh and frozen samples was established only in the "Ohře" cultivar. After the storage in cold temperatures, all mixtures showed a reduction in the antioxidant activity and total content of phenolic substances. The mixture of basil and mint performed the highest values of antioxidant activity and total content of phenolic substances and significantly differed from the other mixtures most often. The addition of basil and its mixtures to food and beverages can substantially increase their biological value and subsequently also the quality of human nutrition.

**Keywords:** Basil; *Ocimum basilicum*; total phenolic content; antioxidant activity; cultivar

## INTRODUCTION

Basil is one of the basic commodities of world gastronomy, particularly Italian. It also plays an important role in Thai, Indonesian, French and Brazilian cuisine. The spread of basil all over the world has driven the development of an immense number of cultivars differing in shape, color, and size of leaves, plant height, taste, and aroma. The place of origin affects the resistance of the plant to higher or lower temperatures or temperature changes. Furthermore, the plants may differ, for example, also in their resistance to infestation. These properties influence the content of chemical substances including phenolic substances or other non-phenolic antioxidants with positive effects on human health.

Basil leaves rich in essential oils are mainly used to add flavor to food and drinks. These essential oils extracted from leaves show many positive impacts on human health. They can lower blood cholesterol, help to lower blood sugar in diabetic patients, and protect the stomach from ulcers **(Dzida, 2010; Ezeani et al., 2017)**. Recently, in connection to the great success of sage seeds (known by its Mayan name chia), basil seeds have also emerged on the market. They are a good source of polyunsaturated fatty acids. Regular consumption of basil, both leaves, and seeds, provides many health benefits.

Basil (*Ocimum basilicum*, OB), also known as great basil, is one of the most common and available species of basil. It is usually an annual plant with a height of up to 60 cm. Apart from a few subspecies that are purple, it is a brightly green plant **(Hiltunen and Holm, 1999)**. Basil has a large number of cultivars and subspecies.

Basil leaves contain over 92% of free or bound water, approximately 5% of tannins and sitosterols, up to 1% of fats, 0.5 – 1.5% of essential oils, 0.17% of oleanolic acid; ursolic acid is present as well.

The composition of seeds is also remarkable, they contain 25% of fat, more than half of which consists of α-linolenic acid, furthermore, minerals, vitamins, antioxidants, and phenolic substances **(Hiltunen and Holm, 1999; Nirali et al., 2017)**.

Antioxidants are a very important group of substances with promising properties that prevent oxidation not only in

food but also in the human body. The main groups of antioxidants in basil occurring primarily in its essential oils are phenolic antioxidants and endiols **(Velíšek and Hajšlová, 2009)**. Endiol antioxidants are principally represented by ascorbic acid and its salts and derivatives. As part of its antioxidant activity, ascorbic acid reacts with reactive oxygen species, which slows down the oxidation of lipids, or with fatty acid radicals and alkoxy radicals. The efficiency of reactions with lipid radicals can be increased by the presence of tocopherols, which, in contrast to ascorbic acid, are fat-soluble **(Velíšek and Hajšlová, 2009)**.

Phenolic compounds can be generally defined as substances containing at least one benzene ring bonding at least one hydroxyl group or their functional derivatives. This group is extensive and diverse with several characteristics in common. Phenolic substances are often secondary metabolites of plants with antioxidant properties which are desirable and valued for a healthy human diet. Hydroxyl group of phenolic substances binds to hydroxy peroxide, superoxide, or hydroxyl radicals and thus prevents radicals from degenerative changes in the body **(Velíšek and Hajšlová, 2009; Skinner and Hunter, 2013)**.

The proportion of individual substances in plant material obtained from basil (*Ocimum basilicum*) is influenced not only by growing conditions and cultivar but also by other factors, such as the method of treatment and storage time. This work aimed to determine the antioxidant activity and total content of phenolic substances in selected cultivars in fresh and frozen leaves of basil (*Ocimum basilicum*).

### Scientific hypothesis

**Hypothesis 1** – Individual basil cultivars differ in the total content of phenolic substances and antioxidant activity.

**Hypothesis 2** – the value of the total content of phenolic substances and antioxidant activity differs in the case of direct processing and within the processing after the storage in a freezer at the temperature of -18 °C.

**Hypothesis 3** – the total content of phenolic substances and antioxidant activity differ between the mixtures prepared from basil (*Ocimum basilicum*) and other plant material (garlic, mint, rocket, spinach).

## MATERIAL AND METHODOLOGY

### Samples

The following basil cultivars were used for the research (*Ocimum basilicum*):

*Ohře (marked as OH) – Czechoslovak cultivar (Figure 1), bright green, pleasant, and typically aromatic, with larger leaves and white flowers **(Muráriková and Neugebauerová, 2018; GRIN Czech, 2021)**.

*Sweet green (marked as SG) – cultivar similar to the cultivar Ohře, medium-sized, shiny, and convex leaves, tastes sweeter, typical.

*Salad leaf (marked as SL) – very broad leaves with a typical green color and taste. However, compared to the Ohře and Sweet Green cultivars, the intensity of the taste is lower.

*Purple opal (marked as PO) – purple color (high content of anthocyanins in leaves **(Simon et al., 1999))**, it is used more for aesthetic purposes than taste (Figure 2).

* Thai (marked as TH)the – different shape of leaves, the coloring of stems red, taste and smell similar to anise or licorice, with better resistance to higher temperatures, but worse resistance to cold **(Asijské bylinky, 2020; Semena.cz, 2020)**.

For selected cultivars, a difference in the analyzed values depending on the type of cultivar was expected.



**Figure 1** *Ocimum basilicum* – cultivar Ohře.



**Figure 2** *Ocimum basilicum* – cultivar Purple opal.

These samples originated from the same locality, were grown under the same conditions (the same soil composition, amount of sunshine,…) and agrotechnical treatments. For this reason, the dependence of these samples on different growing conditions for different sample origins was not expected.

The samples of basil of unknown variety marked as "XX1" and "XX2", were used for the research as well.

Furthermore, the mixtures of basil, cultivar "Ohře", with other plant material were produced with the following composition:

- mix 1 – basil, cultivar "Ohře" (92 wt. %) + garlic (*Allium sativum*) (8 wt. %) (marked as OHG)
- mix 2 – basil, cultivar "Ohře" (95 wt. %) + peppermint (*Mentha peperita*) (5 wt. %) (marked as OHM)
- mix 3 – basil, cultivar "Ohře" (95 wt. %) + rocket (*Eruca vesicaria*) (5 wt. %) (marked as OHR)
- mix 4 – basil, cultivar "Ohře" (90 wt. %) + spinach (*Spinacia oleracea*) (10 wt. %) (labelled as OHS)

**Chemicals**

To prepare samples, an extraction solution in the composition of demineralized water and methanol was used (Methyl alcohol p.a., $CH_3OH$, CAS: 67-56-1, Penta, Chrudim, Czech Republic) in a ratio of 30:70.

The following chemicals were used to determine the total phenolic content:

Folin-Ciocalteu's reagent, (Sigma-Aldrich, St Louis, MO, USA)

Sodium carbonate anhydrous p.a. ($Na_2CO_3$, Sigma Aldrich, St Louis, MO, USA)

Gallic acid (GAE) (Sigma Aldrich, St Louis, MO, USA)

Determination of antioxidant activity was performed using the following chemicals:

DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich, St Louis, MO, USA)

methanol (Methylalkohol p.a., $CH_3OH$, CAS: 67-56-1, Penta, Chrudim, ČR).

All chemicals were of analytical reagent grade or equivalent analytical purity.

Demineralized water – 18.2 MOhm.cm, Milli-Q, Millipore was used throughout the experiment.

**Animals and Biological Material:**

Samples of cultivars Ohře, Purple opal, Sweet green, Salad leaf, and Thai were supplied by the Faculty of Horticulture of Mendel University in Brno, Czech Republic.

The samples were grown in the gene pool areas of this university in Lednice, Czech Republic (approximate GPS 48.79353068254463, 16.798598359226208). The location is in a temperate climate zone. The climate is continental with an average annual temperature of above 9 °C and average annual rainfall of around 520 mm **(ČSÚ, 2016)**.

All other plant materials (samples of basil of unknown variety, garlic, peppermint, rocket, spinach) were purchased in a regular retail chain in Zlín, Czech Republic.

**Instruments**

The following instruments were used for the measurements:

Spectrophotometer Libra S6, Biochrom, Cambridge, UK (Determination of AA),

Spectrophotometer Specord 50 Plus, Analytik Jena, Jena, Germany (Determination of TPC),

Analytical scale Voyager PRO VP214C (Ohaus corporation, Pine Brook USA)

**Laboratory Methods**

***Determination of the total phenolic content***

Folin-Ciocalteu spectrophotometric method was selected to determine the total phenolic content **(Cicco et al., 2009)**. The principle of this method is based on the reaction of the Folin-Ciocalteu reagent with phenolic substances (it reduces itself to a blue-colored mixture). The concentration of the reduced reagent is proportional to the amount of absorbed blue light at the wavelength $\lambda = 765$ nm **(Costa et al., 2019; Albergamo et al., 2020)**.

***Determination of the antioxidant activity***

DPPH spectrophotometric method modified according to Brand-Williams was used to establish the antioxidant activity **(Brand-Williams, Cuvelier and Berset, 1995)**. The principle of this method is based on the reaction of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). In the reaction of DPPH with an antioxidant, a radical is eliminated, and colorless products are formed from purple DPPH. The concentration of antioxidants and their activity is proportional to the decrease of absorbance.

**Table 1** Antioxidant activity (AA) and total phenolic content (TPC) in cultivars of brasil (*Ocimum basilicum*) in fresh and frozen state.

| Cultivars | AA (mg.g$^{-1}$ AA ±*SD*) | | TPC (mg.g$^{-1}$ GAE ±*SD*) | |
|---|---|---|---|---|
| | Fresh | Frozen | Fresh | Frozen |
| **SG** | | 6.9 ±0.4 | | 5.49 ±0.09 $^{a}$ |
| **SL** | | 11.71 ±0.18 $^{a}$ | | 8.93 ±0.13 $^{a, b, c}$ |
| **TH** | 4.2 ±0.3 $^{‡}$ | 5.1 ±0.4 $^{a, b}$ | 3.3 ±0.3 $^{‡U}$ | 2.77 ±0.16 $^{b, d}$ |
| **PO** | | 8.34 ±0.18 $^{b}$ | | 7.00 ±0.11 $^{d}$ |
| **OH** | 8.2 ±0.2 $^{*U,‡}$ | 6.22 ±0.17 $^{*U}$ | 4.23 ±0.16 $^{‡U}$ | 4.7 ±0.3 $^{c}$ |
| **XX1** | 0.630 ±0.007 | | 1.06 ±0.16 | |
| **XX2** | 0.519 ±0.010 | | 1.0 ±0.3 | |

Note: $^{a, b, c, d}$ - the same superscipts indicate a statistically significant difference between the cultivars (frozen samples, Multiple comparison of p values - two-sided),

$^{‡}$ - indicates a statistically significant difference between the cultivars (fresh samples, t-test - two-sided),

$^{‡U}$ - indicates a statistically significant difference between the cultivars (fresh samples, U-test),

$^{*U}$ - indicates a statistically significant difference between the samples analysed on the day of processing and frozen samples (U-test).

The measurement was performed at the wavelength λ = 515 nm **(Brand-Williams, Cuvelier and Berset, 1995; Lee et al., 2005; Thaipong et al., 2006)**.

**Description of the Experiment**

**Sample preparation:** Leaves with a minimal petiole part were separated from the plants and crushed in a ceramic mortar as it maximally prevents the loss of antioxidants. It is also a traditional method. The crushed biological material was processed immediately, stored in the freezer (-18 °C to -20 °C) for approximately 60 days or in the refrigerator (+4 °C to +7 °C) for 4 days. The storage was airtight. After crushing and potential storage, approximately 1 g of the sample was placed into 2 vials and weighted precisely using analytical scales. Then, 10 mL of extraction solution (30% water : 70% methanol) was immediately added to the vials. The sealed vials were placed in a 50 °C water bath for 60 min. After being filtrated, the samples were stored in the refrigerator until the analysis. Due to a technical refrigeration error, it was impossible to analyze some fresh samples.

The parameters of the determination of the differences between the cultivars were as follows:

**Number of samples analyzed:** 4 (fresh), 5 (frozen)
**Number of repeated analyses:** 8x
**Number of experiment replication:** 1x

The parameters of the determination of the differences between the mixtures were as follows:

**Number of samples analyzed:** 4 (fresh), 4 (refrigerated)
**Number of repeated analyses:** 4x
**Number of experiment replication:** 1x

***Determination of the total phenolic content***

Before the analysis, 20% sodium carbonate solution was prepared. Then, 0.1 mL of basil extract was added to a 10 mL volumetric flask with 5 mL of deionized water. 0.5 mL of Folin-Ciocalteu reagent was added to the mixture and shaken. 1.5 mL of 20% $Na_2CO_3$ was added after three minutes and shaken. Demineralized water was added to an overall volume of 10 mL. After the incubation for 30 min at room temperature, the absorbance at 765 nm was determined using a spectrophotometer. To express the results, unit milligrams of Gallic acid (GAE) per 100 grams of fresh weight of the sample $m_g$ were used.

***Determination of the antioxidant activity***

First, a stock methanol solution of DPPH is prepared. For the analysis, 4 mL of the stock solution and 20 μL of the extract are placed into a test tube. If the samples contained a small amount of antioxidants, their volume was increased to either 70 or 210 μL. After 60-min rest in the dark, the absorbance of the sample was measured using a spectrophotometer. The results are expressed as the equivalent of ascorbic acid measured in g per 100 g of fresh weight of the sample $m_a$ using a standard calibration curve.

## 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. Shapiro-Wilk test of normality was performed within all monitored samples.

To establish statistically significant differences between individual basil cultivars, Levene's test of homogeneity of variances was performed. Since the conditions for the calculation by ANOVA analysis did not comply in any of the monitored data sets, a non-parametric Kruskal-Wallis test (α = 0.05) and multiple comparisons of p values (two-sided) (α = 0.05) based on Mann-Whitney U test were performed.

Considering fresh samples, there were only 2 cultivars (TH and OH) to compare, Levene's test of homogeneity was performed after applying the Shapiro-Wilk test of normality. If the conditions for a t-test complied, F-test and a two-tailed t-test (α = 0.05) were executed. Otherwise, the Mann-Whitney U test (α = 0.05) was used. The same procedure was applied to compare fresh and frozen samples.

To determine statistically significant differences between the mixtures, Levene's test of homogeneity was performed after the test of normality. Due to non-compliance with the conditions of normality and homogeneity, the results were further calculated using the nonparametric Kruskal-Wallis test (α = 0.05) and the differences between the groups were monitored using multiple comparisons of p values (two-sided) (α = 0.05).

Levene's test of homogeneity of variances was performed to establish differences between the group processed immediately and the group stored for 4 days in the refrigerator. If the conditions of normality and homogeneity were fulfilled, F-test and the two-tailed t-test were used. Otherwise, the Mann-Whitney U test (α = 0.05) was applied.

## RESULTS AND DISCUSSION

### AA and TPC in the cultivars

As can be seen in Table 1 shows the measurement of fresh cultivars, only OH and TH cultivars could be used for the analysis. There was a statistically significant difference between cultivars for both AA determination using t-test (t = 11.754; sv = 6; $p$ <0.05) and TPC analysis, where it was necessary to employ a non-parametric U-test (U = 10.00; $p$ <0.05). Regarding AA, the OH cultivar performed almost twice the value established in TH. What is more, it showed 15 times higher values if compared to groups purchased in the retail chain. Similar results were achieved in TPC determination. Even though the differences were less considerable, the OH cultivar reached higher values than TH. If compared to the groups purchased in the retail chain, the OH cultivar showed up to 4 times higher values.

As far as a comparison of fresh and frozen samples is concerned, a statistically significant difference was demonstrated only in the OH cultivar within the U-test (U = 0.00; $p$ <0.05). Nevertheless, the trends in the OH values showed a decrease in AA and rice in TPC during freezing. Considering the TH cultivar, contrasting trends were recorded. When comparing the differences in frozen samples, the highest values of TPC and AA were established within the SL cultivar and the lowest within the TH cultivar. The values of antioxidant activity in frozen samples ranged from 5.1 ±0.4 mg.g$^{-1}$ AA FW in TH to 11.71 ±0.18 mg.g$^{-1}$ AA FW in SL. The values of TPC varied between 2.77 ±0.16 mg.g$^{-1}$ GAE FW in TH and 8.93 ±0.13 mg.g$^{-1}$ GAE FW in SL.

**Murárikova and Neugebauerová (2018)** reported the values of the same cultivars OH (0.083 – 0.220 mg.g$^{-1}$ AA FW), PO (0.079 – 0.166 mg.g$^{-1}$ AA FW), and a similar cultivar Lettuce Leaf (0.047 – 0.139 mg.g$^{-1}$ AA FW) which

are a magnitude smaller than the values recorded in this study. The reason may be a different method of cultivation, measuring method and agrotechnics applied.

**Hiltunen and Holm (1999)** state that fresh basil generally contains a large amount of ascorbic acid. **Dzida (2010)** reported the value of 0.26 mg.g$^{-1}$ AA FW measured in the Polish variation of basil ´Kasia´ and the value of 0.204 mg.g$^{-1}$ AA FW established in the variance ´Wala´. A lower value of ascorbic acid in basil (0.119 mg.g$^{-1}$ AA FW) is monitored by **Martyniak-Przebyszewska and Wojciechowski (2004).** Comparing the vitamin C content in basil and rosemary leaves, **Dumbravă et al. (2012)** declared a higher content of vitamin C in basil (0.271 mg.g$^{-1}$ FW) than in rosemary (0.185 mg.g$^{-1}$ FW).

**Szarowská (2012)** presented the antioxidant activity for various aromatic herbs. The highest antioxidant activity was found in the sample of lemon balm (both lyophilized and dried samples), oregano (dried sample), and basil (dried sample). The lowest antioxidant activity was determined in mint (fresh sample), lemon balm (fresh sample), basil (lyophilized sample), and oregano (lyophilized sample). Basil showed the average values depending on the temperature of the leachate and method of processing.

**Chrpová et al. (2010)** examined the antioxidant activity of dried basil in the water extract (70 °C). They determined a value of 55.5 mg.g$^{-1}$ AA DM. **Szarowská (2012)** determined the values of antioxidant activity in the water extract higher than 92.3 mg.g$^{-1}$ AA DM.

**Szarowská (2012)** established TPC in selected varieties of aromatic plants, where the lowest TPC content was monitored in a sample of lavender (lyophilized sample), mint (frozen sample), oregano (lyophilized sample), and chamomile (dried samples). In contrast, the highest TPC was determined in oregano (dried samples), lemon balm (dried and lyophilized samples), and mint (lyophilized samples). The basil reached the average values depending on the temperature of the leachate and method of processing.

**Zheng and Wang (2001)** recorded the value of TPC in the *Ocimum basilicum* of 2.23 mg.g$^{-1}$ GAE FW. The TPC content in the study by **Vábková and Neugebauerová (2010)** was higher in the monitored cultivars – ´Ohře´ 3.63 mg.g$^{-1}$ GAE FW, ´Lettuce Leaf´ 3.47 mg.g$^{-1}$ GAE FW, ´Purple Opaal´ 3.43 mg.g$^{-1}$ GAE FW and ´Compakt´ 2.50 mg.g$^{-1}$ GAE FW. The TPC content was established to be higher in this study (more than twice when evaluating the "Ohre" cultivar). **Cruz et al. (2020)** compared colored basil cultivars Red Basil (25 ± 13 mg.g$^{-1}$ extract), Dark Opal (25 ±17 mg.g$^{-1}$ extract), Basilico Rosso (12 ±3 mg.g$^{-1}$ extract) and one green local landrace Mitikas (9 ±6 mg.g$^{-1}$ extract). Their results revealed a higher level of TPC in colored basil cultivars compared to the examined green local landrace (Mitikas).

**Shan et al. (2005)** examined the TPC of 26 spice extracts, determining 36.4 mg.g$^{-1}$ GAE FW in basil. The highest TPC was established in cinnamon (119 mg.g$^{-1}$ GAE) and oregano (101.7 mg.g$^{-1}$ GAE). The samples of sage (53.2 mg.g$^{-1}$ GAE) and mint (51.5 mg.g$^{-1}$ GAE) contained the average CP values.

**Marcinčák, Popelka and Šoltysová (2008)** determined TPC spectrophotometrically in methanol extracts of 10 selected herbs. The highest TPC was found in the sample of oregano (262.12 mg.g$^{-1}$ GAE DM), basil (249.852 mg.g$^{-1}$ GAE DM) and rapeseed (179.22 mg.g$^{-1}$ GAE DM).

**Chrpová et al. (2010)**, **Shan et al. (2005)** and **Marcinčák, Popelka and Šoltysová (2008)** emphasize the fact that basil is a rich source of TPC. However, as it is consumed only occasionally in small quantities, it has rather insignificant effects on the TPC daily dose **(Velíšek and Hajšlová, 2009)**.

## AA and TPC in the mixtures

Examining the antioxidant activity, the effect of added plant raw materials (garlic, mint, rocket, and spinach) to the cultivar "Ohře" in the fresh state was negative in all. The antioxidant activity of the mixtures decreased by more than 50% - in the mixture with garlic by 54% and in the mixture with rocket by 57%. A decrease of 41% was recorded in the mixture with spinach. The smallest decline in the antioxidant activity was recorded in the mixture with mint (30%), where the smallest decrease in TPC content was determined (11%) as well. The other mixtures showed a significant decrease of more than 45% (OHG mixture 49%, OHR mixture 53%). **Dobrinas, Stanciu and Lupsor (2017)** reported a higher TPC content in the rocket (*Eruca sativa*) (6.20 mg.g$^{-1}$ GAE) than in the cultivar "Ohře"; therefore, an increase in the TPC content of the mixture was expected. The reason for such a drop may be due to differences in the growing and processing conditions. The most significant decline was monitored in the mixture with spinach (61%).

As Table 2 shows, the effect of the storage in the refrigerator for 4 days was significantly reflected in the values of the antioxidant activity.

**Table 2** Antioxidant activity (AA) and total phenolic content (TPC) in mixtures of basil (*Ocimum basilicum*) of „Ohře" cultivar with other raw materials on the day of processing and after 4 days of the storage of the samples in the refrigerator.

| Cultivars | AA (mg.g$^{-1}$ AA ±*SD*) | | TPC (mg.g$^{-1}$ GAE ±*SD*) | |
|---|---|---|---|---|
| | Fresh | Refrigerated | Fresh | Refrigerated |
| **OHG** | 3.80 ±0.13 $^{*}$ | 2.33 ±0.16 $^{*}$ | 2.2 ±0.3 $^{a}$ | 1.3 ±0.3 $^{a}$ |
| **OHM** | 5.7 ±0.3 $^{*, a}$ | 3.98 ±0.07 $^{*, a, b}$ | 3.75 ±0.12 $^{a, b}$ | 3.6 ±0.3 $^{a, b}$ |
| **OHR** | 3.49 ±0.09 $^{*, a}$ | 1.25 ±0.12 $^{*, a}$ | 1.97 ±0.07 $^{*U}$ | 1.31 ±0.8 $^{*U}$ |
| **OHS** | 4.84 ±0.05 $^{*}$ | 1.44 ±0.02 $^{*, b}$ | 1.63 ±0.02 $^{*, b}$ | 0.813 ±0.011 $^{*, b}$ |

Note: * – indicates a statistically significant difference between the samples analysed on the day of processing and samples stored for 4 days in the refrigerator (t-test),
*U – indicates a statistically significant difference between the samples analysed on the day of processing and samples stored for 4 days in the refrigerator (U-test),
a, b – the superscripts indicate a statistically significant difference between the samples of the mixtures (Multiple comparison of p values - two-sided).

All examined mixtures performed a statistically significant reduction in their antioxidant activity. The most significant influence was measured in the mixture of the cultivar "Ohře" with spinach where the content of antioxidants decreased by 70% and a statistically significant difference was recorded ($t = 63.215$; $sv = 6$; $p < 0.05$). Leafy vegetables, such as spinach, are known to contain significant amounts of ascorbic acid together with polyphenols which increase the antioxidant effects and improve the sensory quality **(Kopec, 2010; Martín-Belloso and Soliva-Fortuny, 2010)**. During the storage and processing, losses of antioxidants in the vegetable can occur, particularly by the heat treatment (blanching, pasteurization, cooking, sterilization, preservation, freezing) **(Patras et al., 2009)** or by the oxidation caused by atmospheric oxygen. That is why leaf vegetables should be consumed mostly fresh to preserve antioxidants and not to reduce them as in the OHS mixture.

Rocket, rich in substances with antioxidant effects, is an essential part of the human diet important for detoxication of the body and the protection against oxidative stress **(Barillari et al., 2005; Sarwar et al., 2007; Abbasi et al., 2013)**. The rocket contains mainly vitamin C **(Villatoro-Pulido et al., 2012)**. Similar to the basil-spinach mixture, the antioxidant activity in the OHR mixture decreased by 64%.

Considering fresh mixtures, the highest value of antioxidant activity was established in the OHM mixture ($5.7 \pm 0.3$ mg.g$^{-1}$ AA). Despite a statistically significant decrease ($t = 5.934$; $sv = 6$; $p < 0.05$), it was also the highest value measured after 4 days of storage in the refrigerator with losses of only 23%. Therefore, based on the analysis, the OHM mixture was evaluated as the most stable mixture. That may be caused by the fact that plants, such as mint, are rich in phenolic compounds, polyphenols, and flavonoids synthesized as defensive metabolites of the plant. It has been found that, apart from mint extracts, also mint essential oils perform antioxidative properties as they contain α and β-pinene, cineole, jasmone, isomenthol, isomentone, ledol, limonene, mentofuran, menthol, menton, menthyl acetate, neomenthol, piperitone, pulegone and viridiflorol **(Kozłowska et al., 2015; Peter, 2006; Yadegarinia et al., 2006)**. The difference in the AA established in all extracts from fresh and stored mint samples can be attributed mainly to the species and growing conditions of the herbs **(Kozłowska et al., 2015; Telci et al., 2010)**. Furthermore, the content of antioxidants in mint extracts is also affected by the applied solvent.

The last examined OHG mixture showed a statistically significant decrease ($t = 7.154$; $sv = 6$; $p < 0.05$) of approximately 43%. This decline was lower than in other mixtures of OHR and OHS. The slight decrease seems to be due to the content of antioxidant phytochemicals, which prevents oxidative damage. Also, garlic contains further biologically active substances (S-allyl cysteine (SAC), S-allyl mercapto cysteine (SAMC), allicin, alixin, and selenium) which eliminate hydroxyl radicals in the blood serum and increase the activity of some enzymes with antioxidant effects **(Škrovánková et al., 2018)**. Generally, other influencing factors include the origin, soil conditions, variety, treatment, and thermal treatment **(Borek, 2001)**.

Similarly to the antioxidant activity, the TPC values were reduced after storage in the refrigerator for 4 days. The smallest reduction of 4% occurred in the OHM mixture, the largest reduction of 50% was recorded in the OHS mixture. In this mixture, also a statistically significant difference ($t = 30.670$; $sv = 14$; $p < 0.05$) was determined. The difference ($U = 0.00$; $p < 0.05$) was recorded in the OHR mixture as well, where, however, the U-test had to be applied.

## CONCLUSION

This paper aimed to determine the total content of phenolic substances and antioxidant activity in selected cultivars of basil (*Ocimum basilicum*) in a fresh and frozen state and also in the mixture of basil with other plants in a fresh and refrigerated state. The values of antioxidant activity in frozen samples ranged from $5.1 \pm 0.4$ mg.g$^{-1}$ AA FW to $11.71 \pm 0.18$ mg.g$^{-1}$ AA FW and the total phenolic content varied between $2.77 \pm 0.16$ mg.g$^{-1}$ GAE FW in TH and $8.93 \pm 0.13$ mg.g$^{-1}$ GAE FW. Considering individual cultivars, statistically, significant differences were established between some cultivars in both AA and TPC determinations. Even though differences between cultivars were recorded in both fresh and frozen samples, a statistically significant difference between the fresh and frozen samples was established only in the OH cultivar in AA analysis (U-test; $U = 0.00$; $p < 0.05$).

Fresh and refrigerated mixtures showed statistically significant differences in all mixtures considering AA; the mixture of OHR and OHS also in TPC determination. The most significant differences were found between OHM and other groups, with OHM showing the highest values.

Wider usage of basil in human nutrition as a health-promoting substance could help to suppress the formation of free radicals and their removal from the human body.

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This article does not contain any studies that would require an ethical statement.

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# THE MICROBIOLOGICAL QUALITY OF MINCED PORK TREATED WITH GARLIC IN COMBINATION WITH VACUUM PACKAGING

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## ABSTRACT

The present study aimed to evaluate the microbial quality of minced pork treated with fresh garlic, dried garlic, and garlic oil in combination with vacuum packing. The growth of Total Viable Counts (TVC), Coliform Bacteria (CB), and *Pseudomonas* spp. were evaluated. The microbiological analyses were performed by the plate dilution method. The average value of TVC was 8.45 log CFU.g$^{-1}$ in aerobically packed samples, 5.59 log CFU.g$^{-1}$ in samples treated with garlic oil, 5.36 log CFU.g$^{-1}$ in vacuum packed samples, and samples treated with dried garlic, and 4.98 log CFU.g$^{-1}$ in samples treated with fresh garlic on 8$^{th}$ day of storage. The number of TVC was significantly lower in samples treated with fresh garlic compared to samples treated with dried garlic and garlic oil on the 8$^{th}$ day of storage ($p$ <0.05). The average value of CB was 4.13 log CFU.g$^{-1}$ in aerobically packed samples, 1.82 log CFU.g$^{-1}$ in samples treated with garlic oil, the value of CB in vacuum packed samples, in samples treated with fresh garlic and also with dried garlic was lower than 1.00 log CFU.g$^{-1}$ on 8$^{th}$ day of storage. The number of CB was significantly higher in aerobically packed samples and samples treated with garlic oil compared to vacuum-packed samples, samples treated with fresh garlic and dried garlic on the 8$^{th}$ day of storage ($p$ <0.05). The average number of *Pseudomonas* spp. was 2.45 in aerobically packed samples, count of *Pseudomonas* spp. was lower than 1.00 log CFU.g$^{-1}$ in vacuum packed samples and in the sample with garlic on the 8$^{th}$ day of storage. The number of *Pseudomonas* spp. was significantly higher in aerobically packed samples in comparison with vacuum-packed samples, samples with fresh garlic, dried garlic, and garlic oil ($p$ <0.05).

**Keywords:** packaging; total viable counts; coliform bacteria; *Pseudomonas* spp.; minced pork

## INTRODUCTION

Raw meat is a rich nutrient matrix and is highly perishable. Spoilage of raw meat may occur in two ways during storage: microbial growth and oxidative rancidity **(Sebranek et al., 2005)**. Meat and meat products are highly subject to microbial deterioration, which ultimately leads to safety and quality issues if the meat is not opportunely handled and preserved **(Najjaa et al., 2020)**. This is especially the case for ground meat since ground meat is more sensitive to oxidation because it has more susceptibility to microbial spoilage due to the grounding process **(Esmer et al., 2011)**.

The microbial population that colonizes and spoils minced pork meat is highly variable and depending on which groups of microbial taxa the product has been exposed to **(Zhao et al., 2015)**. The presence of microorganisms on the surface of meat and meat products determines meat spoilage upon their interaction and growth under optimal conditions **(Doulgeraki et al., 2012)**. The most prevailing spoilage organisms in meat are bacteria, yeast, and molds. Due to the ubiquity of microorganisms, they are mostly incorporated in meat from the environment. These organisms can cause spoilage by producing off-odors in meat products. Bacterial spoilage of meat is more prominent as compared to others like yeast and molds **(Sohaib et al., 2016)**. The most common bacteria causing spoilage of refrigerated beef and pork are *Brochothrix thermosphacta*, *Carnobacterium* spp., Clostridium spp., *Enterobacteriaceae*, *Lactobacillus* spp., *Leuconostoc* spp. and *Pseudomonas* spp. Their metabolic activity can cause the defects such as sour flavors, discoloration, gas or slime production, and decreases in pH **(Casaburi et al., 2015)**.

Food packing aims to optimize food quality, to ensure food hygiene and safety, to increase its storage stability, to reduce storage costs, and directly lead to lower use of preservatives during storage **(Łopusiewicz, Jedra and Mizieińska, 2018)**. Vacuum packaging is used in the conditioning of whole pieces or small parts and it protects the meat product from contact with oxygen from the air. Vacuum packaging and storage under refrigerated conditions are often used to prolong the shelf life of fresh meat or cooked meat products **(Pennacchia et al., 2011)**.

The emerging problematics regarding the negative impact exerted by some synthetic preservatives on the health of

consumers versus the benefits imparted by natural antimicrobials and their specificity to foodborne pathogens, all these considerations are leading to the requirement for more systemic research to evaluate the toxicity and mechanism of action of natural preservatives **(Lee and Paik, 2016)**.

Garlic (*Alium sativum* L.) holds an important value due to its prophylactic and therapeutic actions. Sulfur and polyphenols present in garlic respond to the antibacterial, antifungal, and antioxidant activity **(Queiroz et al., 2009)**. Allium vegetables, particularly garlic exhibit broad antibiotic activity against Gram-positive and Gram-negative bacteria **(Whitemore and Naidu, 2000)**. Garlic has a wide spectrum of actions, not only antibacterial, antifungal, and antioxidant, but also it has beneficial effects on the cardiovascular and immune systems **(Harris et al., 2001)**. During the last decade, the antimicrobial activity of garlic, garlic-derived organosulfur compounds, and essential oil was widely investigated against both food spoilage bacteria and foodborne pathogens **(Benkeblia, 2004)**. However, the organosulfur compound volatility and low physic-chemical stability limit the possibility of its application as a food-functional ingredient. Dried garlic powder contains an important amount of alliin (sulfoxide S-allyl cysteine) (approximately 1%). The highest rate of alliin to allicin conversion has been observed in the temperature range of 35–36 °C **(Lanzotti, 2006)**. The antimicrobial activity of freeze-dried garlic and essential oils against various pathogens such as *Bacillus subtilis*, *Pseudomonas aeruginosa, S. aureus*, *E. coli,* and *Proteus* spp. was investigated **(Viswanathan et al., 2014)**.

## Scientific hypothesis

Garlic is known to have numerous beneficial effects on human health. Garlic can be used as a natural preservative to prevent meat spoilage due to bacterial growth during storage. Minced meat treated with garlic, dried garlic, and garlic oil has a longer shelf life compared to untreated meat. Fresh galic, dried garlic, and garlic oil in combination with vacuum packing are effective against many microbial species.

## MATERIAL AND METHODOLOGY

### Samples

The microbiological quality of minced pork meat treated with crushed fresh garlic, dried garlic, and garlic oil was evaluated. Samples were stored for 8 days at a temperature of 4 °C. Analyzes were performed on the $0^{th}$, $2^{nd}$, $4^{th}$, $6^{th}$ the $8^{th}$ day of storage.

### Growth medium

***Plate Count Agar (PCA)***

Plate count agar (PCA) is a bacteriological substrate used for the determination of the total number of live, aerobic bacteria in a sample. The amount of bacteria is expressed as colony-forming units per gram ($CFU.g^{-1}$), in solid samples and per mL ($CFU.mL^{-1}$) in liquid samples. The recommended technique is the pour plate technique. The samples are diluted and appropriate dilutions are added in Petri plates. Sterile molten agar is added to these plates and plates are rotated gently to ensure uniform mixing of the sample with agar. The plates are incubated at 20 or 30 °C in three days. After incubation, the number of colonies is counted on the plate with 25 – 250 colonies, which is considered to give the most accurate result. When calculating the actual number of bacteria in the sample, the dilution factor should be taken into consideration **(Atlas and Snyder, 2014)**.

***Violet Red Bile Lactose Agar (VRBL)***

VRBL, containing Bile and Violet Red dye, is based on MacConkey Agar for the detection and enumeration of lactose-fermenting bacteria and the differentiation of coliforms from non-lactose fermenting organisms in dairy products, water, and foods. Peptone provides nitrogen, vitamins, minerals, and amino acids essential for growth. Yeast extract is a source of vitamins, particularly of the B-group.

***Pseudomonas Agar (PA)***

Pseudomonas Agar Base is designed so that by the addition of the appropriate supplement the medium becomes selective for *Pseudomonas aeruginosa* or *Pseudomonas* spp. generally. Pseudomonas CFC Supplement (SR0103) is recommended for the selective isolation of Pseudomonas spp. generally. **Mead and Adams (1977)** showed that reducing the cetrimide content to 10 $mg.mL^{-1}$ allowed the growth of all pigmented and non-pigmented psychrophilic pseudomonads. To improve its selective action they added cephaloridine 50 $\mu g.mL^{-1}$) and fucidin (10 $\mu g.mL^{-1}$). This combination of agents gave a new and more specific medium for isolating pseudomonads from chilled foods and processing plants.

### Laboratory methods

***Microbiological analysis***

The following groups of microorganisms were determined in samples of minced pork meat:

- Total Viable Count (TVC).
- Coliform Bacteria (CB).
- *Pseudomonas* spp.

The plate dilution method was used for the determination of the number of colonies forming units (CFU) of each group of microorganisms. An amount of 5 g of the minced pork meat was transferred into a sterile stomacher bag containing 45 mL of 0.1% physiological solution (pH 7.0) and homogenized for 30 minutes. Microbiological analyses were conducted under standard microbiological methods.

***Determination of microorganisms***

Plate Count Agar (PCA, Oxoid, UK) was used to isolation of Total Viable Counts (TVC). PCA agar was after inoculation incubated at 30 °C for 48 hours **(STN EN ISO 7218, 2000; STN ISO 4833, 2014)**. Dilutions $10^{-4}$ and $10^{-5}$ were used to determination of TVC.

Violet Red Bile Lactose Agar (VRBL, Oxoid, UK) was used to isolation of Coliform Bacteria (CB). Inoculated agar was incubated at 37 °C for 24 hours **(STN EN ISO 9308 – 1, 2015)**.

Pseudomonas Agar (PA, Oxoid, UK) was used for isolation of *Pseudomonas* spp. Inoculated agar was incubated at 35 °C ±1 °C for 48 hours **(STN 560100, 1968)**. Dilutions $10^{-1}$ and $10^{-2}$ were used to determination of CB and Pseudomonas.

***Samples preparation***

**Preparation of garlic oil**

- 300 mL of sunflower oil,
- 300 mL of extra virgin olive oil,
- 10 cloves (50 g) of garlic.

Sunflower oil and olive oil were poured into a glass cup. The garlic cloves were cleaned and crushed in a mortar. Crushed garlic was added to the oils. Garlic oil was stored in a dark place at room temperature for 7 days. After storage, the oil was filtered and applied to meat samples.

Dried garlic was purchased in the supermarket.

Fresh garlic was extruded using a garlic press.

Samples of meat were prepared in amount 5 g and fresh garlic, dried garlic, and garlic oil were added to the amount 1 g.1 $mL^{-1}$. A total of 150 samples were analyzed (6 samples in each group).

Individual samples were prepared as follows:

- Aerobically packed samples – control group.
- Vacuum-packed samples – control group.
- Vacuum-packed samples with fresh garlic.
- Vacuum-packed samples with dried garlic.
- Vacuum-packed samples with garlic oil.

Vacuum-packed samples were packaged by vacuum packing machine (Concept VA 0010, Czech Republic).

**Enumeration of microorganisms count**

Individual colonies of microorganisms were calculated after cultivation on Petri dishes. The number of microorganisms was calculated according to the following formula:

$$N = \frac{\Sigma c}{\mathrm{V.(N1\ +0,1N2)\ .d}}$$

Where:

N – CFU in 1 g of sample; Σc – the sum of all colonies on selected Petri dishes; V – the volume of inoculum in ml inoculated on each petri dish; $N_1$ - the number of dishes used for the calculation from the first dilution; $N_2$ – the number of dishes used for the calculation from the second dilution; d – dilution factor corresponding to the first dilution.

## Statistical Analysis

The experiments were performed in triplicate and standard deviations for replication were calculated (MS Excel). Analysis of variance (ANOVA) was used to evaluate the results. Comparison of the treatment means was based on Tukey's Honest Significant Difference (HSD) test.

## RESULTS AND DISCUSSION

Minced pork is the basis for different meat preparations (e.g. sausages, meatballs, burgers), but also consumed raw. Contamination of ground meat with microorganisms can occur during the whole processing, though in particular in meat cutting plants or at retail when the meat is cut or minced with more surfaces exposed **(Ejeta et al., 2004)**. Biopreservation is focused on the utilization of natural preservatives from sources like bacteria, fungi, plants, animals, having the ability to ensure food safety due to their antimicrobial activity exerted against a wide spectrum of foodborne pathogens **(Gyawali and Ibrahim, 2014)**.

Plant-based derivatives containing different bioactive compounds can be divided into two major categories: (1) vegetable or essential oils (obtained from the olive, canola, soy, sunflower, linseed, avocado, grape seed, oregano, rosemary, coriander seed), and (2) extracts (obtained from grape seed, green tea, olive leaf, cranberry, pomegranate, broccoli, cocoa leaf, lemon balm) **(Hygreeva et al., 2014)**. Some plants can contain both major derivatives, and it has been reported that garlic can be used like fresh extract, freeze-dried powder, or oil extract **(Sallam et al., 2004)**. **Kačániová et al. (2016)** suggest the possibility of applying the *Pimpinella anisum*, *Mentha spicata* var. *crispa*, *Thymus vulgaris* L., *Origanum vulgare* L. essential oil as natural food preservatives and potential sources of antimicrobial ingredients for the food industry.

The average value of Total Viable Counts (TVC) ranged from 4.59 log $CFU.g^{-1}$ on 0. day to 8.45 log $CFU.g^{-1}$ on 8. day in aerobically packed samples, from 4.59 log $CFU.g^{-1}$ to 5.36 log $CFU.g^{-1}$ in vacuum-packed samples. The average value of TVC ranged from 4.59 log $CFU.g^{-1}$ to 4.98 log $CFU.g^{-1}$ on the 8th day of storage in samples treated with fresh garlic. The highest value of TVC was 4.65 log $CFU.g^{-1}$ on 2. day in samples treated with dried garlic and 5.64 log $CFU.g^{-1}$ on the 4th day in samples treated with garlic oil (Table 1).

Freeze-dried fresh garlic and the spray-dried microencapsulated essential oil can be effectively used in meat and meat products as natural alternatives to synthetic food additives, particularly as effective antimicrobial agents **(Najjaa et al., 2020)**.

**Park and Chin (2014)** compared the antimicrobial activity of 1.4% and 2.8% fresh garlic extract against TVC in minced pork stored at 4 °C for 28 days. They found out, that values of TVC were lower in the samples containing 1.4 and 2.8% of fresh garlic than in the control.

**Gheisari and Ranjbar (2012)** investigated the antimicrobial effects of equivalent concentrations of garlic derivatives in ground camel meat during storage at 4 °C. The antioxidant activities of the various ingredients added followed the order of fresh garlic (FG), garlic powder (GP), and garlic oil (GO). After 14 days of storage, the value of TVC of both FG and GP formulated meat was significantly lower than that of either the control samples. However, the addition of GO resulted in no significant difference in TVC when compared with the control.

**Sallam et al. (2004)** reported that the antimicrobial effects of the fresh garlic were the best out of three preparations, followed by garlic powder and garlic oil. They reported that the low activity of garlic oil and garlic powder compared to fresh garlic can be caused by the losses of organosulfur compounds during sample preparations.

However, **Benkeblia (2004)** reported, that the essential oil extracts of garlic had a marked antibacterial activity against certain pathogens, including *Staphylococcus aureus, Salmonella Enteritidis, Aspergillus niger, Penicillium cyclopium*, and *Fusarium oxysporum*.

**Cao et al. (2013)** examined the effects of 1% or 0.5% chitosan, 10% or 5% aqueous extract of ginger, onion, and garlic and their composite solutions on the quality and shelf life of stewed pork. Microbiological (total viable counts) characteristics were analyzed at 4 °C for 12 days. They found out that chitosan and extract of ginger, onion, and garlic treatments retarded the increases in Total Viable Counts. Chitosan showed better antibacterial activity, but weaker antioxidation compared to ginger, onion, and garlic extract.

The number of TVC was significantly higher in aerobically packed control samples in comparison with samples treated with fresh garlic, dried garlic, and garlic oil

during 8 days of storage. There were significant differences in TVC values between vacuum-packed samples and samples treated with garlic oil on the 4th day of storage. The number of TVC was significantly lower in samples treated with fresh garlic compared to samples treated with dried garlic and garlic oil on the 4th, 6th, and 8th day of storage ($p$ <0.05) (Table 1).

**Huang et al. (2017)** investigated the effect of 2.5% and 5% rosemary powder against TVC in minced pork stored for 7 days at 4 °C. They have shown that a 5% concentration of rosemary powder has a stronger antimicrobial effect than a 2.5% concentration of rosemary powder, and in combination with a supercritical $CO_2$ level may be involved in prolonging shelf life.

**Park et al. (2008)** investigated the addition of onion and garlic powder to pork meat. The value of Total viable counts was lower in samples containing garlic and onion powder compared to the control samples ($p$ <0.05). The garlic powder showed a stronger antimicrobial effect against *Enterobacteriaceae* in comparison with onion powder ($p$ <0.05).

**Krisch et al. (2010)** evaluated the effect of 1% dried garlic and 1% onion powder on TVC in minced pork, which was stored at 5 °C for 72 hours. They reported that the dried garlic would eliminate the contamination of minced pork after 24 hours by 2.00 log CFU.g$^{-1}$. Dried garlic and onion powder also show excellent antimicrobial effects against *Candida* sp. and *E. coli.*

Cold-tolerant species of the family *Enterobacteriaceae,* such as *Serratia liquefaciens, Serratia proteamaculans, Hafnia alvei, Enterobacter agglomerans, Proteus* spp., *Klebsiella* spp., and *Pantoea* spp., contribute to spoilage of chilled meat stored under inadequate conditions **(Odeyemi et al., 2020)**.

The average number of CB ranged from 1.28 log CFU.g$^{-1}$ on the 0th day to 4.13 log CFU.g$^{-1}$ on the 8th day in aerobically packed samples. The highest number of CB was 1.82 log CFU.g$^{-1}$ on the 8th day in samples treated with garlic oil. The number of CB was lower than 1.00 log CFU.g$^{-1}$ in vacuum-packed samples, samples treated with fresh garlic, and in samples treated with dried garlic (Table 2).

**Kaczmarek et al. (2017)** monitored the effect of plant extracts added to fresh pork against different groups of microorganisms. They found out, that the highest inhibitory effect against *Enterobacteriaceae* and *Pseudomonas* bacteria was noted in the meat samples treated with fresh onion.

**Michalczyk et al. (2015)** analyzed the effect of oregano essential oil, freeze-dried garlic, tomato concentrate, and their combination on the shelf life of minced pork meat during 16 days of storage. The effect of oregano essential oil against Total Viable Counts and *Enterobacteriaceae* was was very weak. Adding tomato concentrate, combined with other additives, slowed the rate of microbiological changes. The addition of freeze-dried garlic did not cause a substantial reduction of detectable bacteria to count. The addition of a combination of all three additives resulted in a product with a distinctly longer shelf life.

**Leong et al. (2014)** reported that organosulfur compounds derived from allicin are very unstable, reactive, and decompose rapidly. Therefore, garlic oil prepared by combining fresh garlic and vegetable oil contains a minimal concentration of allicin. On the other hand, essential garlic oil obtained by steam distillation contains compounds soluble in oil, especially alylmetyltrisulphid, which affects antimicrobial activity.

The number of CB was significantly higher in aerobically packed samples compared to vacuum-packed samples, samples treated with fresh garlic, dried garlic, and also garlic oil. The count of CB was significantly higher in samples treated with garlic oil in comparison with vacuum-packed samples, samples treated with fresh garlic, and samples treated with dried garlic on the 4th, 6th, and 8th day of storage ($p$ <0.05) (Table 2).

**Stojanović-Radić et al. (2018)** investigated the effect of the basil and rosemary essential oils as well as their combination on the growth of *Salmonella enterica* subspecies enterica serovar Enteritidis (*Salmonella Enteritidis*) in chicken meat, together with their spoilage protective potential at 4 °C and 18 °C. Food model experiments included investigation of their effect on accompanying microbial flora on fresh meat, while anti-salmonella activity was evaluated on artificially inoculated raw and thermally processed meat. Changes in microflora pointed to a significant effect of both oils against microbial meat spoilage, where various groups were affected by different treatments.

**Park and Chin (2014)** evaluated the effects of fresh garlic on microbial growth in pork patties. The microbial counts for *Enterobacteriaceae* in pork patties without garlic showed growth higher than 7 log CFU.g$^{-1}$ at 28 days of storage, whereas, samples treated with fresh garlic demonstrated growth lower than 6 log CFU.g$^{-1}$ at 28 days of storage.

**Kim et al. (2011)** investigated the antimicrobial effect of dried garlic, at a concentration of 0.5% in pork sausages. found the inhibitory effect of garlic powder against *B. cereus, S. aureus,* and *L. monocytogenes*.

**Fujisawa et al. (2008)** analyzed the instability of garlic (allicin) in aqueous and ethanolic solutions as well as in vegetable oil. Allicin was more stable in 20% alcohol than in water, but unstable in vegetable oil, with an activity half-life of 0.8 h, as estimated from its antibacterial activity toward *Escherichia coli*.

**Stellato et al. (2017)** analyzed the main microflora responsible for the deterioration of raw meat. They reported, that *Pseudomonas* spp. belongs to the dominant aerobic contaminating microflora, especially in pork. *P. fragi* and *P. fluorescens* are responsible for meat degradation by the production of extracellular proteases and lipases at low temperatures.

The average number of *Pseudomonas* sp. ranged from 1.21 log CFU.g$^{-1}$ on the 4th day to 2.45 log CFU.g$^{-1}$ on the 8th. day in aerobically packed samples. The number of *Pseudomonas* sp. was lower than 1.00 log CFU.g$^{-1}$ in vacuum packed samples, samples treated with fresh garlic, with dried garlic, and also with garlic oil (Table 3).

*Pseudomonas* spp. are recognized as the main agents of meat spoilage and are very often isolated from fresh and spoiled meat products **(Rajmohan et al., 2010)**.

The value of *Pseudomonas* sp. was significantly higher on the 4th, 6th, and 8th day in aerobically packed samples compared to vacuum-packed samples, samples treated with fresh garlic, dried garlic, and also samples treated with garlic oil ($p$ <0.05) (Table 3).

**Table 1** Average values of Total Viable Microorganisms in minced meat samples and significant differences.

| Total Viable Counts | | | | | |
|---|---|---|---|---|---|
| | **0. day** | **2. day** | **4. day** | **6. day** | **8. day** |
| **CA** | 4.59 ±0.34 | 5.86 ±0.20 | 6.81 ±0.13 | 7.54 ±0.23 | 8.45 ±0.20 |
| **CV** | 4.59 ±0.34 | 4.60 ±0.24 | 5.24 ±0.22 | 5.34 ±0.19 | 5.36 ±0.17 |
| **FG** | 4.59 ±0.34 | 4.51 ±0.40 | 5.04 ±0.13 | 5.00 ±0.11 | 4.98 ±0.15 |
| **DG** | 4.59 ±0.34 | 4.65 ±0.29 | 5.43 ±0.12 | 5.44 ±0.13 | 5.36 ±0.20 |
| **GO** | 4.59 ±0.34 | 4.64 ±0.10 | 5.64 ±0.19 | 5.62 ±0.20 | 5.59 ±0.15 |
| **Tukey test** | - | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$, CV:GO$^+$, FG:DG$^+$, FG:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$, FG:DG$^+$, FG:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$, FG:DG$^+$, FG:GO$^+$ |

Note: CA – control aerobically packed samples, CV – control vacuum packed samples, FG – samples treated with fresh garlic, DG – samples treated with dried garlic, GO – samples treated with garlic oil, $^+$ significant differences ($p$ <0.05).

**Table 2** Average values of Coliform Bacteria in minced meat samples and significant differences.

| Coliform Bacteria | | | | | |
|---|---|---|---|---|---|
| | **0. day** | **2. day** | **4. day** | **6. day** | **8. day** |
| **CA** | 1.28 ±0.20 | 2.03 ±0.28 | 2.59 ±0.37 | 3.29 ±0.21 | 4.13 ±0.01 |
| **CV** | 1.28 ±0.20 | <1.00 | <1.00 | <1.00 | <1.00 |
| **FG** | 1.28 ±0.20 | <1.00 | <1.00 | <1.00 | <1.00 |
| **DG** | 1.28 ±0.20 | <1.00 | <1.00 | <1.00 | <1.00 |
| **GO** | 1.28 ±0.20 | <1.00 | 1.53 ±0.35 | 1.78 ±0.14 | 1.82 ±0.15 |
| **Tukey test** | | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$, CV:GO$^+$, FG:GO$^+$, DG:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$, CV:GO$^+$, FG:GO$^+$, DG:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$, CV:GO$^+$, FG:GO$^+$, DG:GO$^+$ |

Note: CA – control aerobically packed samples, CV – control vacuum packed samples, FG – samples treated with fresh garlic, DG – samples treated with dried garlic, GO – samples treated with garlic oil, $^+$ significant differences ($p$ <0.05)

**Table 3** Average values of *Pseudomonas* sp. in minced meat samples and significant differences.

| ***Pseudomonas* sp.** | | | | | |
|---|---|---|---|---|---|
| | **0. day** | **2. day** | **4. day** | **6. day** | **8. day** |
| **CA** | <1.00 | <1.00 | 1.21 ±0.07 | 1.72 ±0.77 | 2.45 ±0.47 |
| **CV** | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| **FG** | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| **DG** | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| **GO** | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| **Tukey test** | | | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$ | CA:CV$^+$, CA:FG$^+$, CA:DG$^+$, CA:GO$^+$ |

Note: CA – control aerobically packed samples, CV – control vacuum packed samples, FG – samples treated with fresh garlic, DG – samples treated with dried garlic, GO – samples treated with garlic oil, $^+$ significant differences ($p$ <0.05).

**Zhang et al. (2009)** investigated the antimicrobial effect of clove extract, rosemary extract, licorice extract, and combined rosemary and licorice extract against *Pseudomonas* spp. in raw pork during 28 days. Combined rosemary and licorice extract (RL) showed the strongest inhibitory effect against *Pseudomonas* spp.

**Kunová et al. (2017)** evaluated the effect of essential oils against *Pseudomonas* spp., *Enterobacteriaceae,* and anaerobic plate counts during storage of chicken meat. They found out, that the caraway and thyme essential oils can be used as natural food preservatives and they are also a good source of antimicrobial ingredients for meat.

## CONCLUSION

Within the food industry, meat is one of the main raw materials, which is a valuable source of nutrients for humans. For this reason, it is essential to ensure the availability of high-quality and safe meat for consumers. Minced meat is an ideal growth medium for spoiling and pathogenic microorganisms. Due to the health problems associated with the use of synthetic preservatives, consumers appeal to the use of natural antimicrobial preservatives. Our results show that fresh garlic in combination with vacuum packaging showed the highest efficacy against all groups of microorganisms, garlic oil was not effective against coliform bacteria. Fresh garlic and dried garlic in combination with vacuum packaging are a suitable method of eliminating microorganisms in minced pork.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# POTENTIAL PROBIOTIC YEAST ISOLATED FROM AN INDONESIAN INDIGENOUS FERMENTED FISH (IKAN BUDU)

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## ABSTRACT

Budu is a fermented food resulting from the activities of microorganisms like lactic acid bacteria and yeast. Budu, therefore, serves as a source of probiotics that can have beneficial effects on livestock and humans. Nonetheless, their selection has to be done with caution. The current study purposed to find out whether budu has desirable probiotic properties. This was done by determining its pH, bile acid tolerance, hydrophobicity, and inhibition of pathogens such as *Staphylococcus aureus*, *Salmonella enteritidis*, and *Escherichia coli*. An *in vitro* experiment was conducted using three *Saccharomyces cerevisiae* (coded as SC 11, SC 12, and SC 21) in the preparation of budu. The whole experiment was repeated four times. The budus were tested for their probiotic properties (low pH, bile salts, hydrophobicity, and inhibition of pathogenic bacteria). The results showed that the three *Saccharomyces cerevisiae* survived in gastric juice and bile acid, exhibited good hydrophobicity, and could inhibit pathogenic bacteria, both gram-positive and negative pathogens. They were able to survive at pH 2 for 3 h (40.70 to 55.1%), at pH 2 for 5 h (35.25 to 46.88%), in 0.3% bile acid incubated for 3 h (69.69 to 86.56%), in 0.3% bile acid incubated for 5 h (82.22 to 88.18%) and hydrophobicity ability of 97.0 to 98.1%. The inhibition activity against pathogenic bacteria, that is, *Escherichia coli* was 2.50 to 3.81 mm, *Staphylococcus aureus* was 1.66 to 3.71 mm, and *Salmonella enteritidis* was 1.20 to 2.64 mm.

**Keywords:** probiotic properties; inhibition of pathogenic bacteria; Indonesian fermented fish; Ikan Budu; poultry infection

## INTRODUCTION

In recent times, the use of antibiotic growth promoters (AGP) in livestock business or broiler business, in particular, has been banned. This is due to consumer concerns about the presence of AGP residues in products such as meat, milk, and eggs, because of the potential risk of drug resistance they pose to humans. Farmers always try to find a substitute for AGP with organic compounds such as plant extracts, prebiotics in the form of MOS (mannan oligosaccharides) and FOS (fructooligosaccharides), and probiotics such as giving live microorganisms to livestock **(Davari et al., 2019)**. Microorganisms of the lactobacillus genera are mostly used for the commercial production of probiotics, especially in fermented milk worldwide **(Sharif et al., 2017)**. Probiotics promote the growth of healthy microflora in the gastrointestinal tract **(Rajoka et al., 2018)**.

Probiotics can be from bacteria, fungi, and yeast. Bacteria that are widely used as probiotics are lactic acid bacteria, from fungi are *Rhizopus oligosporus*, while from yeast are *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. The source of microorganisms used as probiotics is essential, which is usually obtained from the digestive tract of livestock because they are already adapted to the intestine. There are not many reports showing that probiotics are isolated from fermented foods such as budu.

Traditional fermented fish, also known as budu is produced mainly in West Sumatra, Indonesia. Budu is usually made from leather skin (*Chorinemus* spp.) and Spanish mackerel (*Scomberomorus* spp.) known as Ikan Talangand Ikan Tenggiri, respectively, in the Indonesian language **(Huda, 2012)**. Budu processing starts with the hanging of fresh fish by the tail fin for four hours under room temperature. It is then eviscerated, washed, and covered with a layer of salt in a traditional container. After which, it is stored for one day at room temperature and sun-dried for five days. Garlic and white pepper can be sprinkled on the fish during the drying process to improve the quality of the budu **(Huda and Ahmad, 2006)**.

**Anggraini et al. (2019)** isolated lactic acid bacteria from budu and found that the LAB produced Gamma-aminobutyric acid (GABA), which served as anti-heat stress for broilers. They also found that yeast undergoes symbiosis with lactic acid bacteria in budu. **Stadie et al. (2013)** reported a symbiotic relationship between yeast and lactic acid bacteria of water kefir origin. Symbiosis

(commensalism or mutualism) widely occurs in fermented foods such as sourdough, milk kefir, and yogurt.

As a probiotic, yeast must be able to withstand gastric pH, bile acids, and pathogenic bacteria. **Brandão et al. (2014)** found that acidic pH was not affected by the fatty acid composition of *S. boulardii*. Yeast is capable of maintaining its internal pH by consuming H+ through a metabolic pathway and by using cell buffer systems. **Ogunremi et al. (2015)** added that *Pichia kudriavzevii* ROM 11, that is, yeast from Ogi, which is a cereal pudding fermented food from Nigeria usually made from corn, sorghum, or millet, had a resistance of 86.36% against bile acids with a concentration of 0.3%.

The purpose of the research was to find yeast present in budu that can serve as a probiotic for potential application for poultry production.

## Scientific hypothesis

As the yeast-derived from fermented foods from West Sumatra such as fermented fish (budu) has not been exploited, especially as a candidate for probiotics, the study hypothesizes that probiotic yeast exists in fermented fish Ikan Budu.

## MATERIAL AND METHODOLOGY

### Samples

A sample of budu was purchased from a traditional producer at Pariaman Regency, West Sumatra, Indonesia. Budu was made from coral reef fish such as red Kakap (*L. campechanus*) and Tenggiri fish (*Scomberomorini*) as shown in Figure 1.

### Chemicals

Chemicals used in this study were NaCl (Merck, Germany), glycerol (Merck, Germany), HCl (Merck, Germany), oxgall (synthetic bile salts) (Merck, Germany), phosphate buffer (Merck, Germany), and lactic acid (Merck, Germany). The media used in this study were yeast universal agar, nutrient broth, and nutrient agar. All media used were also purchased from Merk, Germany.

### Biological Material

Biological material involved in this study was isolated of *Salmonella enteritidis*, *Staphylococcus aureus, Saccharomyces cerevisiae,* and *Escherichia coli.*

### Equipment

The equipment used in this study were microscope (Merck, Germany) spectrophotometer (Mettler Toledo UV Vis, Inggris), incubator (Thomas Scientific, USA), autoclaved (Systec autoclaved company, Japan), and caliper (Misumi, Indonesia).

### Description of Experiments

Isolation of yeast was conducted according to the method of **Bajwa and Sharma (2018)**. The budu (1 g) was added to 9 mL of 0.9% NaCl (saline) solution and mixed thoroughly for 60 s. Serial dilution was then carried out in saline solution and spread plated onto yeast universal agar. The yeast universal agar was composed of 3.0 $g.L^{-1}$ malt extract, 3.0 $g.L^{-1}$ yeast extract, 10.0 $g.L^{-1}$ glucose, 5.0 $g.L^{-1}$ peptone, and 15.0 $g.L^{-1}$ agar. The spread plated yeast universal agar was incubated for 72 h at 28 °C. Presumptive yeast showed white-to-yellow colonies under the microscope. Such isolates were randomly selected and further purified on yeast universal agar. Yeasts showing the typical appearance of *Saccharomyces* (white-to-yellow colonies) were selected. The selected yeast strains were further purified by successive streaking on yeast universal media. Three isolates were maintained at -80 °C in 20% (v/v) glycerol (Hi-Media).

pH resistivity test was done using a modified nutrient broth in which 0.1 N HCl was added to achieve a pH of 2 which corresponds with gastric pH as described by **Ogunremi et al. (2015)**. The yeast extract (1 mL) was inoculated in modified HCl nutrient broth and incubated at 37 °C for 3 and 5 h. After which, the absorbance was read at a wavelength of 600 nm. This research was conducted with three replications. Isolates resistance was also expressed as a percentage, according to **Ogunremi et al. (2015)**.

Bile salt resistivity test was conducted using mixed modified HCl nutrient broth with 0.3% oxygall (synthetic bile salts) and incubated for 3 and 5 h **(Ogunremi et al., 2015)**. The culture was streaked onto a modified nutrient broth and incubated at 37 °C. 1 mL of yeast isolate was streaked onto the bile salt modified nutrient broth medium. Incubation was done at 37 °C **(Ogunremi et al., 2015)**. The results were obtained qualitatively by comparison of the yeast absorbancy of the control (not streaked with yeast) with the streaked modified nutrient broth (0.3% bile salts).

Hydrophobicity test or attachment was carried out by the method of **Vinderola et al. (2004)** using stainless steel plates. Clean and dried stainless-steel plates were marked on one side. One hundred (100) mL of distilled water was used to dissolve 0.8 g of nutrient broth. Growth media and stainless steel were autoclaved at 121 °C for 15 min.



**Figure 1** Fermented fish (budu) made from Tenggiri fish (*Scomberomorini*).

The stainless steel plate was placed in 25 mL nutrient broth inoculated with 1 mL of LAB in an Erlenmeyer and incubated for 24 h at 29 °C. Furthermore, the surface of the stainless steel was evenly wiped with a swab. The swab was put in a tube containing 10 mL of phosphate buffer and homogenized. It was then measured by looking at the absorbance at a wavelength of 600 nm (A). To measure the growth in the liquid phase, 1 mL of liquid was taken from nutrient broth media and diluted in 9 mL of phosphate buffer solution. After which, it was measured at a wavelength of 600 nm (Ao). The percentage hydrophobicity was calculated using the formula of **Fadda et al. (2017)**.

The antimicrobial activity test of 3 yeast isolates against *Salmonella enteritidis*, *Staphylococcus aureus,* and *Escherichia coli* was carried out based on a modification from **Diosma et al. (2013)**. Nutrient agar (10 grams) was added to 500 mL of distilled water, homogenized, heated in a water bath, and autoclaved. The media was allowed to cool (± 45 °C), and 0.2% of test bacteria was added into ±10 mL Petri dishes and allowed to solidify. Meanwhile, a blank antibiotic disk was soaked in the lactic acid bacteria solution for approximately 10 min and was placed on the surface of the nutrient agar medium containing the pathogenic bacterial isolates. It was then incubated aerobically at 37 °C for 24 h. After incubation, the diameter of the inhibition zones was measured using a caliper.

**Statistical Analysis**

Data were subjected to a one-way analysis of variance (ANOVA), and Tukey's test was used for comparison of means using SPSS version 20.0 Software (SPSS Inc., Chicago, IL, USA). A significant difference was defined at $p < 0.05$.

## RESULTS AND DISCUSSION

### Resistance test of yeast isolates to gastric pH

The resistance of yeast to gastric pH was tested at pH 2 because the pH in the proventriculus and gizzard is 2.0 – 3.5 **(Rougie`re and Carre, 2010)**. The gastric pH was tested for 3 h and 5 h, the results for which can be seen in Table 1. The results of the study showed that all yeast isolates could survive at pH 2 with resistance >30%. The resistance of the yeast isolates to pH did not differ significantly ($p > 0.05$) from each other. The three yeast isolates of budu origin grew at pH 2 with the viability of 55.1% for isolate SC 11, 43.70% for isolate SC 12, and 40.70% for isolate SC 21, which were incubated for 3 h. When the time incubation time was increased to 5 h, the percentage viability decreased.

The pH of 2.0 – 3.5 is pH in the proventriculus, where HCl is produced. The probiotic yeast work in the gastrointestinal tract (GIT) by providing nutrients that aid in the digestion of food and inhibition of harmful bacteria. Probiotics are also mixed or added to feed to increase the rate of feed and nutrient utilization **(Markowiak and Śliżewska, 2018)**.

The results in Table 1 show that the yeast isolate that had the highest resistance to gastric juice was SC 11, with resistance >50%. **Nurnaafi, Setyaningsih and Desniar (2015)** explained that good probiotic isolates are those with a survival rate of more than 50% under low pH conditions and are resistant to bile salts. The resistance of isolate SC 11 at 3 h incubation time was 55.10% and decreased at 5 h incubation time to 46.88%, a difference of 8.22%. The results of this study were similar to those of **Kumura et al. (2004),** who found that yeast (*Kluyveromyces lactis* S25) isolated from commercial blue cheese and kefir had a resistance of 54.7%. The results of this study for SC 11 were higher than those of **Diosma et al. (2013)**. They examined yeast isolates from kefir (tested at pH 2.5 with an incubation time of 3 hours) and reported that *Kluyveromyces marxianus* 8116 had 45.5% resistance, *Saccharomyces cerevisiae* 8115 had 40.5% resistance, and *Saccharomyces boulardii* had 45.5% resistance.

**Tovar et al. (2002)** reported that when yeast isolates enter the digestive tract of poultry, they must be able to survive at low pH because the proventriculus and gizzard have a pH of 2.0 – 4.5. **Zubaidy and Khanda (2014)** added that *Saccharomyces cerevisiae* var *boulardii* (S.b32) was able to survive at low pH. Glucomannan, chitin, mannoprotein, and beta-glucan make up the cell component of *Candida* sp. **Drabikova et al. (2009)**. However, beta-glucan forms the largest (50 – 60%) component of the inner layer of the cell wall, while chitin forms 1 – 10%. Mannoproteins form mainly 30 – 40% of the outer layer of the cell wall. They play a major role in interactions with the host, determine the nature of the cell surface and cell-to-cell recognition **(Vickova et al., 2004)**.

### Resistance test of yeast isolates to bile salts

Resistance of the yeast isolates to bile salt was not significantly different ($p > 0.05$). The results in Table 1 shows that isolate SC 11 had a resistance ability of 69.69% at an incubation time of 3 hours and increased to 82.02% at an incubation time of 5 hours, a difference of 12.33% increase. The results of this study are comparable to those of **Chen et al. (2010)**, who examined yeast isolated from fresh milk on Beijing and Heilongjiang farms against 0.3% bile salts and reported that *Pichia fermentans* HJ15 isolate had 79% resistance, *Pichia kudriavzevii* BY10 isolate had a resistance of 25.9%, and *Yarrowia lipolytica* HJ6 isolate had a resistance of 62.9%.

Yeast can survive in bile salt solutions because of its extreme environmental resistance **(Chen et al., 2010)**. **Chen et al. (2010)** explained that yeast develops resistance properties in stressful environments (salt, acids, and sugars), and in competition with other microbial yeasts, they can live a normal life. The difference in the results of this study can be due to the differences in the type of yeast tested against 0.3% bile salts. Yeast cell walls are mostly composed of beta-glucan **(Lee et al., 2001)**. **Ooi and Liu (2000)** reported that beta-glucan is a linear polysaccharide that contains monomers of glucose that are linked by glycosidic bonds. Beta-glucan is water-soluble, and a small concentration will produce high viscosity **(Suzuki et al., 2001)** and will form a gel in the digestive tract to increase the excretion of bile acids. By this, the fat is not emulsified and absorbed in the stomach.

### Inhibition test against pathogenic bacteria

The ability of yeast to act antagonistically is due to changes in medium pH, competition for nutrients, secretion of antimicrobial agents, and production of ethanol in high concentrations. *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella enteritidis* were chosen because they are among the pathogenic bacteria associated with poultry and other animals. The results of the study (Table 2)showed that the inhibitory zone produced by isolate SC 11 against *Escherichia coli* was 3.81 mm, and greater than the inhibition zone of 3.71 mm produced against *Staphylococcus aureus*. However, the inhibition of the yeast isolates to *Staphylococcus aureus,* and *Escherichia coli* did not differ significantly ($p$ >0.05). The differences in the bacterial cell walls could not cause significant differences in their resistance to yeast. *Staphylococcus aureus* is gram-positive bacteria, while *Escherichia coli* is gram-negative bacteria. **Saidi et al. (2019)** reported that gram-negative bacteria have a thinner layer of peptidoglycan (5 – 10 cm), while gram-positive bacteria have a thicker layer of peptidoglycan (20 – 80 cm). Therefore, it was more difficult for yeast isolates to penetrate the cell wall of *Staphylococcus aureus* bacteria than the cell wall of *Escherichia coli* bacteria.

Table 2 shows that the inhibition of *Salmonella enteritidis* by yeast was numerically lower than that of *Staphylococcus aureus* and *Escherichia coli.* This might be influenced by antigens present in *Salmonella*. According to **Wang et al. (2020)**, *Salmonella* has three main antigens, namely: somatic antigens or O antigens, flagellate antigens or H antigens, and capsule antigens or Vi antigens; which produce enterotoxins and cytotoxins, making it difficult for yeast to inhibit their growth.

The inhibitory strength possessed by yeast varies; therefore, different yeast species will produce different inhibition and metabolite activities during fermentation. **Freimoser et al. (2019)** studied the antagonistic activity of *Kloeckera* and *Kluyveromyces* species against bacteria and found that they produce intracellular and extracellular antimicrobial compounds that inhibit the growth of gram-negative and gram-positive bacteria. Research by **Suzuki et al. (2001)** and **Marquina et al. (2002)** found yeast to produce antagonistic activity due to the production of killer toxins or mycotoxins. These toxins are extracellular proteins or glycoproteins that can damage the cell membrane. The antimicrobial activity of yeast through the secretion of organic acids and antimicrobial peptides has been reported **(Boirivant and Strober, 2007; Vanderpool, Yan and Polk, 2008; Ciorba, 2012)**. *S. boulardii* secretes mainly capric acid, a medium-chain fatty acid which showed bioactivity against *Candida albicans* and formation of biofilms **(Krasowska et al., 2009; Murzyn et al., 2010)**. *S. cerevisiae* secretes antimicrobial peptides (saccharomycin), which inhibits the growth of pathogenic bacteria **(Hammami et al., 2013)**. Antimicrobial peptides inhibit bacteria growth by absorbing the cell membrane receptors, destructing cell membrane permeability and alteration of intracellular pH **(Rizk et al., 2018)**.

**Table 1** The resistance of yeast isolates towards acid and bile salt conditions.

| Isolates yeast | Time (3 h)(%) | Time (5 h)(%) |
|---|---|---|
| **Acid condition** | | |
| *Saccharomyces cerevisiae* (SC) 11 | 55.10 ±2.19 | 46.88 ±1.82 |
| *Saccharomyces cerevisiae* (SC) 12 | 43.70 ±1.35 | 39.36 ±0.80 |
| *Saccharomyces cerevisiae*(SC) 21 | 40.70 ±0.87 | 35.26 ±0.38 |
| **Bile salt condition** | | |
| *Saccharomyces cerevisiae*(SC) 11 | 69.69 ±0.14 | 82.02 ±0.53 |
| *Saccharomyces cerevisiae*(SC) 12 | 84.54 ±1.37 | 87.43 ±1.91 |
| *Saccharomyces cerevisiae*(SC) 21 | 86.56 ±1.71 | 88.18 ±1.72 |

Note: values were reported as means ±*SD* of triplicate groups.

**Table 2** The resistance of yeast isolates towards pathogenic bacteriaassociated with poultry.

| Yeast isolates | Diameter inhibition zone (mm) | | |
|---|---|---|---|
| | *Escherichia coli* | *Staphylococcus aureus* | *Salmonella enteritidis* |
| *Saccharomyces cerevisiae* (SC) 11 | 3.81 | 3.71 | 2.64 |
| *Saccharomyces cerevisiae* (SC) 12 | 2.50 | 2.56 | 2.52 |
| *Saccharomyces cerevisiae*(SC) 21 | 1.63 | 1.66 | 1.20 |

Note: values were reported as means ±*SD* of triplicate groups; mean values in the same column with different lowercase were significantly different ($p$ <0.05).

**Table 3** The hydrophobicityof yeast on stainless steel plates.

| Yeast Isolates | Hdrophobicity (%) |
|---|---|
| *Saccharomyces cerevisiae* (SC) 11 | 97.00 ±0.24 |
| *Saccharomyces cerevisiae* (SC) 12 | 97.96 ±0.72 |
| *Saccharomyces cerevisiae* (SC) 21 | 98.71 ±0.19 |

Note: values were reported as means ±*SD* of triplicate groups.

### Hydrophobicity using stainless steel plates

Table 3 shows the hydrophobicity of the yeast isolates. From Table 3, all the yeast isolates had a hydrophobicity percentage >90%. The hydrophobicity of yeast isolates was not significantly different ($p$ >0.05).

The results of this study were higher than those of **Sourabh et al. (2011)**, who showed that *Saccharomyces cerevisiae* isolated from traditional West Himalayan fermentation food, Sc01 had a hydrophobicity percentage of 59.65%. **Fadda et al. (2017)** found that *Saccharomyces boulardii* isolated from codex had a hydrophobicity ability of 55.9%, *Kluyveromyces lactis* isolated from kefir had a hydrophobicity ability of 74.1 – 79.4% and *Kluyveromyces marxianus* had a hydrophobicity ability of 75.9%.

The ability of microorganisms to attach to the digestive tract becomes one of the selection criteria for probiotics. The formation of colonies in the digestive tract is influenced by the ability of microorganisms to attach to the digestive tract that is specific to the host **(Suzuki et al., 2001)**. **Tovar et al. (2002)** reported that some yeast species can synthesize and secrete polyamine molecules which can stimulate the development of the digestive tract and the production of digestive enzymes.

## CONCLUSIONS

The results indicated that all the isolates identified were *Saccharomyces* sp., (SC 11; SC 12 and SC 21) and showed notable potential probiotic properties. They exhibited a better survival in gastric juice and bile acid, showed high hydrophobicity, and the ability to inhibit pathogenic bacteria (gram-positive and negative pathogens) associated with poultry. They were able to live at pH 2 for 3 h (40.70 to 55.1%), pH 2 at 5 h (35.25 to 46.88%), in bile acid 0.3% incubated for 3 h (69.69 to 86.56%), and at 5 h (82.22 to 88.18%), and hydrophobicity ability of 97.0 to 98.1%. The inhibition zones produced by *Saccharomyces* sp. against *Escherichia coli* was 2.50 to 3.81 mm, *Staphylococcus aureus* was 1.66 to 3.71 mm, and *Salmonella enteritidis* was 1.20 to 2.64 mm.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE ANTIMICROBIAL ACTIVITY OF POLYFLORAL HONEY AND ITS AWARENESS AMONG URBAN CONSUMERS IN SLOVAKIA

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## ABSTRACT

The current interdisciplinary research studies the antimicrobial activity of selected polyfloral kinds of honey (n = 30) against three microorganisms (gram-positive bacteria *Enterococcus faecalis*, gram-negative bacteria *Salmonella enterica,* and one yeast *Candida krusei*) as well as investigates consumer behavior and awareness towards honey healing properties. Consumer research involved 617 honey consumers living in urban areas. T-test for Equality of means, non-parametric tests, and descriptive statistics were applied. Results showed that antimicrobial activity was found in all honey samples with a concentration of 50%. Nevertheless, better activity was obtained in honey samples from urban beekeepers compared to samples from retail stores. Results of consumer research showed that honey is mostly used as food (sweetener in beverages, ingredient in the recipe, or direct consumption) and as medicine mainly during the winter period. The consumer awareness towards honey healing effects was very high (97%), however, 1/3 of respondents were not able to list any specific examples, and only 11 % mentioned antibacterial activity. Furthermore, more than 70% of respondents did not know to explain the term "medical honey" and more than 50% of respondents are not aware of the maximum temperature used for heating honey without decreasing its biologically active compounds.

**Keywords:** consumer research; consumer perception; honey consumer; antimicrobial activity; Slovak honey

## INTRODUCTION

In modern nutrition, honey has an irreplaceable place, as it is considered a valuable dietary food, sweetener, or medicine **(Ahluwalia et al., 2020; Meo et al., 2017; Samarghandian, Farkhondeh and Samini, 2017; Kumar et al., 2010)**. **Selmi, Irnad and Sistanto (2020)** emphasize, that honey is used for nutritional and medicinal purposes, as well as honey is required by industries, especially by pharmaceutical and cosmetic companies. In recent years, honey has been considered an important commodity in the international market **(Buba, Gidado, and Shugaba, 2013)**. In the context of the above mentioned it could be stated that the popularity of honey is increasing, and natural honey is becoming a sought-after product among consumers. The increased demand can be justified by the awareness of consumers towards the unique properties of honey, which are attributed to the influence of the various groups of substances it contains **(Puścion-Jakubik, Borawska and Socha, 2020; Yeow et al., 2013)**.

**Escuredo et al. (2013)** focus on the fact that honey is a food that contains about 200 substances. Honey contains a mixture of two monosaccharides (glucose and fructose) and is a source of proteins, minerals, vitamins, organic acids, flavonoids, phenolic acids, and enzymes **(Keskin and Keskin, 2021; Gündoğdu, Cakmakci and Şat, 2019)**.

The composition of honey is not uniformed and it has a very complex composition, which depends on the different factors. The most important determinants influencing the composition of honey are, in particular, the botanical and geographical origin, climatic conditions, and weather during harvest. Another important determinant is also beekeeping management, which is associated with the collection and storage of honey, as well as its conditions **(Escuredo and Seijo, 2019; Da Silva et al., 2016; Escuredo et al., 2014; Karabagias et al., 2018; Otero and Bernolo, 2020)**. **Ranneh et al. (2021)** add that honey contains macro and micronutrients which depend on bee type, floral source, but also on environmental and processing factors.

Honey can be classified as a superfood thanks to its unique natural composition, and its consumption has a positive effect on the health of consumers. Honey can boost the immune system to fight infection **(Scepankova, Saraiva and Estevinho, 2017)**, honey has proven antiviral effects **(Kala et al., 2020)**, honey is also beneficial for sore throats, coughs, and colds **(Kumar et al., 2010)** and honey is a valuable cure against pathogenic respiratory agents, including viruses that cause cough **(Al-Hatamleh et al., 2020)**. **Abbas et al. (2019)** showed that honey in a combination with other substances has also a relatively high

efficiency in patients with asthma. According to **Idrus et al. (2020)**, honey can even act as a protective agent in cardiovascular disease. **Khalil and Sulaiman (2010)** add that honey has anxiolytic, antidepressant, anticonvulsant, and antinociceptive effects and ameliorates the oxidative content of the central nervous system. Moreover, **Güneş and Rn (2007)** indicate that honey has beneficial effects in the treatment of diabetes. In addition, **Samarghandian, Farkhondeh and Samini (2017)** stated that honey could be able to act preventively against cancer, for example, breast cancer, carcinoma, melanoma, colon carcinoma, hepatic cancer, and bladder cancer. However, they also add that there are necessary more studies to improve understanding of the positive effect of honey and cancer.

In the context of the above, it could be concluded that honey is one of the most complete foods for humans, due to its therapeutic, antioxidant, antimicrobial, antitumoral, anti-inflammatory, antiviral, and activities **(Bueno-Costa et al., 2015)**. Nowadays, however, the antibacterial effects of honey are also highlighted. Antibacterial activity is considered the most investigated biological property of honey **(Bucekova et al., 2019)**. Natural unheated honey has broad-spectrum antibacterial activity honey is specific for its antibacterial activity, which it also shows by tests against pathogenic bacteria, oral bacteria as well as food spoilage bacteria **(Lusby, Coombes and Wilkinson, 2005; Mundo, Padilla-Zakour and Worobo, 2004)**. The antibacterial activity of honey is not derived from one mechanism, resp. the action of one chemical, but is based on multifactorial action. The factors responsible for the antibacterial activity of honey are the high sugar concentration, which participates at osmotic pressure, low pH value, and water activity, as well as the 1,2-dicarbonyl compound methylglyoxal (MGO) and the cationic antimicrobial peptide bee defensin-1 **(Kwakman et al., 2010; Bucekova et al., 2020)**. **Mandal and Mandal (2011)** emphasize that identification of the antibacterial purpose of honey can be beneficial and can provide relevant information related to honey quality as well as therapeutic potentials against health diseases of people.

## Scientific Hypothesis

Assumption No. 1: We assume that there exist significant differences in antimicrobial activity between honey samples from urban beekeepers and those purchase from retail stores.

Assumption No 2: We assume that there exists a statistically significant dependence between annual honey consumption and respondent´s age.

Assumption No. 3: We assume that there exists statistically significant dependence between honey usage and respondent´s age.

Assumption No. 4: We assume that urban consumers evaluate the factors affecting the purchase of honey differently.

Assumption No. 5: We assume that more than 40% of respondents are not aware of the max. temperature for heating honey without decreasing biological active compounds.

## MATERIAL AND METHODOLOGY

### Samples

30 samples of polyfloral honey (15 samples were directly from urban beekeepers and 15 samples were purchased from retail stores).

### Chemical

Muller Hinton broth, Mueller Hinton agar, Sabouraud dextrose broth, Sabouraud dextrose agar, blanc discs, antibiotics: tigecycline (30 µg per disc), chloramphenicol (30 µg per disc), fluconazole (30 µg per disc) (Oxoid, Basingstoke, UK).

### Animals and Biological Material

One species of Gram-positive bacteria *Enterococcus faecalis* CCM 4224, one subspecies of Gram-negative bacteria *Salmonella enterica* subsp. *enterica* CCM 4420, and one species of yeast *Candida krusei* CCM 8271. All tested microorganisms were collected from the Czech Collection of microorganisms (Brno, Czech Republic).

### Instrument

Densitometer (Biosan DEN-1, Riga, Latvia).

### Laboratory Methods

#### *Antimicrobial susceptibility testing*

Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method according to CLSI criteria. The inoculums of bacteria were prepared in sterile Muller Hinton broth and yeast in Sabouraud dextrose broth. The optical density of microorganisms to 0.5 McFarland turbidity with a densitometer (Biosan DEN-1, Riga, Latvia) was used. The test microorganisms were uniformly seeded over the Mueller Hinton agar (Oxoid, Basingstoke, UK) resp. Sabouraud dextrose agar (Oxoid, Basingstoke, UK). Diameters of the zone of inhibition around the discs were measured using a ruler in mm. The antibiotics tested were for Gram-positive bacteria tigecycline (30 µg per disc), for Gram-negative bacteria chloramphenicol (30 µg per disc), and yeasts fluconazole (30 µg per disc) as a positive control.

#### *Antimicrobial activity of honey*

Susceptibility testing was performed by Kirby–Bauer disk diffusion method according to criteria by CLSI, 2016. The inoculums of each microorganism were prepared with a sterile wire loop and suspended in sterile Mueller-Hinton broth resp. Sabouraud dextrose broth. The optical density of microbial suspension ware determined with densitometer on McFarland turbidity 0.5. The test microorganism was uniformly seeded over the Mueller–Hinton agar resp. Sabouraud dextrose agar on the surface. Using a sterile cork borer (6 mm diameter, 4 mm deep, and about 2 cm apart), wells were made in the agar medium. Using a micropipette, 50 µL of honey with a concentration of 50%, 25%, 12.50%, and 6.25% was added to the wells in the plate. The plates with bacterial strains were incubated at 37°C for 24 h and with yeasts at 25 °C for 24 h. The mean diameters of inhibition zones were measured in mm, and the results were recorded. Sterile distilled water is used as negative control and antibiotics as a positive control. The experiment was repeated triplicate for each strain.

### Description of the Experiment

### Sample preparation:

Thirty honey samples were used for antimicrobial testing. Hundred percent pure honey (100% v/v) was obtained after filtered using sterile gauze. To get 50% honey solutions (v/v), 0.50 mL of honey was diluted in 0.50 mL sterilized Muller Hinton broth (Oxoid, Basingstoke, UK) and yeast in

Sabouraud dextrose broth (Oxoid, Basingstoke, UK). Further serial dilutions of 0.25 mL of each, 0.125 mL and 0.0625 mL of honey, and 0.50 mL of sterile Muller Hinton broth and yeast in Sabouraud dextrose broth were added to obtain 50%, 25%, 12.5%, and 6.25% honey solutions (v/v), respectively.

**Number of samples analyzed**: 30
**Number of repeated analyses**: 3
**Number of experiment replication**: 3

## Consumer research

The second part of the research was based on a questionnaire survey conducted in 2020. The survey was carried out online in Google forms and was disseminated via emails and social media (mostly Facebook groups) by applying a snowball sampling strategy. The research sample comprised 617 honey consumers living in urban areas. The socio-demographic profile is described in Table 1. The questionnaire involved both close-ended questions and open-ended questions regarding consumption patterns, purchasing behavior, and consumer´s awareness of the healing properties of honey.

**Table 1** Socio-demographic profile of research sample.

| Demographic variable | | (%) |
|---|---|---|
| Gender | male | 36.63 |
| | female | 63.37 |
| Age | 18 – 30 years | 47.81 |
| | 31 – 50 years | 36.95 |
| | >50 years | 15.24 |
| Education | secondary | 51.70 |
| | university | 48.30 |
| Economic status | employed | 66.29 |
| | unemployed | 1.13 |
| | student | 22.37 |
| | pensioner | 5.51 |
| | maternity leave | 4.70 |
| Individual income per month (Netto) | up to 400 € | 21.39 |
| | 401 – 600 € | 13.13 |
| | 601 – 800 € | 20.42 |
| | 801 – 1,000 € | 19.12 |
| | >1,000 € | 25.93 |

## Statistical Analysis

The antimicrobial activity of honey samples was conducted in triplicate and recorded with standard deviations. T-test for Equality of means was used for testing differences between honey samples from stores and urban beekeepers. In consumer research, there were applied non-parametric tests such as the Chi-square test of Independence, Friedman test, and multiple pairwise comparisons using Nemenyi's procedure. The significance level was set to 0.05. All statistical analysis was carried out in statistical software SPSS Statistics v.25, IBM.

# RESULTS AND DISCUSSION

## Antimicrobial activity of honey samples

Results showed that antimicrobial activity was found in all tested kinds of honey (50% concentration). The highest antimicrobial activity in commercial kinds of honey was found against *E. faecalis* followed by *S. enteritidis* and *C. krusei*, while in kinds of honey from urban beekeepers was the order as follows: *E. faecalis* > *C. krusei* > *S. enteritidis* (see Table. 2). In addition, we formulated the first hypothesis, which assumes that there exist significant differences in antimicrobial activity between honey samples from urban beekeepers and those purchase from retail stores. T-test for Equality of Means confirmed statistically significant differences in all three types of microorganisms ($p \leq 0.001$).

**Table 2** Antimicrobial activity of analyzed honey samples at concentration 50% (mm).

| Microorganisms | *S. enteritidis* | *E. faecalis* | *C. krusei* |
|---|---|---|---|
| Beekeeper´s kinds of honey 50% | 5.13 ±0.81 | 9.78 ±0.72 | 5.49 ±0.89 |
| Commercial kinds of honey 50% | 3.89 ±0.73 | 4.62 ±0.78 | 3.69 ±0.78 |

Note: mean (n = 15) ±standard deviation.

Moreover, the antibacterial activity of kinds of honey with a concentration of 25% was found only in one type of examined microorganism and only of few honey samples. Eight honey samples from urban beekeepers inhibited *E. faecalis* (10.75 ±0.43) while only two honey samples from stores inhibited *S. enteritidis* (4.5 ±0.23). Antibacterial activity of honey with a concentration of 12.5% was found only in the case of 5 samples from urban beekeepers and it was against *E. faecalis* (6.9 ±0.97). Antibacterial activity is the most evaluated and investigated biological activity of honey and its presence has been proven in several tested samples of honey from the different botanical and geographical origin that were the object of examination of numerous studies which were orientated on the antibacterial activity **(Al-Jabri et al., 2003; Al-Waili, 2004; Bucekova et al., 2019; Bucekova et al., 2018; Cilia et al., 2020)**. The mentioned statement confirms our achieved results related to antimicrobial activity. The similar results with samples of Slovak honey were obtained by **Šedík et al. (2018)** and by **Kačániová et al. (2012)**.

## Results of consumer research

The questionnaire survey showed that Slovak consumers living in urban areas have the following annual consumption patterns: 39.7% consume only up to 1 kg and honey is mostly consumed occasionally or only during illness; 32.4% consume 1 or 2 kg in certain frequencies (few times per month or week). The rest 27.8 % consume ≥3 kg mostly every day or few times per week. Based on the realized survey in Romania **Pocol (2011)** identify that 11.0% of consumers do not consume honey, while consumption of honey at the level of a maximum of 750 g per year is recorded in a group of approximately 35% of the population. **Pocol (2011)** also added that the average consumption, between 750 g and 2 kg per year, has a rate of approximately 27%, while 20% of the population consumes over 2 kg of honey per year. Furthermore, by applying the Chi-square test of independence we confirmed ($p$-value = <0.0001) the statistically significant differences in honey consumption per year among different age segments (H2). Lower honey consumption (only up to 1 kg) is more

frequent for younger consumers (18 – 30 years). The highest annual consumption has consumers older than 50 years (see Figure 1). **Kopała, Balcerak and Kuźnicka (2019)** also found that elder respondents declared honey consumption more often than young people. Moreover, **Żak (2017)** realized the survey in Poland and reports that 83% of Poles over 65 years consume 500 g of honey in a month. In the context of the above **Pocol and Moldovan-Teselios (2012)** stated that young people up to 30 years consume rather small quantities of honey, consumers in the age category 32 – 45 years present a "normal" consumption behavior which is similar that the entire population, consumers between 46 – 60 years prefer consumption of average and large quantities of honey, being under-represented among non-consumers and the last category of consumers, consumers older than 61 years, consume medium quantities of honey.

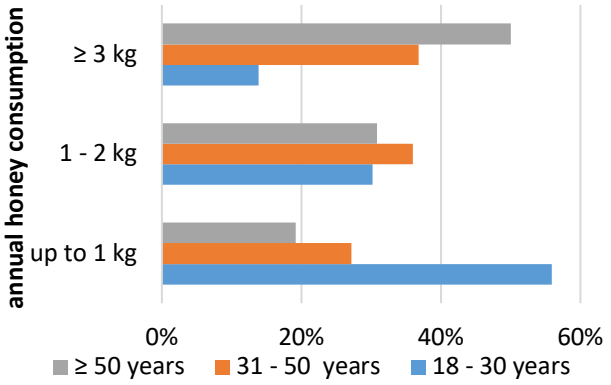


**Figure 1** Annual honey consumption based on respondent´s age.

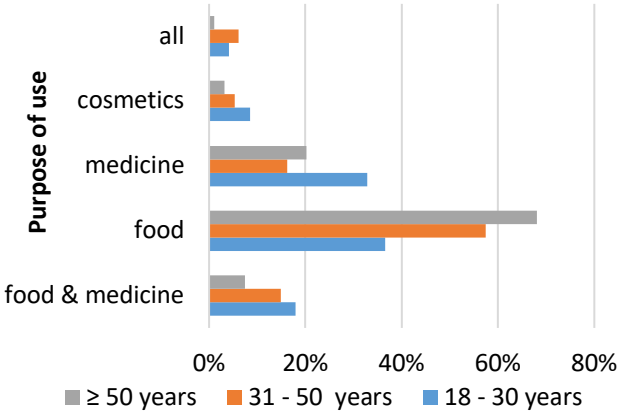


**Figure 2** Honey usage based on respondent´s age.

Approximately 78% of respondents answered that honey is consumed by the whole family. The household consumption of honey is on average 6,5 kg per year. The results of the study of **Roman, Popiela-Pleban and Kozak (2013)** showed that 7.2% of respondents declared that honey is not consumed in their households and emphasized that only 20.6% of respondents stated a daily consumption of this product while almost 39% of respondents stated that they consumed it only occasionally. Regarding the purpose of honey, it can be stated that honey as food is used by 68% and as medicine by 44% of consumers. Only 10% indicated cosmetics purpose. The purpose of honey also confirmed the survey realized by **Ismaiel et al. (2014)** and stated that the major motives of consumers for purchasing and consuming honey were to use it as medicine, food, a sweetener, or for other uses. In addition, the Chi-square test of independence confirmed (H3) significant dependence between respondent's age and purpose of use ($p$-value = <0.0001). Young consumers use honey more as medicine while the older generation uses it more as food (see Figure. 2).

Honey is mostly consumed as follows: sweetener in beverages > ingredient in the recipe > directly from a jar. Around 1/2 of respondents consume this product all over the year and around 43% only during the winter period to support their health. Moreover, 31% use honey regularly as a healthier alternative to sugar, and approximately 60% only sometimes.

Distribution of honey takes place primarily through producer-consumer or manufacturer-retailer-consumer channels **(Borowska, 2011; Kumar, Sharma and Singh, 2012)**. Results of our study showed that the honey is mostly purchased as follows: directly from beekeeper > shops and retail stores > farmer markets > specialty shops. The least frequent place of purchase was e-shops. These results are also confirmed by **Roman, Popiela-Pleban and Kozak (2013)** who found that more than 60.0% of respondents prefer to buy honey from beekeepers, and by **Ciric, Ignjatijević and Cvijanovic (2015)** who identified more than 40% of consumers has a habit of purchasing honey directly from the manufacturer of honey. **Pocol and Bolboaca (2013)** concluded, that local producers were preferred to purchase honey, but on the other hand, respondents also purchase honey and bee products in regional markets, hypermarkets or supermarkets, fairs and exhibitions, specialized shops for organic products, and other places. **Marzec (2003)** in her research showed that 84% of consumers purchase honey at the store and only 21% directly from the beekeeper. **Krystallis, Petrovici and Arvanitoyannis (2007)** state that the most usual channel for frequent food purchases for more than half of the sample is small local stores and open markets, while supermarkets are preferred for occasional food purchases. The optimal price per 1 kg of honey was considered 7 € (22%), 8 € (20%), or 6 € (17%). In comparison with other studies, we could state that consumers from other countries also most prefer honey up to 10 € per 900 g **(Kos Skubic, Erjavec and Klopčič, 2018)**. In addition, respondents evaluated the importance of selected factors during honey purchase using 7 points scale, where 1 = the most important and 7 = the least important. Based on the Friedman test ($p$-value = <0.0001) it can be stated that there exist statistically significant differences in the evaluation of selected factors (H4). These differences were identified by Nemenyi´s procedure and are illustrated in Table 3. The most important factors are the following: honey quality > honey taste > honey origin > hone type > consistency. The least important were honey packaging and price.

In the context of quality as the most important factor during honey purchase is important to emphasize that honey quality is connected with food safety, credence dimension, quality mark, honey taste, as well as nutritional value **(Borodin, Arion and Muresan, 2013; Röhr et al., 2005)**. According to **Ványi, Csapo and Karpati (2010)**, the most important properties in the process of honey purchasing are the taste, quality, and color of honey.

**Table 3** Results of Nemenyi's procedure applied to selected purchasing factors.

| Sample | Mean of ranks | Groups | | | | |
|---|---|---|---|---|---|---|
| quality | 2.74 | A | | | | |
| taste | 3.07 | A | B | | | |
| origin | 3.26 | | B | | | |
| type | 4.22 | | | C | | |
| consistency | 4.29 | | | C | | |
| price | 4.78 | | | | D | |
| packaging | 5.64 | | | | | E |

Note: questionnaire research, 2021.

**Oravecz et al. (2020)** found that consumers consider honey as a trusted product, which is confirmed by the fact that the source and the quality are the most important factors that influence the purchase of honey. The results of a study conducted by **Wu et al. (2015)** emphasizes that the origin of honey is a very important attribute when choosing purchased honey and that even consumers are willing to pay a higher price for local honey, which also confirms our result that price is not a decisive factor in the process of honey purchase. On the one hand, **Gyau et al. (2014)** realized the descriptive analysis of the main attributes of honey and they showed that price, packaging, and color are the three key attributes that strongly influence a consumer's choice of honey. On the other hand, they emphasized that quantity, taste, and the origin of the honey have a moderate influence on the choice, whereas the production process does not influence consumer preferences.

In addition, the survey focused also on consumer awareness and perception towards honey and its biological properties. Approximately 97% of respondents think that honey has healing properties, however, 1/3 of them were not able to answer the specific ones. The interesting result is that more than 40 % of them were younger than 30 years. The rest of the respondents listed various properties and effects. The most frequent were the following ones: immunity booster (22.7%), healing effects in case of cold, flu, or sore throat (12.5%), antibacterial activity (11.2%), anti-inflammatory (8.4%), and others. Based on the results, it can be concluded that the consumer´s awareness about the antibacterial activity is very low. A similar situation is with medical honey which is unknown for 72% of urban consumers. The rest of them perceived it as honeydew honey, honey used in hospitals, in medicine, or as clean, pure honey used for healing wounds.

In general, consumers are interested in the liquid consistency of honey, which was proved by **Cosmina et al. (2016)**. Due to this fact, crystallized honey is usually liquified by applying thermal treatment. Increasing temperature of honey can decrease its antibacterial activity **(Pimentel-González et al., 2015)**. The last hypothesis (H5) assumes that more than 50% of respondents are not aware of the maximum temperature for heating honey without decreasing biological active compounds. The survey showed that only 18% knows the correct answer – 42 degree. The rest of them either do not know (48%) or has incorrect information (34%).

## CONCLUSION

Slovak multi floral kinds of honey with a concentration of 50% were able to inhibit the growth of all three microorganisms (*S. enteritidis, E. faecalis, C. krusei*). A better antimicrobial activity was found in honey samples from urban beekeepers. Consumer research showed that consumers in Slovakia use honey as food (sweetener in beverages or ingredients in the recipe) and as medicine mostly during the winter to support their health. Approximately 97% think that honey has healing effects, however, only 11% are aware of its antibacterial activity and 1/3 was not able to identify specific effects. The rest of them mostly listed immunity booster, healing properties in terms of cold or sore throat.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# MICROBIAL, PHYSICOCHEMICAL AND SENSORY CHARACTERISTICS OF SALAMI PRODUCED FROM WILD BOAR (*SUS SCROFA*)

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## ABSTRACT

The wild boar (*Sus scrofa*) population in central Italy has strongly increased in the last decades. The meat of the game is characterized by high-quality value and the manufacture of food products from game meat could represent a remarkable added value for the local market promoting local gastronomic specialties and traditions. Adult animals were hunted with the waiting method and the carcasses were processed into the game processing center. Five batches of salami were produced with different amounts of wild boar meat and pork meat. The microbiological, physicochemical, rheological, and sensory evaluations were performed. The microbiological analyses indicated that the salami is safe to consume as *Listeria monocytogenes* and *Salmonella* spp. were undetectable in the end products. The *Enterobacteriaceae* count was below 3 log CFU.g$^{-1}$ attesting to the adequacy of hygienic characteristics of the process. The chemical composition analyses showed lower lipid content in comparison to pork salami, while the rheological characteristics were equivalent among products. The sensory evaluation highlighted that the consumers' appreciation of wild boar salami is comparable to that of traditional pork salami.

**Keywords:** game meat products; fermented meat products; food safety; textural characteristics; wildlife

## INTRODUCTION

Wildlife population, especially wild boar (*Sus scrofa*), has been characterized by a wide geographical and demographic expansion across Europe in the last decades, and although is difficult to precisely esteem boars population density **(Fernández-Llario et al., 2004)**, available data on the hunting bags reveal that in Italy the number of harvested wild boars has grown from less than 50,000 in 1985 to approximately 30,0000 in 2015 **(Massei et al., 2015)**. Particularly, in the Umbria region (central Italy) wild boar is the main hunted game species with several 40,000 animals killed per year **(Sebastiani et al., 2015)**. The meat of the game is characterized by very good chemical composition **(Šnirc et al., 2017)**, low-fat content **(Pedrazzoli et al., 2017)**, a favorable ratio of unsaturated/saturated fatty acids **(Quaresma et al., 2011)**, high protein content and good protein composition **(Okuskhanova et al., 2017)**, the outstanding value of mineral content **(Skobrák et al., 2011)** and peculiar taste and aroma **(Neethling, Hoffman and Muller, 2016)**. Multiple factors are responsible for the high quality of game meat, such as the natural and slow growth of animals, wildlife habits, the natural diet, the absence of pharmacological treatments, and farming-related stress conditions **(Babicz et al., 2018)**. Consumers are increasingly interested in healthier food products characterized by a high nutritional value and balanced nutritional profiles; furthermore, their concern about the environmental issues has increased and consequently they tend to prefer low input products, such as organic and natural products **(Petrescu, Vermeir and Petrescu-Mag, 2020)**. All the mentioned aspects have diffusely enhanced interest in game meat **(Paleari et al., 2002; Marescotti et al., 2019)**.

A crucial aspect to consider is represented by the compliance of these food products with the EU food safety regulation: it is essential indeed that game meat products reach quality and safety standards comparable to those of farmed animals produced on an industrial scale **(Fettinger, Smulders and Paulsen, 2011)**. There are several factors potentially affecting the quality and safety characteristics of wild boar meat, like the hunting techniques, the hunter's ability in terms of precision of the shot, the weather conditions, and the post-mortem activities such as transport and treatments of carcasses, as well as those of storage and meat processing **(Ranucci et al., 2019a; Paulsen, Vali and Bauer, 2011)**.

Besides, the game meat plays a key role in the promotion of local gastronomic specialties and traditions, concurring, therefore, to culinary and cultural touristic promotion with a great potential of economic return for the regional economies **(Branciari et al., 2020)**. A growing number of activities have been promoted to strengthen and diversify the "local direct market" of game meat that has a long

tradition in Central Europe: hunters directly supply to consumers or food local businesses **(Paulsen, Vali and Bauer, 2011)**. For this direct supply sector, as well as for game meat companies, the manufacture of food products from game meat could represent a remarkable added value **(Paulsen, Vali and Bauer, 2011)**. In particular, wild boar raw meat marketing can be limited by low color stability and short shelf-life **(Neethling, Hoffman and Muller, 2016)** and frequently is commercialized underpriced. Therefore, growing attention is focused on the manufacture of wild boar meat products **(Paleari et al., 2003; Żochowska-Kujawska et al., 2007)**. The interest addressed to this meat in central Italy regions is attested by the presence on the market of some game meat-based products and by its presence in the menu of restaurants and local festivals. Despite the significant expansion of wildlife populations throughout Europe, limited literature has been produced concerning the production of wild board meat products and the related safety, quality, and sensory aspects, especially for dry-cured fermented foodstuffs **(Zochowska- Kujawska et al., 2007; Schimpl, Bauer and Paulsen, 2010)**.

### Scientific hypothesis

This study aimed to evaluate the microbial, chemical, rheological, and sensory characteristics of experimentally produced salami made with different percentages of pork and wild boar meat. Hygienic characteristics of game meat products are expected to be equivalent to those of products from intensively farmed animals. Furthermore, this approach will likely allow for the profitable use of a frequently undervalued game species and for the production of salami appreciated by consumers.

## MATERIAL AND METHODOLOGY

### Hunting method

Adult wild boars were hunted from May 2018 to May 2019 in the municipality of Gubbio in the Umbria region (central Italy). The animals were hunted by selection hunters applying the waiting method according to which the hunters stay still in a selected location and wait for the animal to come towards them. This hunting strategy is characterized by a remarkably low level of antemortem stress experienced by animals.

### Salami production

After the hunting, animals were promptly exsanguinated and eviscerated on-field then transferred to a collection center for carcass storage and kept at 2 °C for 1 to 6 days (average 4 days). The carcasses were subsequently transferred to a game processing center for skinning and sectioning and then to a local producer for fermented meat (salami) production (Fazi Carni, Gualdo Tadino, Italy). Salami were made following the regional traditional recipe. Firstly, the deboned wild boar meat was combined with swine cuts (belly and shoulder) and grinded twice with a 6 mm plate. The meat batter was then added with salt (2.2%), ground black pepper (0.3%), black pepper grains (0.2%), ground garlic (0.05%), and starter cultures (Eurostarter MI Rapid, MEC Import, Roma, Italy; a mix of *Staphylococcus xylosus* and *Staphylococcus carnosus* + *Lactobacillus sakei* in a 2:1 ratio). Antioxidants and preservatives were not used. After resting overnight at 4 °C, the meat batter was stuffed into previously rehydrated dry-salted natural casings (swine intestine) to a final weight of approximately 1 kg for each salami. The products were subjected to a drying process inside controlled hot chambers for 10 days with different temperature (T) and relative humidity (RH) as follows: 22 °C and 62% RH for 48 h, 19 °C and 66% RH for 76 h followed by 1 °C T reduction and 1% RH increase every 24 h until reaching 15 °C and 72% RH. Afterward, the salami were ripened in controlled seasoning rooms at 13 °C and 75% RH for 60 days. The meat products were obtained by mixing wild boar and pork meat in different percentages: 50% wild boar meat and 50% pork meat (high percentage: BH), 40% wild boar meat and 60% pork meat (low percentage: BL), and 100% pork meat (control: C). The trial consisted of five batches for each group of which, to perform all the analytical determination, immediately after stuffing (T0) and after 7 (T1), 14 (T2), and 60 days (T3) samples from each experimental group (BL, BH, C) were collected and transported to the laboratory under refrigeration condition.

### Physicochemical analyses

The physicochemical determinations were performed, according to the sampling plan, on two samples per batch. Proximate composition and salt content (% NaCl) were determined according to the AOAC method **(AOAC, 2000)**.

Water activity ($a_w$) was measured at 25 °C with the $a_w$ recorder AquaLab, series 3, Model TE (Decagon Devices Inc., Pullman, WA, USA) as reported by **Ranucci et al (2019b)**. pH was measured according to **Branciari et al. (2016)** using a pH-meter Crison pH25 (Crison, Barcelona, Spain). The color of meat products was determined on the surface of sample slices using a Minolta Chromameter (CR 400, Minolta, Osaka, Japan). Lightness (L* value), redness (a* value), yellowness (b* value), Chroma (C), and Hue angle (H) were determined according to CIE Lab System (Commission Internationale de l'Eclairage) **(CIE, 1976)**.

### Microbiological analysis

At each time of the sampling protocol adopted, three samples for each group were tested in triplicate for Enterobacteriaceae count following the ISO 21528-2 method **(ISO 21528-2: 2004)**; for *Enterococcus* spp. count by plating diluted samples on Slanetz–Bartley agar (Biolife Italiana, Milan, Italy) and incubated at 37 °C for 48 h; for CNC enumeration by plating diluted samples on mannitol salt agar (Biolife Italiana, Milan, Italy) and incubation at 37 °C for 24 h. Furthermore, samples were also analyzed for *Lactobacillus* spp. counted on De Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) anaerobically incubated at 37 °C for 48 and *Lactococcus* spp. on M17 agar (Oxoid, Basingstoke, UK) aerobically incubated at 37 °C for 48 h.

Counting results were reported in terms of log $CFU.g^{-1}$. The detection of pathogens *Listeria monocytogenes* and *Salmonella* spp. were performed according to the ISO 11290-1 **(ISO 11290-1:2017)** and **ISO 6579-1:2017** procedures, respectively.

### Texture profile analyses (TPA)

The texture profile analyses of the products belonging to each experimental group were assessed at the end of the ripening time by the excision of three cylinders of height and diameter of 2.5 cm using a core drill as reported. A texture analyzer (TVT 6700, Perten Instrument, Sweden) was equipped with a cylindrical probe (3 cm in diameter) and set accordingly to a compression rate of 30% and a probe speed rate of 2 mm/s. The parameters considered were hardness, resilience, cohesiveness, gumminess, and chewiness.

### Sensory analyses

At the time of the end of ripening the products of the three different groups considered were evaluated through a ranking test according to the ISO methodology 8587 **(ISO 8587:2006)** set of three samples for general preference. At least 92 untrained panelists were recruited for the execution of the ranking test. For each panelist, samples were assigned random 3-digit numbers and sample order was randomized **(Branciari et al., 2017)**. The lowest rank (1) corresponded to the least preferred, whereas the highest rank (3) corresponded to the most preferred. Data analysis was based on the sum of ranks obtained by each sample.

### Statistical Analysis

Analytical results were subject to analysis of variance (ANOVA) (GMP, SAS Institute, Cary, NY, USA), considering the microbiological and physicochemical data assessed over sampling time (T0, T1, T2, and T3) and across different compositions (BH, BL, C). For the analysis performed exclusively on the finished products (T3), a one-way ANOVA was used with the salami composition as a fixed factor (BH, BL, C). Tukey's post hoc test was used to determine whether the values obtained were different with a significance level of 0.05. The results of the sensory analyses were evaluated using the Friedman Test.

## RESULTS AND DISCUSSION

### Chemical composition and physicochemical characteristics

The results of the chemical composition of the experimentally produced salami are reported in Table 1. The addition of wild boar meat in the product formulation significantly affected the fat and the ash content ($p$ <0.05). The fat content decreased along with the decrease of the pork meat varying from over 34% for pure swine products to 32% for BH salami. The fat content for all the groups is consistent with the type of product, indeed it has been reported, that fermented meat products should contain fat ranging from 25 to 45% to meet consumer expectations **(Yilmaz et al., 2009)**. The registered fat content is similar to what already found for similar game meat products (mean value 33%) **(Ranucci et al., 2019a)** and it was lower than that of wild boar salami (48%) registered by **Paulsen, Vali and Bauer (2011)**. Other results in the literature showed that the meat of wildlife is leaner than pork meat due to a more natural diet and periods of prolonged locomotion **(Szmanko et al., 2007)**. Concerning the ash content, the values recorded decreased with the increase of pork meat in the composition of salami, as shown in Table 1. No difference in the values between groups was observed for moisture, protein, and salt content and the data align with those reported by **Chakanya et al. (2018)** for a similar product produced with different game meats.

The pH and $a_w$ values are reported in Figure 1a and Figure 1b, respectively. The pH of all the products decreased as storage progressed from T0 to T1 ($p$ <0.001) followed by a increase in the subsequent sampling times ($p$ <0.001); however, no differences were recorded among experimental groups ($p$ >0.05) indicating that the meat batter composition does not affect such parameter following results reported in the literature by **Chakanya et al. (2018)** for similar products. This trend in pH was expected, as the bacterial starter culture contained lactic acid bacteria that metabolize carbohydrates into lactic acid lowering pH during the first days of maturation **(Fontana et al., 2012)**. Tissue and microbial (e.g. CNC) proteases are responsible for the subsequent increase of pH values in the latter stages of maturation **(Amadoro et al., 2011)**. It has been reported that antemortem stress can affect pH values hunting strategy using dogs to drive the animals toward the hunters and those in which the hunters stalk the prey can cause high levels of stress for the quarry resulting in meat with higher pH values (pH >6.0) that can frequently be classified as dark, firm and dry (DFD) **(Daszkiewicz et al., 2012)**. However, in the present study, the waiting hunting strategy was adopted causing no stress to the animals and enabling meat to acidify normally.

As shown in Figure 1b, $a_w$ values gradually decrease during seasoning time from T0 to T3 ($p$ <0.001), however no significant differences between the experimental group were registered ($p$ >0.05) indicating that $a_w$ trend is influenced by the ripening environment regardless the salami composition. This result is under what was reported for similar meat products in previous studies regarding salami produced with different amounts of wildlife meat **(Chakanya et al., 2018)**. The gradual loss of humidity from the salami along the ripening period results in less water available for the microorganism growth improving the safety characteristics of the final product.

The instrumental texture evaluation was conducted on the different salami at the end of the ripening time (T3) testing five different attributes. The results reported in Table 2 show no difference among the experimental groups for the attributes considered. This finding is in contrast with what was reported in a previous study by **Ranucci et al. (2019a)** for similar products. The author reported that salami with 50% roe-deer and 50% pork meat showed higher values of hardness and gumminess and lower values of chewiness in comparison with 33% roe-deer/ 67% pork salami and 100% pork salami. In the mentioned study, these differences have been related to the differences highlighted in the chemical composition of the three products **(Ranucci et al., 2019a; Gómez and Lorenzo, 2013)**. In the present study, however, such differences in chemical composition have not been recorded, likely explaining the discrepancy in the results for textural determinations.

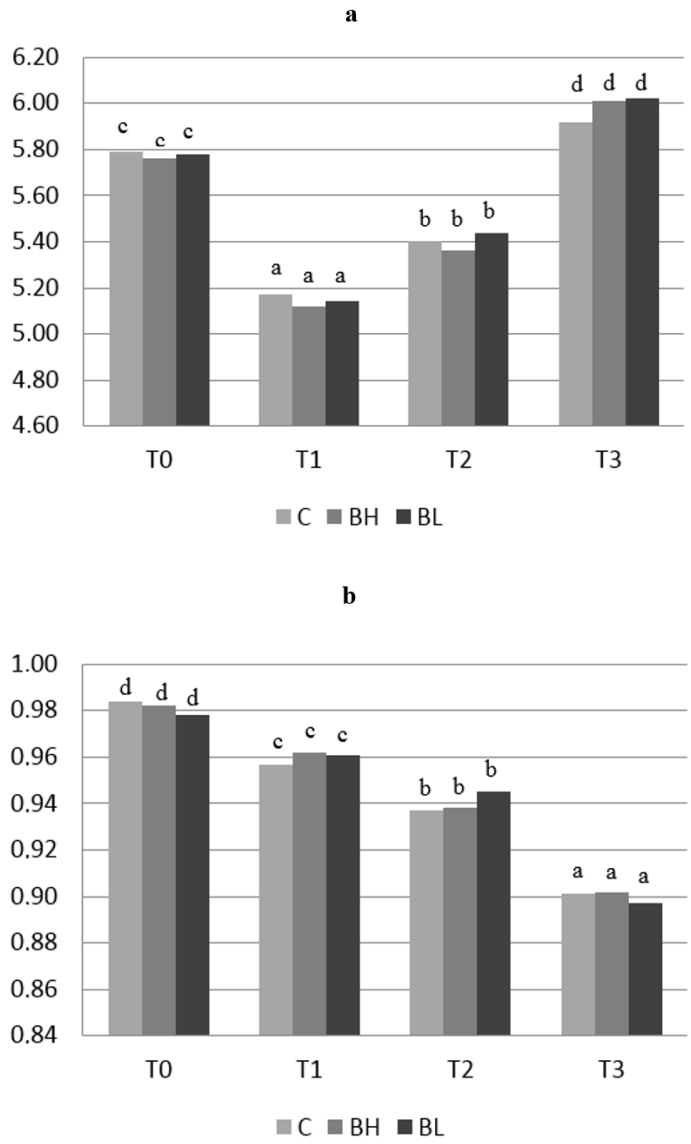


**Figure 1** Values of pH (a) and $a_w$ (b) of wild boar and pork salami.
Note: C (control: 100% pork meat); BL (low percentage: 40% wild boar meat and 60% pork meat); BH (high percentage: 50% wild boar meat and 50% pork meat). Different superscripts indicate differences in the mean values during sampling times ($p$ <0.001).

**Table 1** Chemical composition of pork salami and wild boar salami.

| | Salami formulation | | | SEM | *p*-value |
|---|---|---|---|---|---|
| | **C** | **BH** | **BL** | | |
| Moisture (g.100 $g^{-1}$) | 29.35 | 29.18 | 30.35 | 0.23 | 0.080 |
| Fat (g.100 $g^{-1}$) | 34.79b | 32.01a | 33.28 | 0.45 | 0.025 |
| Protein (g.100 $g^{-1}$) | 27.36 | 28.11 | 27.26 | 0.18 | 0.118 |
| Ash (g.100 $g^{-1}$) | 8.50a | 10.61b | 9.11ab | 0.37 | 0.042 |
| NaCl (g.100 $g^{-1}$) | 4.59 | 4.66 | 4.63 | 0.10 | 0.972 |

Note: C (control: 100% pork meat); BL (low percentage: 40% wild boar meat and 60% pork meat); BH (high percentage: 50% wild boar meat and 50% pork meat); SEM (standard error of the mean). Different letters in the same row reveal a difference in the mean values at *$p$ <0.05.

**Table 2** Results of texture profile analyses of wild boar salami and pork salami.

| Attribute | C | BH | BL | *SEM* | *p-value* |
|---|---|---|---|---|---|
| Hardness (g) | 4776.44 | 4463.93 | 4827.73 | 380.67 | 0.911 |
| Springiness | 0.59 | 0.58 | 0.55 | 0.009 | 0.105 |
| Cohesiveness | 0.52 | 0.52 | 0.45 | 0.016 | 0.056 |
| Gumminess (g) | 2517.56 | 2095.33 | 2187.53 | 176.39 | 0.662 |
| Chewiness (g) | 1601.67 | 1345.73 | 1244.27 | 126.39 | 0.570 |

Note: C (control: 100% pork meat); BL (low percentage: 40% wild boar meat and 60% pork meat); BH (high percentage: 50% wild boar meat and 50% pork meat).

**Table 3** Colour attributes of wild boar salami and pork salami.

| | L* | a* | b* | *C* | *H* |
|---|---|---|---|---|---|
| C | 41.70b | 10.71 | 4.91 | 11.78 | 24.62 |
| BH | 37.87a | 11.58 | 5.06 | 12.65 | 23.68 |
| BL | 38.80a | 11.57 | 5.12 | 13.21 | 22.84 |
| *SEM* | 0.71 | 0.24 | 0.13 | 0.35 | 0.45 |
| *p-value* | 0.050 | 0.219 | 0.806 | 0.244 | 0.274 |

Note: C (control: 100% pork meat); BL (low percentage: 40% wild boar meat and 60% pork meat); BH (high percentage: 50% wild boar meat and 50% pork meat). L* (Lightness value); a* (redness value), b* (yellowness value), C (Chroma) and H (Hue angle). Different letters in the same row reveal a difference in the mean values at $*p < 0.05$.

Concerning the color of salami (Table 3) no significant differences were found between the three experimental groups for a*, b*, C, H indices. However, the values of L* indicate that products containing wild boar meat (BL and BH) show lower levels of lightness in comparison to the 100% pork salami. The inherent darker color of wild boar meat is attributable to a higher content of myoglobin **(Young and West, 2001)**. Wild animals, indeed, have darker muscles than domestic animals due to a higher concentration of red muscle fibres as a result of their intense physical activity **(Daszkiewicz et al., 2012)**.

Generally, fermented meat products are considered microbiologically stable nevertheless, when initial contamination of raw material is too high or production phases are conducted inadequately, the safety of such products can be compromised **(Maksimovic et al., 2018)**. In few cases fermented meat products have been associated with *Salmonella* spp. and *Listeria monocytogenes* foodborne outbreaks **(Pierre, 2014)**. The microbiological determination performed on salami revealed the absence of *L. monocytogenes* (absent in 25 g) in all samples regardless of the product's composition and the sampling time, in compliance with the requirement for food safety criteria fixed by European Regulation (EC) n. 2073 **(EC, 2005)**. Concerning *Salmonella* spp., the analyses showed the presence of *Salmonella enterica* serovar Typhimurium in the meat batter and in T1 salami samples of the group BH of batch 1, however, the presence of the pathogen was no longer detected (absent in 25 g) in salami sampled in correspondence of the following sampling times (T2 and T3). Similarly, *Salmonella enterica* serovar Rissen was found in the meat batter of the BH experimental group of batch 4, nonetheless, the microorganism was undetectable (absent in 25 g) in all the subsequent salami samples (T1, T2, and T3). For both mentioned cases of the presence of *Salmonella* spp. it was infeasible to determine the specific source of contamination due to the thorough mixing procedure that the pork meat and the wild boar meat were subjected to.

The presence of *Salmonella* spp. in pork meat dry-cured products has been already reported in the literature **(Scavia et al., 2013; D'Ostuni et al., 2016; Bonardi et al., 2017)**, furthermore pork meat and products were responsible for 4.5% of 1241 food-borne outbreaks caused by *Salmonella* spp. in 2018, representing, therefore, the meat most frequently related with salmonellosis in humans **(EFSA, 2019)**. On the other hand, *Salmonella* spp. has also been found in wild boar species in central Italy with an incidence varying from 2.4 to 10.9% **(Russo et al., 2017)**. Conversely, other studies in the literature report the absence of *Salmonella* spp. in wild boar carcasses and meat **(Ludwiczak et al., 2019; Stella et al., 20l9)**. However, despite the detection of S*almonella* spp. in the meat batter and the T1 salami, it is important to highlight that in the finished products the pathogen was undetectable (absent in 25 g) in compliance with the requirement for food safety criterion of Regulation (EC) n. 2073 **(EC, 2005)**, confirming that the experimentally produced wild boar salami meet the safety standards required for large scaled industrialized products.

**Table 4** Microbial counts (log CFU.g$^{-1}$) of wild boar and pork salami at different times of storage.

| | | Days of Storage | | | | SEM | *p* value | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | | T | S | TxS |
| *Enterobacteriaceae* | C | 3.97b | 3.76ab | 3.22ab | 2.83a | 0.16 | <0.001 | 0.073 | 0.963 |
| | BH | 3.72b | 3.58ab | 2.83ab | 2.24a | | | | |
| | BL | 3.46b | 3.62ab | 3.16ab | 2.44a | | | | |
| *Lactobacillus* spp. | C | 6.00a | 8.51b | 8.76b | 8.56b | 0.04 | <0.001 | 0.358 | 0.051 |
| | BH | 6.08a | 8.70b | 8.61b | 8,67b | | | | |
| | BL | 6.26a | 8.59b | 8.59b | 8.63b | | | | |
| *Lactococcus* spp. | C | 6.08a | 8.04b | 8.60b | 8.88b | 0.07 | <0.001 | 0.123 | 0.813 |
| | BH | 6.25a | 7.94b | 8.57c | 8.99c | | | | |
| | BL | 6.33a | 8.33b | 8.66bc | 8.97c | | | | |
| CNC | C | 6.53$^{ab}$ | 6.65$^{Wab}$ | 6.97$^{Wa}$ | 6.29$^{Wb}$ | 0.08 | <0.001 | <0.001 | <0.001 |
| | BH | 6.58$^{a}$ | 7.72$^{Xb}$ | 7.86$^{Xb}$ | 7.58$^{Xb}$ | | | | |
| | BL | 6.47$^{a}$ | 7.08$^{Zb}$ | 7.53$^{Xb}$ | 7.20$^{Xb}$ | | | | |
| *Enterococcus* spp. | C | 2.80 | 3.15 | 3.31 | 2.96 | 0.08 | 0.595 | 0.173 | 0.308 |
| | BH | 3.06 | 2.93 | 3.04 | 2.92 | | | | |
| | BL | 2.98 | 2.81 | 2.83 | 2.84 | | | | |

Note: C (control: 100% pork meat); BL (low percentage: 40% wild boar meat and 60% pork meat); BH (high percentage: 50% wild boar meat and 50% pork meat); SEM (standard error of the mean). Different letters in the same row reveal a difference in the mean values at * $p$ <0.005. T (time); S (samples). CNC = coagulase negative cocci.

A summary of the Log counts of some non-pathogenic microorganisms detected in salami produced with different amounts of wild boar meat and control salami (100% pork meat) were reported in Table 4. The values of *Enterobacteriaceae* registered at T0 were slightly below 4 log CFU.g$^{-1}$for all experimental groups (Table 4). Afterward, the level of such microorganisms decreased by approximately 1 log CFU.g$^{-1}$ for all products more likely due to the evolution of physicochemical characteristics and to the proliferation of competitive microflora **(Paleari et al., 2002)**. No difference between groups was registered suggesting that the salami formulation had no impact in the presence of such bacteria confirming therefore that the presence of *Enterobacteriaceae* is principally related to fecal contamination during slaughter operations **(Chakanya et al., 2018)**. These results are in line with **Chakanya et al. (2018)** who found in wild boar matured salami a level of such bacteria ranging from <1.0 to 3.9 log CFU.g$^{-1}$. However, it is reported that unacceptable counts of *Enterobacteriaceae* are found in 33% of wild boar and deer fermented sausages made without the use of starter cultures and nitrites, confirming the importance of adequate production strategies **(Maksimovic et al., 2018)**.

Regarding *Lactobacillus* spp. count the initial values were around 6 Log CFU.g$^{-1}$ as the result of the addition of the starter culture to the meat batter, subsequently as expected, the values increased significantly during the ripening time similarly for all the salami formulations to a final mean value of 8.62 log CFU.g$^{-1}$. This evolution of *Lactobacillus* spp. occurs typically in fermented meat products, such as salami, and it represents a crucial aspect for the safety characteristics of products **(Miraglia et al., 2017)**.

Concerning the evolution of *Lactococcus* spp., the trend shown by the microorganisms is similar to that reported for *Lactobacillus* spp. with a constant increase until the

last day of sampling (T3) to a final mean value of 8.94 log CFU.g$^{-1}$. The trend of such microbial population was consistent between experimental groups during the entire ripening period.

The coagulase-negative cocci (CNC) count appeared already high in correspondence of the day of salami production (T0) in all the experimental groups irrespective of the meat batter composition with a mean initial value of 6.53 log CFU.g$^{-1}$, likely as a consequence of the use of starter culture. Subsequently, the population grew slightly for groups BH (final value 7.58 log CFU.g$^{-1}$) and BL (final value 7.20 log CFU.g$^{-1}$) while remaining stable in the control group (C). Similar trends are reported in different traditional Italian salami and mainly in those made with lean meat **(Amadoro et al., 2011; Miraglia et al., 2017)**.

In regards to *Enterococcus* spp. counts (Table 4), no differences were registered between the samples or along the sampling period, and the registered values ranged between 2.81 and 3.31 log CFU.g$^{-1}$. The values were lower than those registered in some traditional Italian salami **(Branciari et al., 2016)**.

Concerning the sensory analysis, the ranking test assessed the general preference of assessors regarding the experimental salami investigated in the present study. The sum of ranks attributed to the tested samples was 188 for C salami, 181 and 175 for HB and BL salami, respectively. The results showed no difference ($p$ >0.005) in the appreciation of panelists suggesting that the products containing different amounts of wild boar meat (BH and BL) and traditional 100% pork salami (C) can be similarly valued by consumers.

## CONCLUSION

The results exposed in the present study confirm that wild boar meat represents a valuable alternative to meat and meat products obtained from factory-farmed animals. This type of meat not only can attend to the needs of health-conscious consumers attentive to its high nutritional value, but it has also shown to be suitable for dry-cured fermented meat products manufacture. The fat content for the formulation with a higher amount of wild boar meat was 2% lower than of that pure swine products. Both safety and quality standards reached by the two experimental wild boar salami are comparable to salami produced from intensively farmed animals (pork) and fully comply with EU standards (*Salmonella* spp. undetectable in 25 g). Therefore, the manufacture of game meat products should be encouraged, pursuing the production of healthier, safe, and sustainable foodstuff characterized by a strong bond with local traditions and that also concur with the correct management of increasing wildlife.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This article does not contain any studies that would require an ethical statement.

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# A POLICY CONSTRUCTION FOR SUSTAINABLE RICE FOOD SOVEREIGNTY IN INDONESIA

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## ABSTRACT

As an agricultural country with large natural resources, Indonesia still has considerable problems in managing food security. This is evidenced by the lack of agricultural land and human resources that can support people's food needs so that the government still imports food from other countries. These issues as; it are feared that population growth and high food consumption power, social exclusion (marginalization of agricultural laborers and agricultural land) will cause food security vulnerability in the future. This Mixed method quantitative and qualitative method by statistical and in-depth interview study involving 169 respondents from many stakeholders such as politicians, academicians, farmers, and students to determine policy construction for sustainable rice food sovereignty in Indonesia. The statistical study shows the rice consumption in Indonesia is correlated to education than the age and gender of the respondents. The study indicating problems such as; the welfare of farmers, the application of price limits for staple goods, and improving the quality of agriculture both natural resources, human resources, and the provision of agricultural equipment assistance have not been resolved properly. To build food security, it should be balanced with the application of the concept of food sovereignty which is realized by aligning and maximizing competence between political resources, environmental resources capacity, and environmental diplomacy.

**Keywords:** food security; food sovereignty; political resources; environmental resources; environmental diplomacy

## INTRODUCTION

Indonesia is an archipelagic country that stretches widely and has quite extensive agricultural land. Indonesia is also located in a tropical area with a climate suitable for extensive agricultural business so that Indonesia is an agricultural country. The majority of Indonesian people's staple food is rice **(Davidson, 2018; Maraseni et al., 2018; Purba et al., 2020)**. However, in reality, Indonesia does not has rice food sovereignty yet **(Davidson, 2018)**. This is the reason why Indonesia still imports rice **(Warr, 2005)**. The theoretical perspective and the results of previous research that the author has done show that there is no concept or model or theory on the relevant sustainable rice food sovereignty policy implemented in Asia **(Sharma and Daugbjerg, 2020)** or even in Indonesia itself **(Davidson, 2018)**. Therefore, this study focuses on how to construct a model for sustainable rice food sovereignty in Indonesia.

From the data of the United Nations (UN), 80% of the world's population who suffer from hunger live in rural areas and of the 1 billion people who suffer from poverty in the world, 75% live and work in rural villages **(Yeboah-Assiamah et al., 2015)**. Likewise, Indonesia was also affected by the global food crisis in 2007 – 2008 **(Hossain, 2018)**. The food crisis has led to an understanding in the world community that "agriculture must be the main agenda in economic development **(Deichmann et al., 2011)**. Agriculture should be the main agenda in development planning because it is related to fulfilling national food security **(Kotykova, Babych a Krylova, 2020; Neilson and Wright, 2017)**. Fulfillment of national food needs has always increased due to growth in food consumption **(Szűcs, Szabó and Bánáti, 2013)** and population growth. In talking about rice, it will always be a complex situation in a strategic position in the process of agricultural development in Indonesia. Rice has become a political commodity and controls the life of the Indonesian people. The community has made rice a staple food so that it has become a strategic economic sector for the economy and national food security.

The Indonesian Central Statistics Agency (BPS) (Figure 1) shows that the macro indicators of the Indonesian agricultural sector, especially rice production in 2015, reached 75.3 million tons, while the rice production target was 82 million tons in 2017. Even though it has increased by about 1.5% compared to 2014 production, the target for this increase is not easy. Rice production in 2016, according to the estimated figures of BPS and the Ministry of Agriculture, reached 79.1 million tons of milled dry unhulled rice (GKG). This figure is up 4.97% compared to the 2015 production which reached 75.3 million tons of GKG. If the production data is correct, there should be a rice surplus of tens of millions of tons in 2017.

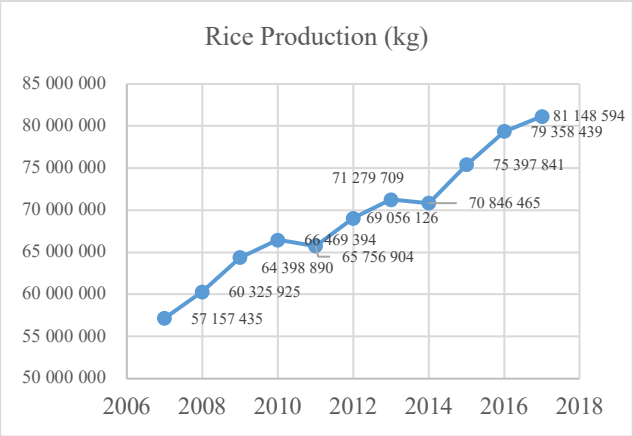


**Figure 1** Rice Production in Indonesia

Apart from the potential for exports, the stock surplus should push down prices. However, in reality, there was a rice shortage of 5.8 million tons in 2015 and is predicted to increase to 7.49 million tons of rice by 2020. To produce this amount of rice, a harvest area of around 13,500 – 15,000 ha of rice fields is needed or an area of around 9,000 paddy fields. - 10,000 ha **(Nugroho et al., 2018)**.

Indonesia is very dependent on carbohydrate food sources such as rice and wheat/wheat flour **(Davidson, 2018)**. This condition is evidenced by the continuous import of rice **(Patunru, 2019; Warr, 2005)** carbohydrates that exceeds the quota set by the government, which is more than 62%. The country's dependence on rice food is a reflection of the people's food consumption pattern (food habits) which tends to rice, even though non-rice food sources are still abundant, such as sweet potatoes, corn, sago, soybean, and other tubers. In response to such conditions, the government is trying to encourage food diversification to reduce the burden of carbohydrate consumption on other food commodities that are cheaper and more affordable.

The government's role in agricultural development towards strengthening food security is quite a lot. Various policies and programs in the agricultural sector have been implemented to increase agricultural production, such as the use of superior seeds, provision of fertilizers and medicines to agricultural land processing. The Ministry of Agriculture (KEMTAN) of the Republic of Indonesia has realized the expenditure budget for the period 2013 – 2017 from 17.8 trillion to IDR 23.9 trillion for the 2017 State Budget, although there was a decrease compared to the 2016 State Budget for the Ministry of Agriculture of the Republic of Indonesia amounting to IDR 31.5 trillion. Increasing the portion of the Ministry of Agriculture's budget allocation for the Republic of Indonesia during the 2013 – 2017 period is still related to the efforts in improving people's welfare, namely in the form of job creation, especially in rural areas, and economic growth and realizing national food security. The level of food availability is at least 90% of domestic needs. This is to secure food self-sufficiency, even though the most basic agricultural problems are the availability of land and agricultural infrastructure. On the agricultural infrastructure side, most of the irrigation networks are in poor condition. In 2017, the budget allocation for water resources management reached Rp. 6.3 trillion, while the program for the provision and development of agricultural facilities and infrastructure was IDR 2.7 trillion. Although the irrigation network has continued to increase from 2010 – 2017, according to **(Sugiyanto, Djumadin and Digdowiseiso, 2018)** there are still around 52% of the irrigation network was damaged. This condition can reduce the capacity of the agricultural sector to increase production yields, in addition to the availability of land, farming infrastructure which is also an important issue for increasing agricultural productivity. Food sovereignty should give freedom to farmers as producers to make choices independently and without coercion in developing food security and independence by the capacity and potential of local agricultural resources so that farmers can increase their production and welfare. The role of the government is needed as a regulator and facilitator in the agricultural sector through various policy instruments, regulations, and food programs that support these efforts for food sovereignty. For food commodities with political content such as rice, the government needs market intervention at or before the main harvest season. The government sets a base price for rice at the lowest price level to improve farmers' welfare. Meanwhile, during the planting season, the government sets an upper price to protect consumers, including farmers. This government policy can later have a significant influence on the direction and activities of Indonesia's food program.

## Scientific Hypotheses

The hypotheses are measured based on the literature review from several researchers, in the quantitative method **(Worsley et al., 2004)**, the measurement will measure people in Indonesia demographic conditions of gender, age, and education, the relations between three parameters with Rice Consumptions, the Hypotheses-0 ($H_0$) in this research is gender, age and education have a significant relation to rice consumptions and the Hypotheses-1 ($H_1$) in this research is gender, age and education do not have a significant relation to rice consumptions. The significance level is 0.05%, or in another word, the value of asymptotic significance <0.05% means the significant relationship is indicated between two parameters, and if the asymptotic significance <0.05% the relationship is not significantly indicated between two parameters. The other hypotheses in this research are, food sovereignty policy in Indonesia is supported by environmental management, stakeholder, the capacity of environmental resources, environment ecology, and policy foundation.

## MATERIAL AND METHODOLOGY

### Samples

However, this research is a mixed-method Qualitative and Quantitative based research **(Hashmi and Carlson, 2012; Schoonenboom and Johnson, 2017)**, specific samples are assigned by a purposive sampling method **(Serra, Psarra and O'Brien, 2018)**. To determine the food security model as modeled by **(Shoaib et al., 2021)**, samples are targeted to the person who aware of this matter. This study aims to sample academicians, politicians, food resilience experts, and students. Questions in this research are using structured interviews and questionnaires to explore the food security model, a total of 169 samples are targeted in this research.

**Table 1** Demographics of Respondents (Gender).

| | Freq | Valid % | Cumulative % |
|---|---|---|---|
| Male | 75 | 44.4 | 44.4 |
| Female | 94 | 55.6 | 100.0 |
| Total | 169 | 100.0 | |

**Table 2** Demographics of Respondents (Age).

| | Freq | Valid % | Cumulative % |
|---|---|---|---|
| 15 – 29 | 101 | 59.8 | 59.8 |
| 30 – 39 | 54 | 32.0 | 91.7 |
| 40 – 49 | 7 | 4.1 | 95.9 |
| 50 and above | 7 | 4.1 | 100.0 |
| Total | 169 | 100.0 | |

**Table 3** Demographics of Respondents (Education).

| | Freq | Valid % | Cumulative % |
|---|---|---|---|
| High-School/ Middle-School | 53 | 0031.4 | 31.4 |
| College Degree | 99 | 58.6 | 89.9 |
| Postgraduate Degree | 17 | 10.1 | 100.0 |
| Total | 169 | 100.0 | |

**Table 4** Gender and Rice Consumption.

| Count | | Rice Consumption | | Total |
|---|---|---|---|---|
| | | Yes | No | |
| GENDER | Male | 70 | 5 | 75 |
| | Female | 90 | 4 | 94 |
| Total | | 160 | 9 | 169 |

Aimed for policy construction for food security sovereignty, a mix method research with an exploration of relations between respondents demographic and rice consumptions (quantitative method) to its view to food sovereignty and also qualitative method as suggested by several researchers such as **(Bush-Kaufman et al., 2019; Katikireddi, Bond and Hilton, 2014)** who explore strategies. To measure the significance of the relation. IBM SPSS Statistics 22.0 is used to measure the *Chi-Square* value between two parameters. To support the research, more method is suggested by using Qualitative research method by providing an in-depth interview to its policy understanding and can understandable by its respondents. Questions related to staple foods, food locations, food substitutions, etc. In this research, statistical tests were two-sided, and significance was assessed at the a = 0.05 level. Statistical analyses were performed in R **(Gossai et al., 2017)**.

As we can see in Table 1, from the total of 169 respondents 55.56% of the respondents are females and 44.4% are males.

Table 2 below, from a total of 169 respondents more respondents (59.8%) are aged 15 – 29 years old (yo) and 32% are 30 – 39 yo and the rest of them are up to 40 years old.

The demographic table (Table 3) also shows more educated respondents in this research, while 58.6% of the respondents are graduated from college, and the smaller portion (31.4%) are graduated from high school. A very small portion (10.1%) are graduated from postgraduate school.

With better education, it is assumed the respondents will have a better understanding of this topic to answer the research questions.

## RESULT AND DISCUSSIONS

The Food Sovereignty Policy Model is raised by several researchers **(Beuchelt and Virchow, 2012; Shilomboleni, 2017)**, especially in food sufficiency to the Asian society such as rice. Refers to the basic principles of environmental science, namely sustainability **(Helms, 2004)**.

Sustainability itself means that there is a process of natural life systems in which humans and other living things carry out their life and the welfare of humans and other living things **(Scerri, 2010)**. This scope in which there are humans and living things is limited by factors that are accessible to humans, for example, natural, political, economic, and social factors **(Yu and Wu, 2018)**. Also, the use of natural resources to drive economic growth **(Ji, Magnus and Wang, 2014)** is carried out without sacrificing the carrying capacity, supporting capacity, and environmental productivity **(Yang et al., 2016)**.

Sustainable development in the field of food sovereignty **(Katikireddi, Bond and Hilton, 2014)** consists of the social environment, the artificial environment, and the natural environment in which the three subsystems interact **(Rahmann et al., 2020)**. The resilience of each subsystem will improve a balanced and dynamic condition and this condition can assure sustainability which in turn will increase the quality of food production **(Bosona and Gebresenbet, 2018)**.

Based on the results of distributing questionnaires to 169 respondents, the authors see various conditions related to conditions and future expectations for developing a food sovereignty policy model. This food sovereignty policy model will later be a novelty combined with the theories used in research. A total of 169 respondents were drawn from distributing questionnaires.

## Relations between Gender and Rice Consumption

Several researchers such as: **(Kunto and Bras, 2018; Wennberg et al., 2012)** placed Gender as an important role in food consumption. From these results, it can be seen that people in Indonesia still depend on rice as their daily staple food **(Surahman, Soni and Shivakoti, 2018)**. The amount of agricultural land that has existed in Indonesia until now and in the future is decreasing. This is due to massive development in terms of industry, offices, and housing, which has resulted in the erosion of agricultural land in Indonesia **(Schreer and Padmanabhan, 2020)**. The erosion of agricultural land has resulted in Indonesia importing rice from neighboring countries, such as Vietnam, Thailand, China, India, Pakistan, the United States, Taiwan, Singapore, Myanmar, and other countries **(Yuniarti, 2010)**. As we can see from Table 4 and Table 5, both males and females are consuming rice for their main meals (Table 4 and Table 5).

Based on the data of the respondents the value or asymptotic significance count >0.05% means the less significant relationship is indicated between two parameters (gender and rice consumption).

Staple food other than rice should be a lot **(Gao et al., 2019)**. The food starting from sago, cassava, bread, potatoes, corn, and yams **(Utami, Cramer and Rosenberger, 2018)**. However, Indonesian people, in general, are familiar with the term "if you haven't eaten rice, it's a sign that you haven't eaten yet". This term has continued until recently. So that the current trend in the future is that the Indonesian people are in an emergency for rice food. This is inversely proportional to the current agricultural conditions in Indonesia. Until now, the land has been increasingly eroded by industrial development like happens in India **(Balakrishnan and Lalithambika Devi, 1984)**, both small and large scale, as well as the massive development of exploration for mines or other natural resources. Also, other developments are for housing or office infrastructure.

## Relations between Age and Rice Consumption

From previous research **(Chanpiwat and Kim, 2019)**, it is not suggested for unproductive age to consume rice (40 years old and up) since rice mostly contain high carbohydrate (50 g per 180 g serving size) **(Haimoto et al., 2018)**, most of those ages statistically prone to diabetes disease. Age is playing an important role in rice consumption, as we can see from Table 6 and Table 7, the theories are not supported, even more people of older age still consuming rice as their main food source (Table 6 and Table 7).

This figure has an indirect impact made the rice import figures for the public get higher and higher. There is a paradox based on the results of distributing questionnaires given to 169 respondents. Most of the respondents consume the staple rice in their daily lives, at least 2 times meals per day. However, the respondents answered that there was no need to import rice for the Indonesian people. This is a finding that when the community's staple food is rice, there has been no concrete action yet on how to sustain the rice food in Indonesia.

In terms of age based on the data of the respondents, the value of asymptotic significance count >0.05% means the less significant relationship is indicated between two parameters (age and rice consumption).

## Relations between Education and Rice Consumption

In terms of Education based on the data of the respondents the value or asymptotic significance count <0.05% means both parameters have a significant relationship (Table 8 and Table 9) (education and rice consumption) **(Di Leo and Sardanelli, 2020)**.

The data measurement is answering the hypotheses. Indonesia demographic conditions of gender, age, and education, the relations between three parameters with rice consumptions, the hypotheses-0 ($H_0$) in this research is gender, age and education have a significant relation to rice consumptions. Based on the measurement, only gender and age can accept this hypothesis, and the relations between education and rice consumption have significant relations, the result of asymptotic significance is 0.035 or count <0.05% means both parameters have a significant relationship. This result means rejecting hypotheses-1 ($H_1$) in this research is gender, age and education do not have a significant relation to rice consumptions. This research is supported by the previous researcher **(Utami, Cramer and Rosenberger, 2018; Worsley et al., 2004)**, who investigate a relationship between education and rice consumption.

## Policy Construction to Rice Sovereignty

Rice imports, which have been carried out by the Government of the Republic of Indonesia, are carried out to maintain the stability of food availability for the community. However, the rice import policy must be in the last order to be implemented. The entry of rice imports in the last sequence is considered to maximize the potential that exists in the country.

**Table 5** Gender and Rice Consumption Chi-Square measurement.

| | Value | df | Asymptotic Significance (2-sided) | Exact Sig. (2-sided) | Exact Sig. (1-sided) |
|---|---|---|---|---|---|
| Pearson Chi-Square | .481[a] | 1 | .88 | | |
| Continuity Correction[b] | .122 | 1 | .727 | | |
| Likelihood Ratio | .477 | 1 | .490 | | |
| Fisher's Exact Test | | | | .512 | .361 |
| Linear-by-Linear Association | .478 | 1 | .489 | | |
| N of Valid Cases | 169 | | | | |

**Table 6** Age and Rice Consumption.

| | | Rice Consumption | | |
|---|---|---|---|---|
| | | Yes | No | Total |
| AGE | 15 – 29 | 94 | 7 | 101 |
| | 30 – 39 | 52 | 2 | 54 |
| | 40 – 49 | 7 | 0 | 7 |
| | 50 and above | 7 | 0 | 7 |
| Total | | 160 | 9 | 169 |

**Table 7** Age and Rice Consumption Chi-Square measurement.

| | Value | df | Asymptotic Significance (2-sided) |
|---|---|---|---|
| Pearson Chi-Square | 1.585[a] | 3 | .663 |
| Likelihood Ratio | 2.320 | 3 | .509 |
| Linear-by-Linear Association | 1.508 | 1 | .219 |
| N of Valid Cases | 169 | | |

It can be seen from the results of the questionnaire respondents that most people still buy rice from markets or traditional markets.

This is evidenced by the increase in rice imports from year to year. The government through the Ministry of Trade (Kemendag), in 2018, imported 500,000 tons of rice from Vietnam and Thailand. This rice import was carried out due to the addition of rice stocks which had decreased over time. The government thinks that with a population of more than 250 million people, rice is needed as a staple food for Indonesians.

Besides, based on data from the Central Statistics Agency (BPS), Indonesia has always imported rice from 2000 to 2015, or for fifteen years. For 2016 and 2017, the government has temporarily stopped importing rice. In 2018, Indonesia again imported rice. So that during this period, Indonesia has imported rice as much as 15.39 million tons of rice with the largest volume of rice imports in 2011 with a volume of 2.75 tons. Meanwhile, the smallest volume in 2005 was 189,616 tons. In terms of funds spent on rice imports amounting to 15.39 million tons, reaching the US $5.83 Billion or converted into rupiah amounting to IDR 78.70 trillion (exchange rate of IDR 13,500).

Based on the results of respondents in the interview, for the location of buying rice, most people still depend on traditional markets, even though people also buy their rice food needs at mini-markets/supermarkets/malls. It occupies the last position if people buy directly from farmers or bring rice from their hometowns. Even though it ranks last in terms of purchasing rice, this is a potential that people are still trying to be able to buy rice food from domestic products.

Based on Table 10. This shows that Indonesian people do not need to import rice. This is because the potential that exists in the domestic community still can produce food in the form of rice. Besides, the public thinks that importing rice, has the potential to increase the amount of foreign debt held by the state.

Table 11 shows that rice consumption per week and family is around 66.036 kilograms. So it can be seen that the dependence on consuming rice for Indonesian people is still very high based on this figure.

**Table 8** Education and Rice Consumption.

| | | Rice Consumption | | Total |
|---|---|---|---|---|
| | | Yes | No | |
| Edu | High-School/Middle-School | 53 | 0 | 53 |
| | College Degree | 90 | 9 | 99 |
| | Postgraduate Degree | 17 | 0 | 17 |
| Total | | 160 | 9 | 169 |

**Table 9** Education and Rice Consumption Chi-Square measurement.

| | Value | df | Asymptotic Significance (2-sided) |
|---|---|---|---|
| Pearson Chi-Square | 6.722[a] | 2 | .035 |
| Likelihood Ratio | 9.982 | 2 | .007 |
| Linear-by-Linear Association | 1.163 | 1 | .281 |
| N of Valid Cases | 169 | | |

**Table 10** Urgency of Importing rice.

| | | Freq | Valid % | Cumulative % |
|---|---|---|---|---|
| Valid | Needed | 25 | 14.8 | 14.8 |
| | Not-Needed | 144 | 85.2 | 100.0 |
| | Total | 169 | 100.0 | |

**Table 11** Respondents' Rice Weekly Consumption.

| | N | Min | Max | Mean |
|---|---|---|---|---|
| Weekly consumption | 169 | 1.00 | 125.00 | 66.036 |
| Valid N (listwise) | 169 | | | |

**Table 12** Price of Rice according to Respondents.

| | | Freq | Valid % | Cumulative % |
|---|---|---|---|---|
| Valid | Very Cheap | 21 | 12.4 | 12.4 |
| | Cheap | 22 | 13.0 | 25.4 |
| | Average | 42 | 24.9 | 50.3 |
| | Expensive | 78 | 46.2 | 96.4 |
| | Very Expensive | 6 | 3.6 | 100.0 |
| | Total | 169 | 100.0 | |

The community's dependence on rice is not comparable to the community's ability to buy rice.

The increasing number of rice imports based on BPS data every year shows that Indonesia, with a population of more than 250 million, still depends on the availability of rice food. There need to be more contributions to overcome the problems that exist today to achieve food security **(Timmer, 2014)** for the people of Indonesia. In addition to the need related to the existence of rice food, the author tries to explore most of the people shopping for their rice food needs anywhere.

This can be seen in Table 12 that the price of rice according to respondents is still expensive. The high price of rice has made the health and economic conditions of the people deteriorate over time.

Based on Table 13, it is stated that as many as 76 respondents said they wanted to replace their staple food like rice. And almost as many as the previous respondents, as many as 93 respondents did not want to replace the staple food rice. This can be assumed that the Indonesian people still cannot replace their staple food, namely, rice, because since childhood and from a long time ago, people have been accustomed to consuming rice.

**Table 13** Substitute Rice according to Respondents.

| | | Frequency | Valid % | Cumulative % |
|---|---|---|---|---|
| Valid | Needed | 76 | 45.0 | 45.0 |
| | Not-Needed | 93 | 55.0 | 100.0 |
| | Total | 169 | 100.0 | |

**Table 14** The main food consumed other than rice according to the respondent.

| | | Frequency | Valid % | Cumulative % |
|---|---|---|---|---|
| Valid | Wheat | 2 | 1.2 | 1.2 |
| | Cassava | 100 | 59.2 | 60.4 |
| | Kentang | 13 | 7.7 | 68.0 |
| | Sago | 18 | 10.7 | 78.7 |
| | Others | 36 | 21.3 | 100.0 |
| | Total | 169 | 100.0 | |

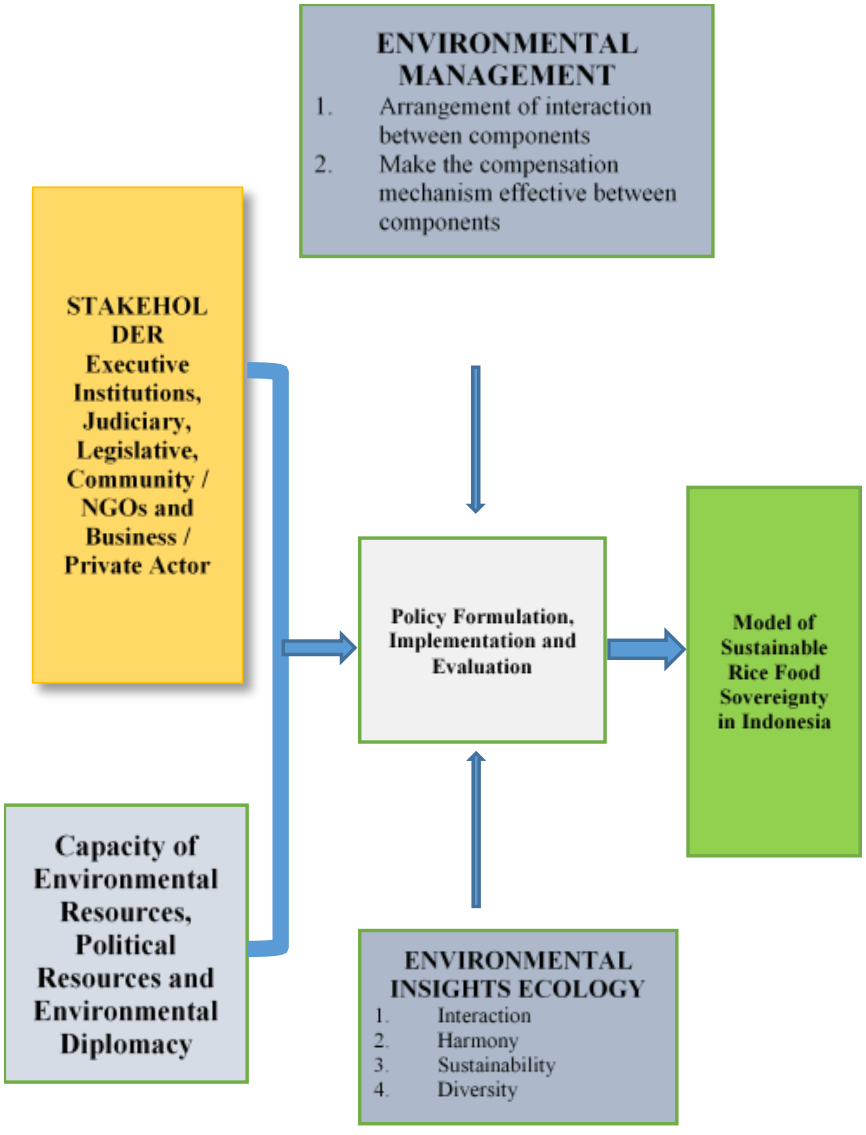


**Figure 2** Model of Sustainable Rice Food Sovereignty in Indonesia.

And based on Table 14, the food other than rice that most respondents eat is cassava. Thus, cassava becomes an alternative to eat if there is no rice or a staple food other than rice.

Based on the data that has been collected by the author, both primary data from respondents and secondary data based on literature studies, the authors have compiled a Sustainable Rice Food Sovereignty Policy Model. This model is based on the results of analysis both from the capacity of political resources, the environment, and environmental diplomacy.

The author has mentioned the results obtained from the questionnaire distribution process, secondary data related to food sovereignty policies and environmental resource capacity. And has conducted an analysis based on three aspects, namely political resources, environmental diplomacy, and environmental resources themselves.

Food sustainability in the rice food policy model is under the definition of the environment **(Fischer, 2017)**, which means the spatial unity between all objects, forces, conditions, and living things including humans and their behavior, which affects nature itself, the continuity of life, and the welfare of humans and other living things. This food sustainability includes the existence of political resources, environmental diplomacy, and the capacity of environmental resources embodied in the food policy model. Political resources are part of the social dimension related to the rice food policy process. Environmental diplomacy, how an environmental policy, especially on rice food, can be profitable economically in Indonesia. As well as the capacity of environmental resources by looking at the carrying capacity, and accompanied by supporting technology that can be applied to achieve food security, especially rice food.

These three dimensions, namely the social, economic, and environmental dimensions in the rice food policy model, strive to achieve sustainable development. This sustainable development is environmentally sound, which is not only profitable economically and socially but also benefits the natural environment. Besides, this policy model by the dimensions of sustainable development must include Economically Profitable, Socially Acceptable, and Environmentally Sustainable.

So far, according to the data that the authors have collected, rice food security policies have not focused on the use of agricultural land or the development of new land for agriculture to create rice food production that can be available every year. If there is an increase in the price of rice per kilogram or liter, as well as limited sources of rice food in Indonesia, the government will make a policy to import rice. On the positive side, the rice import policy does not have a bad impact in general. This is because it can meet the food needs of the Indonesian people and maintain the stability of rice food prices in Indonesia.

The long-term side of the rice import policy tends to get worse over time. First, the amount of rice food production in Indonesia is decreasing over time. This condition is due to the rice import policy which has been running routinely and the use of agricultural land to become industrial land or oil palm plantation and housing. Second, there is a growing dependence on rice and is not accompanied by sustainable rice food production. Third, the lack of innovation in developing rice substitutes, which so far can be an alternative.

Also, food security is important because of the problems that have been a top priority both in Indonesia and in the world. These problems are divided into three components, namely food availability, access to food, and the benefits of food itself. The rice food sovereignty policy model that the author tries to provide is based on these three components of the problem. Food availability in Indonesia is seen in how much and the type of food it can produce, how food can be distributed, and how much food can be traded.

So far, the type of food produced in Indonesia has focused on rice food. This is because so far for the majority of people in Indonesia, the staple food is rice. Besides, there is a tendency that rice production will decrease each year due to the availability of agricultural land. Based on the results of distributing questionnaires to 169 respondents, it shows that the Indonesian people want to change their staple food to other than rice if rice is indeed scarce. The results showed that most of the substitute food other than rice for the staple food was cassava. The current condition of cassava is only a complementary food or snack. This potential should be an important concern so that it can be developed, not only as a snack but as a potential substitute for the staple rice food if rice food is experiencing a dire condition, namely scarcity.

The food distribution process also becomes an important point related to the process of rice food sovereignty. This food distribution includes the cost of fuel, distribution time, and quality of rice food to customers. The increase in fuel costs or the increase in vehicle fuel is the main key concerning the selling price of rice. The higher the fuel price, the higher the selling price of rice. Apart from that, the distribution process also includes the possibility of weather conditions during the distribution trip to the market which is then purchased by the customer. The condition of the rice can be damaged due to moisture or the presence of rice lice. This has resulted in a decrease in rice sales and distribution of rice to customers.

How much food can be bought and sold also depends on the production and distribution processes that have been carried out so far? Weather conditions and soil conditions that exist in this process are important to note. The government has been anticipating the provision of adequate facilities in the production process and additional distribution facilities to support the increasing demand for rice food. Anticipation according to the author apart from the Government by providing welfare for farmers. So far, farmers do not own their land and work on other people's land, so the income of these farmers is very minimal.

The components of access to food include affordability, allocation, and preference. Affordability is defined as the purchasing power of households or people for rice food. The current conditions vary widely to the condition of society regarding the purchasing power of rice food, which is distinguished from the middle to lower class and upper-middle class. Based on the results of the questionnaire that was given during data collection, it can be seen that most people buy rice in the range of 2 to 5 kg per week to buy rice. Per month it can be estimated that each family consumes between 8 s.d. 20 kg. Other results also show that some buy 50 kg of rice per week and some buy 0.5 kg of rice per week. These results indicate that there are middle to

lower-class people who cannot buy enough rice to support their families and there are those who buy rice excessively because the family is classified as the middle to the upper class.

The government has anticipated the provision of subsidized rice for the lower middle class. This is done to make the process of distributing food evenly distributed throughout society, not only for the upper-middle class but also for the lower middle class. However, the subsidized rice provided by the government usually has low conditions so that the content in rice is very low. The anticipation that according to the author can be done to realize food sovereignty, especially rice, is to continue to provide rice subsidies, but not reduce the quality of the rice itself. So that the nutrition obtained by the Indonesian people is evenly distributed and is not differentiated by certain groups.

The components of the allocation of sustainable rice food sovereignty are economic, social, and political mechanisms, when, where and how food can be consumed by the community. The purpose of this mechanism is that each region has policies that differentiate it from other regions. One example is that the demand for rice in urban areas is greater than that in rural areas. This is due to the large population in urban areas compared to rural areas, which makes the demand for rice in urban areas much greater than in rural areas. This allocation component is related to the preference component.

Preference components are defined as social, religious, and cultural norms and values that influence consumer demand for certain types of food. In several regions in Indonesia, rice is not their staple food. Like some areas where the staple food is sago, and that includes the social and cultural norms that have been applied from generation to generation. Therefore, in these areas, there is little or no demand for rice food, because they own land or produce Sago for their daily staple food needs. This condition usually occurs in rural areas, not in urban areas, so that the allocation components and preference components are related to one another.

In the food benefit component, it is seen from the nutritional value, social value, and food security. The nutritional value in question is the number of calories, vitamins, protein, and nutrients found in food consumed by consumers. In the process of producing rice food based on this nutritional value, it must pay attention to the content or ingredients during the process of planting to harvest. Often farmers use hazardous substances or pesticides in the process of planting rice seeds until the harvesting process. So that it can endanger consumers who consume rice. Therefore, there have been various breakthroughs in organic rice without preservatives or hazardous substances in the rice food production process. However, organic rice is traded at a higher price than ordinary rice, and only the middle and upper class can buy it. The solution that can be given by the author is to carry out a strict monitoring process starting from the initial process of planting rice seeds to harvesting. Besides, assist farmers in terms of seeds and supporting facilities to produce good rice food production.

Social value has a meaning almost the same as a preference, concerning social, religious, and cultural functions, and the benefits provided by these foods. The purpose of this social value is that not all people have the staple food of rice. This can be seen based on the culture and history that has been applied from generation to generation. In addition to the nutritional value and social value in the food benefit component, the safety of the food produced is also seen. Food safety is seen in the presence of potential toxins during production, distribution, and others. This potential can occur if some elements or farmers prioritize the maximum profit by not meeting normative standards in the rice food production process. The solution that can be given by the author is to be given a very strict monitoring process in the production process, to minimize these potentials.

The problems previously mentioned are based on these three main components, in addition to developing political resources, there is a need for environmental diplomacy and environmental resource capacity that need to be met to realize a Sustainable Model of Rice Food Sovereignty. The environmental diplomacy that the author provides is not in the realm of rice imports, which has been mostly carried out by the Indonesian government so far. The environmental diplomacy referred to by the author is the transfer of knowledge related to the sustainability of rice food in Indonesia.

This environmental diplomacy can create opportunities for cooperation, build trust, and resolve conflicts related to issues of the environment and shared natural resources. Environmental diplomacy in the domain of knowledge transfer provides several alternatives, including the provision of agricultural tools, provision of superior rice seeds, application of fertilizers, and provision of rice paddy land. Based on respondents, as many as 111 respondents showed that providing agricultural tools and pressing paddy field resistance were priorities to achieve rice food sovereignty.

Regarding Environmental Diplomacy between Indonesia and other countries, cooperation can only be done by providing agricultural equipment. The provision of agricultural equipment can be done by cooperating with developed countries that have the best facilities or tools in terms of the rice food production process. This environmental diplomacy is not only beneficial for the country, but it can also benefit Indonesia because it gets a transfer of knowledge related to supporting facilities towards good rice food production. The solution for granting rice paddy land, according to the author, cannot be done with Environmental Diplomacy. This is because granting rice paddy land can only be done by the Indonesian government itself.

Granting rice paddy land can be done by building new land for farming and keeping the agricultural land continuing every year. The current condition has the potential for damage to land planted for rice food production as a capacity of environmental resources. The capacity of environmental resources for rice food production can be overcome by establishing irrigation channels, using superior rice seeds, maintaining soil conditions at all times, and having a land restoration process so that the land can be used continuously to maintain rice food production for the Indonesian people. So that dependence on rice imports is increasingly minimized.

The analysis that the author has provided is related to the sustainable rice food sovereignty policy model, enabling the author to compile a Sustainable Rice Food Sovereignty Policy Model. This model looks at Political Resources,

Environmental Diplomacy, and Environmental Resource Capacity. These three things are to achieve Socially Acceptable, Economically Profitable, and Environmentally Sustainable.

### Political Resources

The Rice Food Policy based on the Results of Literature Study and Questionnaire Distribution must pay attention to four aspects, namely: the fulfillment of community food needs, monitoring, and evaluation of food production, understanding of food other than rice, and policies for food diversification other than rice.

This policy must be written in-depth and comprehensively, both in urban and rural areas, as well as farmers and consumers. This deep and comprehensive process is part of a socially acceptable. The initial process of food production starting from the initial process to the final process (towards consumers) needs to be accepted by the community. What is accepted by the community is the existence of good quality and nutritious rice food, easy access to food for the community, and affordable prices for the community. As previously mentioned, based on the theory that the author uses, food sovereignty takes precedence before achieving food security.

Food sovereignty in terms of political resources can be realized by fulfilling the existing components of political resources in the form of policies for the welfare of the community so that rice food needs are met. Also, these policies were formulated not only to make rice food available to the public but by providing other food innovations as a substitute for rice. The potential for developing rice substitute food innovations, especially cassava based on the results of questionnaires to respondents. Cassava food development innovation policies can be realized to meet the daily staple food needs of the community.

### Environmental Diplomacy

The economic aspect, which is economically profitable, can be realized by environmental diplomacy between Indonesia and other countries. Environmental diplomacy as well as other diplomacy seeks to realize an economic level, both at the state economy level in general and the economic level of society in general. The current condition of Indonesia's economy is based on the latest information, that Indonesia is increasing its foreign debt for the sake of development in Indonesia.

One of the ways to reduce or minimize the increase in foreign debt is to take advantage of the potential that exists in Indonesia, both in terms of human resources and in terms of natural resources. Indonesia, which is currently a developing country has limitations in several fields, especially in the field of rice food development which is the focus of this research. The limitations are the lack of rice fields, lack of functioning irrigation channels, lack of superior seeds, and lack of supporting facilities (for example tractors or agricultural technology).

The limitations of a developing country like Indonesia need to be addressed through the environmental diplomacy process with other countries. The process of environmental diplomacy is carried out by transferring knowledge related to the development of sustainable food production, especially rice, by applying environmentally friendly technology and providing superior seeds. However, the environmental diplomacy process must be adapted to the land conditions in Indonesia which have a tropical climate. It is because, if we don't see this condition, the environmental diplomacy process will not run well and it will not be beneficial for both parties.

### Environmental Resource Capacity

Environmental resources are the final component to be able to create a model of sustainable food sovereignty (Figure 2). The stability of the land for producing rice food needs to be maintained because often after several years, the condition of the land decreases, and the yield of rice production is increasingly obedient and has less good results. Apart from seeing the land conditions, it is necessary to pay attention to the weather aspect. The weather aspect is related to climate change that has occurred so far, not only in Indonesia but also in almost all countries.

In terms of land conditions, treatment can still be given to recover by carrying out land management that follows environmental principles. The environmental principles are sufficient supporting facilities, one of which is an adequate irrigation channel. Other environmental principles are not to use pesticides or other chemicals, because they are not only harmful to consumers but also harmful to the condition of rice plants and the land. However, related to weather conditions, namely climate change, of course, it can be overcome, but still the effects of climate change cannot be completely fixed instantly.

Based on this, the capacity of environmental resources for rice food production needs to be managed properly from the start of production until the product reaches consumers. Apart from the capacity of environmental resources, this model of rice food sovereignty cannot be separated from the contribution of political resources and environmental diplomacy. This is because as a unit to achieve sustainable development.

In short, we can see the model of Sustainable Rice Food Sovereignty in Indonesia as shown in the diagram above, can be implemented and evaluated if the policy formulation is included the four aspects, such as; Environmental Management, Environmental Insight Ecology, Stakeholder and the Capacity of Environmental Resources, Political Resources, and Environmental Diplomacy are combined.

## CONCLUSION

Food security has become a crucial issue in the last few decades. Not only in Indonesia, but almost all over the world are also experiencing various food crises to meet the food needs of the population whose growth is not accompanied by a significant increase in the amount of food availability. Various world institutions have been trying to find solutions and alternatives to cover the low growth in food security which tends to decline. There are many causes of these problems, including reduced human resources, inadequate availability of natural resources, and or starting to experience degradation, as well as natural disasters and climate change.

The government is expected not only to apply regulations that tend to solve short-term problems. It is hoped that this problem can find a solution to the root of the main problem

that causes the decline in national food security. The problems mentioned above cannot be overcome by simply importing rice, subsidizing the cost of fertilizers, or providing agricultural equipment which in the end do not have a significant effect. The implementation of the problem should be thoroughly searched to the smallest corner, then determining the solution to the problem, in the end, can be done. This of course tends to be somewhat difficult considering that Indonesia is still included in the category of developing countries which have limitations in several ways.

Building a local food system means fighting for the rights of citizens to have food sovereignty. The principle of food sovereignty is different from food security regardless of where the food is produced. Food sovereignty tends to uphold the rights of every citizen and local community as a whole to produce, distribute and fulfill food needs above all other interests, including trade. However, the concept of food sovereignty in no way contradicts the principle of food security, the two complement each other. Efforts to build food security without being followed by efforts to uphold food sovereignty will create new social problems, such as dependence on imported rice and low productivity of farmers. In Indonesia, there is no concept or model, or theory on sustainable rice food sovereignty policies that are relevant for implementation. Therefore, this research is expected to be able to provide solutions to food problems that exist in Indonesia.

Two main things will be implemented in constructing a model of sustainable rice food sovereignty in Indonesia; first, to generally analyze the capacity of political resources, environmental resources, and environmental diplomacy, and to create a model for sustainable rice food sovereignty policies. With the design of the rice food sovereignty policy model, it is hoped that it will be able to contribute empirically to the concept of sustainable development based on environmental/ecological principles to support the implementation of Indonesia's environmental diplomacy in national strategic programs, while still incorporating environmental and sustainable development principles so that in the end it will have implications for the achievement of Indonesia's 'food sovereignty and the clarity of Indonesia's bargaining position on global environmental issues in the context of environmental diplomacy. Then, the formulation of food sovereignty policies based on the use of resource capacity as a determinant of the success of policy performance and the impact of these policies will provide a conducive and constructive picture for stakeholders, especially business actors (private sector) in implementing sustainable environmental policies, especially aspects of natural resource management, protection. and strategic natural environment conservation and ensuring the people's welfare in the food sector.

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**Conflict of Interest:**
The authors declare no conflict of interest.

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# INVESTIGATION OF DEPENDENCES OF THE MORPHOLOGICAL COMPOSITION OF BODY AND AMINO ACID COMPOSITION OF TROUT MEAT PROTEINS (*ONCORHYNCHUS MYKISS*) ON LEVELS OF THE ENERGY VALUE OF FEEDS

*Vadym Kondratiuk, Volodymyr Otchenashko*

## ABSTRACT
The article studies the effect of using complete compound feeds with different energy levels on the morphological composition of the body and the amino acid composition of trout meat proteins. The experiment aimed to establish the influence of different levels of energy nutrition of commercial rainbow trout on the morphological composition of their body and the amino acid composition of meat proteins. For this purpose, five experimental groups were formed using the analog method. The study lasted 210 days and was divided into two periods: comparative (10 days) and main (200 days). During the comparative period, the study fish consumed compound feed of the control group. During the main period, the energy level in experimental compound feeds for different experimental trout groups ranged from 16 to 20 mJ per 1 kg. It was found that with an increase in the mass of two-year-old trout, the mass of muscle tissue and the yield of edible parts probably increases. Feeding fish with an increased amount of metabolic energy (20 $mJ.kg^{-1}$) resulted in a significant change in the weight of internal organs, including the heart, liver, and kidneys. Increasing the metabolic energy in the compound feeds of fish from 18 $mJ.kg^{-1}$ to 19 – 20 $mJ.kg^{-1}$ leads to a significant increase in bowel mass by 13.3 – 5.0%. An increase in the level of metabolic energy in rainbow trout diets from 18 $mJ.kg^{-1}$ to 19 – 20 $mJ.kg^{-1}$ contributed to a likely increase in the methionine content in meat. A similar pattern was observed for the tryptophan content. It was found that the content of most essential amino acids in the protein of rainbow trout meat exceeds the corresponding values in the "ideal" protein, except for the content of isoleucine and leucine, which refers them to limiting amino acids.

**Keywords:** rainbow; feeding; compound feed; commodity quality; amino acid; Oncorhynchus mykiss

## INTRODUCTION
Trout meat is a product that has benefits for human health. This is due to its composition, which contains many important substances **(Archibisova and Suslov, 2018)**. Trout meat is the richest source of easily digestible animal protein, vitamins A, D, group b vitamins, as well as trace elements such as selenium, zinc, iodine, magnesium, potassium, calcium, and phosphorus. In cooking, trout is very much appreciated due to the high taste qualities of its fatty meat **(Mushtruk, et al., 2020a)**. Trout meat contains a lot of unsaturated fatty acids, which can reduce the amount of "harmful" cholesterol in the blood, preventing the development of atherosclerosis and its complications in the form of coronary heart disease, as well as heart attacks and strokes **(Makarenko et al., 2021)**.

Trout meat contains many essential amino acids, which confirms the benefits of trout for people with anemia, diseases of the cardiovascular system, and people suffering from debilitating, long-term diseases **(Orel, 2020)**.

Since rainbow trout is one of the most valuable fish species for humans, the need for the Ukrainian consumer market for this product is growing, which encourages producers to develop fish farms, increase production capacity, and encourage increased productivity and product quality **(Kozakevych, 2017; Mushtruk, et al., 2020b)**. The most effective and efficient way to achieve the goals is to choose the right feed and feeding strategy. Analysis of literature sources has shown that the most effective way to achieve goals is to select the appropriate feed and feeding strategy. The consumption of a part of the feed for energy needs is often variable and requires a detailed study, taking into account all the features of the study object **(Buchtová and Ježek, 2011; Sukhenko et al., 2017)**.

So, the study of the influence of different energy nutrition of rainbow trout on the morphological composition of the body and the amino acid composition of meat proteins is necessary to determine the energy needs of fish for the successful operation of fish farms and meet the needs of consumers in a high-quality product.

## Scientific hypothesis

Morphometric parameters can serve as an integral characteristic of the living conditions of fish in a particular water body. At the same time, among the relevant studies, this area remains the least studied. Therefore, research is aimed at establishing the influence of the main factors of energy supply and their impact on the morphological composition of the body and the amino acid composition of trout meat proteins.

## MATERIAL AND METHODOLOGY

The research was conducted in the spring, summer, and autumn periods from 2017 to 2019 in ponds based on the training-research-production laboratory of fish farming of the Shipot farm in Perechinskyi District of Zakarpattia (Transcarpathian) region.



**Figure 1** Two-year-old rainbow trout (*Oncorhynchus mykiss*), which was obtained in the autumn of fish farming of the Shipot farm in Perechinskyi District of Zakarpattia (Transcarpathian) region.



**Figure 2** Collection of ichthyological materials of fish farming of the Shipot farm in Perechinskyi District of Zakarpattia (Transcarpathian) region.



**Figure 3** Conducting a morphometric analysis of two-years of the rainbow trout (*Oncorhynchus mykiss*).

**Samples**

Using the analog method, five experimental groups were formed, from which fresh fish was sampled for further study in the amount of five specimens with an average weight for the group (Figure 1, 2, and 3).

**Chemicals**

Formaldehyde ($CH_2O$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Formalin (water solution formaldehyde, producer «Inter-Synthesis» Limited Liability Company, Ukraine).

**Animals and Biological Material**

Experimental studies were performed on two-year-old rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792).

During the morphological analysis, 200 samples of rainbow trout were caught, and during the morphological analysis, 100 specimens were processed.

**Instruments**

Electronic laboratory scales (TBE-0.15-0.001-a-2, producer «Inter-Synthesis» Limited Liability Company, Ukraine)

Technical electronic scales (BTHE-6-H1K-1, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Binocular microscope (XSP-139B LED Ulab, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Stereoscopic microscope (MBS-9, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Amino acid analyzer (Biotronik LC 2000 (Germany), producer "Laboratory equipment" Limited Liability Company, Ukraine)

**Laboratory Methods**

To achieve the task of the experiment used standard methods of morphometric analysis **GOST (1985)**.

Processing of ichthyological materials was performed according to standard methods generally accepted in ichthyology **(Sabaj, 2020)**.

The size composition of fish was carried out on live material according to the scheme developed by I. F. Pravdin for salmon fish **(Souza et al., 2019)**.

The mass fraction of amino acids – by ion-exchange chromatography on the automatic amino acid analyzer Biotronik LC 2000 (Germany), the proportion of tryptophan - by the colorimetric method after alkaline hydrolysis of the studied samples. Biometric processing of the study results was carried out according to N.A. Plokhinskyi **(Park et al., 2018)**.

**Description of the Experiment**

The scientific and economic experiment aimed to establish the influence of different levels of energy nutrition of commercial rainbow trout on the morphological composition of the body and the amino acid composition of trout meat proteins.

For this purpose, five experimental groups were formed using the analog method (Table 1). During the comparative period of the experiment, which lasted 10 days, the experimental fish consumed compound feed of the control group.

During the main period of the experiment (200 days), the level of metabolic energy in the trout feed of the experimental groups was regulated by changing individual components of compound feed (using combined mathematical methods for optimizing the calculation using the AgroSoftWinOpti program).

Feeding of rainbow trout during the research period was carried out 4 – 6 times a day, in the daytime at regular intervals. The required amount of feed was calculated following the indicators of individual fish weight and ambient temperature at the time of feeding.

**Table 1** Scheme of scientific and economic experiment.

| Group | Density at the beginning of the experiment, exe./ $m^2$ | Average mass at the beginning of the experiment, kg | Experiment periods | |
|---|---|---|---|---|
| | | | comparative (10 days) | main (200 days) |
| | | | exchange energy content in 1 kg of compound feed, mJ | |
| **1-control** | 50 | 0.0502 ±1.72 | | 18.0 |
| **2-experiment** | 50 | 0.0507 ±2.41 | | 16.0 |
| **3-experiment** | 50 | 0.0505 ±3.14 | 18.0 | 17.0 |
| **4-experiment** | 50 | 0.0509 ±1.53 | | 19.0 |
| **5-experiment** | 50 | 0.0503 ±2.83 | | 20.0 |

**Table 2** Content in 1 kg of compound feed, %.

| Indicator | Group | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| **Exchange energy, mJ** | 18.00 | 16.00 | 17.00 | 19.00 | 20.00 |
| **Crude protein** | 48.00 | 48.00 | 48.00 | 48.00 | 48.00 |
| **Crude fat** | 18.00 | 18.00 | 18.00 | 18.00 | 18.00 |
| **Crude fiber** | 2.50 | 2.72 | 2.40 | 2.56 | 2.44 |
| **Calcium** | 1.80 | 1.80 | 1.80 | 1.80 | 1.80 |
| **Total phosphorus** | 1.20 | 1.20 | 1.20 | 1.20 | 1.20 |
| **Lysine** | 2.70 | 2.70 | 2.70 | 2.70 | 2.70 |
| **Methionine** | 0.90 | 0.90 | 0.90 | 0.90 | 0.90 |
| **Vitamin A, ths. MO** | 10 | 10 | 10 | 10 | 10 |
| **Vitamin $D_3$, ths. MO** | 3 | 3 | 3 | 3 | 3 |
| **Vitamin E, mg** | 200 | 200 | 200 | 200 | 200 |

The nutritional value of experimental production compound feeds is shown in Table 2.

Experimental trout were weighed every 10 days. The weighing of fish was carried out on electronic scales in a tared container with water, with an accuracy of 0.1 g. Cultivation of commercial two-year-olds was carried out in ponds with an area of 100 $m^2$ with a density of 50 sp./$m^2$, and the water level in them is 1 m. The total number of trout individuals in experimental studies was 25 thousand specimens.

The assessment was carried out according to the main fish-ichthyological parameters: body weight, industrial body length, head length, the largest and smallest body height, and the length of the caudal fin. Measurements were made with a centimeter tape with an accuracy of 0.1 cm. The individual weighing was carried out on electronic commodity scales with an accuracy of 1.0 g. For the study, fresh fish was used in the amount of 5 specimens with an average weight in the group.

Commercial qualities of rainbow trout were determined by the main indicators of the size and mass composition of raw materials-the mass of fish, head, fins, bones, muscle tissue, bones, skin, and internal organs. Based on the obtained weighing data, the ratio of the mass of individual parts of its body to the mass of a whole fish was determined **GOST (1985)**.

Based on the obtained indicators, the amino acid score of essential amino acids was calculated for a more complete and versatile characterization of raw materials and products:

$$AC_i = \frac{AK_{pr}}{AK_{st}} \cdot 100$$

Where:

$AC_i$ is the score of essential amino acid, %; $AK_{pr}$ - essential amino acid content in 1 g of the test protein, g.100$g^{-1}$; $AK_{st}$ -the content of the same essential acid in 1 g of "Ideal" protein, g.100$g^{-1}$.

Limiting acid was considered the one with the lowest speed.

Studies of the growth rate of rainbow trout were carried out based on the results of control catches. At least 100 sp. of each group was weighed on an electronic scale.

**Description of the Experiment**

**Sample preparation:** For two years, 500 specimens of rainbow trout (*Oncorhynchus mykiss*) of different sizes and weight groups were studied.

**Number of samples analyzed:** According to the results of experimental studies in the first year, 200 samples were analyzed, and in the second year of research, 300 samples, respectively.

**Number of repeated analyses:** All measurements of instrument readings were performed 5 times.

**Number of experiment replication:** The number of repetitions of each experiment to determine one value was also 5 times.

## 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 (version 2019.3.2). Values were estimated using mean and standard deviations. We calculated the arithmetic mean (unweighted) value ($M$), the arithmetic mean error ($\pm m$), which made it possible to estimate with a certain probability the deviation of the arithmetic mean deviation, Fulton fatness rate. The statistical reliability of the results of the research was provided by analyzing samples with the number of fish from 10 to 25 specimens.

## RESULTS AND DISCUSSION

Today, the whole world sets up its production according to the principles of sustainable development, in Europe farms try to develop, adhering to its basic principles, but at the Open Data Science (ODS) summits, each time the question is raised about the problem of incomplete implementation of important points of the principle **(FAO, 2016)**. This encourages researchers to improve production technology, scientifically substantiating their developments and proposals. Many studies suggest using interesting alternatives in feeding and keeping rainbow trout **(Voorhees et al., 2019; Voorhees et al., 2018; Jones et al., 2020; Huysman et al., 2019)**. Working through an array of scientific publications, it became obvious that the range of available study objects is extremely wide. Scientists have studied many indicators, starting with the etiology (behavior) of trout, ending with the mortality rate **(Crank, Voorhees and Barnes, 2019; Keshavarzi et al., 2018)**. Choosing the subject and object of our study, the decisive argument was the consumer's need for a high-quality product, which focused our attention on the growth rate and high-quality composition of trout meat.

Comparison of the efficiency of energy use in fish feeding has been studied by many foreign specialists, whose scientific works confirm the influence of the studied factor on the object of the study **(Brooks et al., 2019; De Sá, et al., 2018; Mao et al., 2018)**.

The data of our studies indicate that commercial trout fed with compound feeds with different energy nutritional values had different masses in all time periods of the main period of the experiment. It was found that at the end of the experiment (7th month), the highest mass was reached by two-year-olds who were fed with compound feed with an exchange energy content of 19 and 20 mJ.$kg^{-1}$, which prevailed over analogs who were fed with feed with an exchange energy level of 18 mJ.$kg^{-1}$by 0.015 and 0.0334 kg ($p$ <0.05), respectively, or by 5.2 and 11.5%. At the same time, trout fed with compound feed with an exchange energy content of 16 and 17 mJ.$kg^{-1}$ were inferior to their peers fed with feed with an exchange energy level of 18 mJ by 0.0292 ($p$ <0.01) and 0.0128 kg, respectively, or by 10.1 and 4.4%. A direct correlation between the content of metabolic energy and the bodyweight of fish was also recorded in studies by foreign scientists **(Carozza, Bianchi and Galbraith, 2019; Hicks et al., 2019; Houk et al., 2018)**.

It is known that the level of metabolic energy is one of the most significant factors influencing the intensity of tissue development and biosynthetic processes in the fish body, affecting both the level of productivity and the exchange of other compounds **(Rubio-Gracia et al., 2020; Sheiko et al., 2019; Jutfelt, 2020)**.

The mass composition of fish, that is, the ratio of fish body parts is one of the important characteristics for developing norms for the consumption of raw materials and the yield of finished products.

To make rational use of fish raw materials and establish yield standards for semi-finished products and finished products, we studied the mass composition of rainbow trout, depending on the influence of part typical factors, in particular feeding conditions (energy nutrition).

The results of the conducted studies show that different levels of metabolic energy in compound feed for rainbow trout during cultivation affect its commercial qualities in different ways (Table 3).

Analysis of data on the mass composition of rainbow trout indicates certain stability of the relative mass of the skin and internal organs. When achieving high mass indicators, there is an increase in the relative mass of muscle tissue and a slight decrease in the yield of inedible parts (group 5). This, in turn, leads to an increase in the yield of edible parts, the difference between the control and the indicators of the 5th group is statistically significant ($p$ <0.001).

In general, it was found that with an increase in the mass of two-year-old trout, the mass of muscle tissue and the yield of edible parts increases.

A more complete description of rainbow trout grown in industrial conditions using compound feeds of various energy nutritional values were obtained after studying the internal organs (Table 4).

Analysis of the obtained data revealed changes in internal organs depending on the content of metabolic energy in experimental compound feeds. Feeding fish with an amount of metabolic energy (20 mJ.kg$^{-1}$) resulted in a significant change in the weight of internal organs, including the heart, liver, and kidneys.

The use of compound feeds with a high content of metabolic energy in feeding rainbow trout led to a significant change in intestinal mass. As you know, the digestive system of trout reacts quite quickly to changes in the physicochemical characteristics of the feed. During the research of M.A. Shcherbina, it was found that the stomach of rainbow trout also takes an active part in the absorption of nutrients. At the same time, the main place of absorption is the anterior part of the intestine and the part adjacent to the pyloric appendages, where up to 90% of proteins, fats and carbohydrates can be absorbed with optimal feeding **(Hu et al., 2018; Liu et al., 2019; Kasozi et al., 2017)**.

As a result of the analysis of the morphological body composition of rainbow trout, it was found that an increase in the amount of metabolic energy in fish feed to 19 mJ.kg$^{-1}$ (group 4) and 20 mJ.kg$^{-1}$ (group 5) leads to an increase in intestinal mass by 5.0% and 13.3% compared to the same control indicator. The difference is statistically significant ($p$ <0.05; $p$ <0.01).

It is known that the level of energy nutrition affects the need for amino acids in fish **(Raji et al., 2020; Jia et al., 2017; Smetanska et al., 2021)**, but to characterize the nutritional and biological value of fish raw materials, it is important to study changes in its amino acid composition (Table 5).

Analysis of the amino acid composition of fish raw materials shows that meat proteins (amino acid composition of muscle tissue proteins) of rainbow trout have a high biological value and contain all essential amino acids, among which lysine predominates (2.23 – 2.74 mg.100g$^{-1}$), leucine+isoleucine (1.79 – 2.03 mg.100g$^{-1}$), threonine (1.14 – 1.69 mg.100g$^{-1}$). In scientific studies **(Rajabzadeh et al., 2018; Roohani et al., 2019; Nogales-Mérida et al., 2019)**, the amino acid composition of various fish raw materials was analyzed and only the lysine content was determined.

Study results show that increasing the energy level of feeding rainbow trout contributes to an increase in the accumulation of essential amino acids. Thus, the amount of methionine in the meat of fish of the experimental groups was in the range of 0.47 – 0.87 mg per 100 g of product. However, it should be noted that an increase in the level of metabolic energy in the diets of fish of the experimental groups from 18 mJ.kg$^{-1}$ (control group) to 19 – 20 mJ.kg$^{-1}$ (4th and 5th groups) contributed to an increase in the content of methionine in meat by 33.8 – 47.5 % ($p$ <0.01, $p$ <0.001). The highest methionine content was found in trout meat of the 5th experimental group, which received compound feed with an increased (up to 20 mJ.kg$^{-1}$) content of metabolic energy.

Analyzing the data on the content of methionine in fish meat, the diet of which was with a reduced content of metabolic energy, it should be noted that reducing its level to 16 mJ.kg$^{-1}$ (2nd experimental group) contributed to a decrease in the content of methionine from 0.59 to 0.48 mg.100g$^{-1}$ of the product, and up to 17 mJ.kg$^{-1}$ – led to a slight increase in its level, compared with analogs of the control group. In scientific studies **(Rogoskii et al., 2020; Čapla et al., 2020)** analyzed the content of methionine in fish meat, it should be noted that scientists observed a decrease in its level to only 10 mJ.kg$^{-1}$, which helped reduce the content of methionine from 0.42 to 0.38 mg.100g$^{-1}$ of product.

The same pattern was observed for the tryptophan content. In the muscles of rainbow trout of the 3rd, 4th, and 5th experimental groups, the tryptophan content was 11.5 – 65.4% higher than in the analogs of the control group. The highest content of tryptophan was in the meat of fish of the 5th experimental group and amounted to 0.43 mg.100g$^{-1}$ of the product, which is 48.3% and 22.8% more compared to the indicators of fish of the 3rd and 4th experimental groups.

Amino acids such as serine, arginine, and threonine are hydrophilic amino acids that determine the moisture-retaining ability of fish meat. As can be seen from the conducted experimental studies, proteins contain a sufficient amount of these amino acids, which explains the rather high moisture-retaining ability of meat.

The results of the studies showed that the sum of essential amino acids in the muscle tissue of rainbow trout of the 3rd, 4th, and 5th experimental groups, whose compound feed contained an exchange energy content of 17 – 20 mJ.kg$^{-1}$, exceeded the indicator of the control group by 4.8%, 14.4%, and 23.5%, respectively. This is due to the predominance of essential amino acids such as lysine, methionine, threonine, phenylalanine, and tryptophan.

The sum of non-essential amino acids in trout meat of the studied groups ranged from 11.21 mg.100g$^{-1}$ of protein (2nd experimental group) to 15.45 mg.100g$^{-1}$ of protein (5th experimental group). The greatest amount of non-essential amino acids was found in trout meat, whose diet contained an increased content (20 mJ.kg$^{-1}$) of metabolic energy.

Thus, the sum of non-essential amino acids in the studied trout samples of the 5th experimental group was 25.2% higher than in the control group and amounted to 15.45 mg.100g$^{-1}$ of protein.

Among the non-essential amino acids, aspartic acid (2.41 mg.100g$^{-1}$ of protein), alanine (2.07 mg.100g$^{-1}$ of protein), arginine (1.79 mg.100g$^{-1}$ of protein), and glycine (1.62 mg.100g$^{-1}$ of protein) were dominant in trout meat of the 5th experimental group.

**Table 3** Commercial qualities of rainbow trout, (n = 5).

| Indicator | Group | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| **Fish mass, kg** | 0.2903 ±8.14 | 0.2611 ±7.26$^{*}$ | 0.2775 ±8.72 | 0.3053 ±7.33 | 0.3237 ±9.38$^{*}$ |
| **Head mass, kg** | 0.0482 ±2.13 | 0.04386 ±1.96 | 0.0444 ±3.04 | 0.0512 ±4.36 | 0.0543 ±3.65 |
| **Fins mass, kg** | 0.0181 ±1.87 | 0.01697 ±1.98 | 0.0555 ±2.06 | 0.0198 ±1.21 | 0.0209 ±0.96 |
| **Bones mass, kg** | 0.0193 ±0.98 | 0.01749 ±1.06 | 0.01859 ±1.10 | 0.0206 ±1.32 | 0.0221 ±0.87 |
| **Muscles mass, kg** | 0.1518 ±6.98 | 0.13547 ±7.03 | 0.14153 ±6.74 | 0.1596 ±5.62 | 0.1716 ±3.21$^{*}$ |
| **Skin mass, kg** | 0.01974 ±1.02 | 0.01775 ±2.01 | 0.01887 ±1.99 | 0.02084 ±1.24 | 0.02108 ±1.03 |
| **Internal organs mass, kg** | 0.01626 ±0.98 | 0.01462 ±0.78 | 0.01388 ±1.01 | 0.01703 ±1.03 | 0.01838 ±0.87 |
| **Mass of edible parts, kg** | 0.17418 ±5.23 | 0.15196 ±6.85$^{*}$ | 0.1665 ±7.02 | 0.19936 ±6.54$^{*}$ | 0.21752 ±6.24$^{***}$ |
| **Yield of edible parts, %** | 60.0 ±3.01 | 58.2 ±3.69 | 59.3 ±3.87 | 65.3 ±2.0 | 67.2 ±2.67$^{*}$ |
| **Mass of inedible parts, kg** | 0.11612 ±3.21 | 0.10914 ±3.47 | 0.1091 ±4.65 | 0.10594 ±2.45$^{*}$ | 0.10618 ±2.33$^{*}$ |
| **Yield of inedible parts, %** | 40.0 ±1.97 | 41.8 ±2.06 | 40.7 ±1.99 | 34.7 ±1.09$^{*}$ | 32.8 ±1.41$^{*}$ |

Note: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001 compared to the 1st group.

**Table 4** Mass of internal organs of two-year-old rainbow trout, kg (n = 5).

| Indicator | Group | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| **Heart** | 0.00095 ±0.005 | 0.0008 ±0.002$^{***}$ | 0.0008 ±0.04$^{***}$ | 0.0010 ±0.07$^{***}$ | 0.00110 ±0.005$^{***}$ |
| **Liver** | 0.00403 ±0.09 | 0.00368 ±0.04$^{***}$ | 0.0034 ±0.04$^{**}$ | 0.00426 ±0.11 | 0.00460 ±0.06$^{***}$ |
| **Kidneys** | 0.00181 ±0.24 | 0.00162 ±0.22 | 0.00154 ±0.33 | 0.00187 ±0.21 | 0.00202 ±0.18 |
| **Stomach** | 0.00428 ±0.63 | 0.00399 ±0.42 | 0.00361 ±0.39 | 0.00443 ±0.29 | 0.00483 ±0.36 |
| **Intestines** | 0.00519 ±0.14 | 0.00434 ±0.13 | 0.00448 ±0.27 | 0.00545 ±0.11$^{*}$ | 0.00588 ±0.13$^{**}$ |

Note: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001 compared to the 1st group.

**Table 5** Amino acid composition of rainbow trout meat proteins mg per 100 g of product, (n = 5).

| Indicators | Group | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| **Essential amino acids (EAA)** | **8.98** | **8.78** | **9.41** | **10.27** | **11.09** |
| Valin | 1.71 ±0.03 | 1.64 ±0.05 | 1.71 ±0.02 | 1.78 ±0.04 | 1.82 ±0.03$^{*}$ |
| Leucine+isoleucine | 1.82 ±0.05 | 1.79 ±0.06 | 1.84 ±0.09 | 1.98 ±0.02$^{*}$ | 2.03 ±0.07$^{*}$ |
| Lysine | 2.23 ±0.09 | 2.36 ±0.12 | 2.41 ±0.14 | 2.65 ±0.09$^{*}$ | 2.74 ±0.07$^{*}$ |
| Methionine | 0.59 ±0.01 | 0.48 ±0.01 | 0.66 ±0.01 | 0.8 ±0.02$^{***}$ | 0.87 ±0.04$^{***}$ |
| Threonine | 1.14 ±0.04 | 1.08 ±0.06 | 1.21 ±0.09 | 1.33 ±0.05 | 1.69 ±0.06$^{***}$ |
| Phenylalanine | 1.23 ±0.05 | 1.19 ±0.08 | 1.29 ±0.07 | 1.39 ±0.04$^{*}$ | 1.51 ±0.04$^{**}$ |
| Tryptophan | 0.26 ±0.04 | 0.24 ±0.06 | 0.29 ±0.05 | 0.35 ±0.03$^{*}$ | 0.43 ±0.01$^{**}$ |
| **Non-essential amino acids (NEAA)** | **12.34** | **11.21** | **13.46** | **14.07** | **15.45** |
| Alanine | 1.87 ±0.06 | 1.77 ±0.04 | 1.91 ±0.09 | 1.99 ±0.09 | 2.07 ±0.06$^{**}$ |
| Arginine | 1.51 ±0.05 | 1.41 ±0.02 | 1.64 ±0.07 | 1.69 ±0.10 | 1.79 ±0.01$^{*}$ |
| Histidine | 0.93 ±0.01 | 0.83 ±0.01 | 0.99 ±0.02 | 1.11 ±0.01 | 1.23 ±0.03$^{*}$ |
| Proline | 0.74 ±0.01 | 0.64 ±0.01 | 0.94 ±0.01 | 1.06 ±0.02 | 1.24 ±0.08$^{***}$ |
| Serin | 1.34 ±0.09 | 1.21 ±0.08 | 1.40 ±0.10 | 1.44 ±0.05 | 1.64 ±0.09$^{*}$ |
| Glutamic acid | 0.62 ±0.01 | 0.52 ±0.01 | 0.73 ±0.02 | 0.78 ±0.03 | 0.92 ±0.04$^{***}$ |
| Aspartic acid | 2.11 ±0.09 | 2.01 ±0.10 | 2.21 ±0.11 | 2.21 ±0.14 | 2.41 ±0.08$^{*}$ |
| Glycine | 1.32 ±0.08 | 1.12 ±0.06 | 1.44 ±0.13 | 1.49 ±0.12 | 1.62 ±0.06$^{*}$ |
| Cystine | 0.96 ±0.01 | 0.86 ±0.01 | 1.06 ±0.06 | 1.11 ±0.14$^{*}$ | 1.26 ±0.09$^{*}$ |
| Tyrosine | 0.94 ±0.01 | 0.84 ±0.01 | 1.14 ±0.11 | 1.19 ±0.16$^{*}$ | 1.27 ±0.06$^{***}$ |
| **NEAA-to-EAA ratio** | **0.73:1** | **0.78:1** | **0.70:1** | **0.73:1** | **0.72:1** |

Note: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001 compared to the 1st group.

**Table 6** Assessment of compliance of the amino acid composition of rainbow trout proteins with the ideal protein with the FAO/WHO scale, g.100g$^{-1}$ of protein, (n = 5).

| Amino acid | Group | | | | | FAO/WHO ideal protein |
|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | |
| Valin | 8.02 | 8.20 | 7.48 | 7.31 | 6.86 | 5.0 |
| Isoleucine | 3.09 | 3.20 | 2.93 | 2.92 | 2.75 | 4.0 |
| Leucine | 5.44 | 5.75 | 5.12 | 5.22 | 4.90 | 7.0 |
| Methionine+cystine | 7.27 | 6.70 | 7.52 | 7.81 | 8.03 | 3.5 |
| Threonine | 5.35 | 5.40 | 5.29 | 5.46 | 6.37 | 4.0 |
| Phenylalanine+tyrosine | 10.18 | 10.16 | 10.63 | 10.60 | 10.47 | 6.0 |
| Tryptophan | 1.22 | 1.20 | 1.27 | 1.44 | 1.62 | 1.0 |
| Lysine | 10.46 | 11.81 | 10.54 | 10.89 | 10.32 | 5.5 |
| **Total** | 51.03 | 52.42 | 50.78 | 51.65 | 51.32 | **36** |

**Table 7** Amino acid score of rainbow trout meat proteins, %.

| Amino acid | Group | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| Valin | 160 | 164 | 150 | 146 | 137 |
| Isoleucine | 77 | 80 | 73 | 73 | 69 |
| Leucine | 77 | 82 | 73 | 73 | 70 |
| Methionine+cystine | 207 | 191 | 215 | 223 | 229 |
| Threonine | 134 | 135 | 132 | 137 | 159 |
| Phenylalanine+tyrosine | 169 | 169 | 177 | 177 | 175 |
| Tryptophan | 122 | 120 | 127 | 144 | 162 |
| Lysine | 190 | 215 | 192 | 198 | 188 |
| **Total** | 1136 | 1156 | 1139 | 1173 | 1189 |

So, as a result of experimental studies, it was found that an increase in the levels of metabolic energy in compound feeds of rainbow trout causes noticeable changes in the amino acid composition of meat, which improves its biological value.

An important indicator of the biological value of proteins is the correspondence of the content of essential amino acids to the ideal protein (Table 6).

The data in Table 6 show that the sum of essential amino acids in the protein of rainbow trout meat, regardless of the factor under study, exceeds their amount in the "ideal" protein. However, the content of isoleucine and leucine turned out to be lower than such an "ideal" protein, that is, these amino acids can be attributed to limiting ones.

Thus, the results of experimental studies established that the content of isoleucine in the proteins of fish raw materials of rainbow trout was in the range from 2.75 to 3.2 g.100g$^{-1}$ of protein. The smallest amount of it was found in the protein of fish of the 5th experimental group, which were fed with compound feed with an exchange energy content of 20 mJ.kg$^{-1}$. A similar pattern was found in the leucine content. At the same time, trout meat has a high content of valine, methionine + cystine, threonine, phenylalanine + tyrosine, tryptophan, and lysine, which are quite important for the human body.

Results of calculating the amino acid score (Table 7) indicate a fairly high biological value of rainbow trout meat proteins.

The limiting amino acids in trout meat are methionine + cystine, phenylalanine + tyrosine, lysine, and valine. Analysis of experimental data showed that changes in the nutritional value of rainbow trout feed accompanied noticeable changes both in the amino acid composition of proteins and in the calculations of the amino acid score. In particular, an increase in the level of metabolic energy in fish feed to 19 – 20 mJ.kg$^{-1}$ contributed to a significant increase in the level of essential amino acids. The dominant amino acids in fish meat protein of the 4th and 5th experimental groups were methionine + cystine (223% and 229%), lysine (198% and 188%), and phenylalanine + tyrosine (177% and 175%).

It was also found that trout use of compound feeds with exchange energy of 16 mJ.kg$^{-1}$ (2nd experimental group) leads to a decrease in the amount of methionine + cystine, tryptophan in meat protein, and an increase in the content of lysine and valine compared to the control.

Trout cultivation on compound feed with a metabolic energy content of 17 mJ.kg$^{-1}$ was accompanied by a decrease in the amount of amino acids such as valine, isoleucine, leucine, and threonine in meat protein. The limiting amino acids in trout meat protein of the 3rd experimental group were methionine + cystine (215%), phenylalanine + tyrosine (177%), and lysine (192%).

So, the analysis of experimental data showed the influence of the level of metabolic energy on the quality indicators of rainbow trout meat. The relationship of energy nutrition of fish with the intensity of metabolism and improvement of the quality of its meat is proved.

## CONCLUSION

It was found that at the end of the experiment, the highest mass was reached by two-year-olds who were fed with compound feed with an exchange energy content of 19 and 20 mJ.kg$^{-1}$, which prevailed over analogs who were fed with feed with an exchange energy level of 18 mJ.kg$^{-1}$ by 0.015 and 0.0334 kg ($p$ <0.05) (5.2 and 11.5%), respectively. Trout fed with compound feed with an exchange energy content of 16 and 17 mJ.kg$^{-1}$ were inferior to their peers fed

with feed with an exchange energy level of 18 mJ by 0.0292 ($p$ <0.01) and 0.0128 kg, respectively, or by (10.1 and 4.4%). It was found that an increase in the amount of metabolic energy in fish feed to 19 mJ.kg$^{-1}$ and 20 mJ.kg$^{-1}$ leads to an increase in intestinal mass by 5.0% and 13.3%, respectively, compared to the same control indicator ($p$ <0.05; $p$ <0.01). An increase in the level of metabolic energy in the diets of fish of experimental groups from 18 mJ.kg$^{-1}$ to 19 – 20 mJ.kg$^{-1}$ contributed to an increase in the content of methionine in meat by 33.8 – 47.5% ($p$ <0.01; $p$ <0.001). The content of most essential amino acids in the protein of rainbow trout meat exceeds the corresponding indicators in the "ideal" protein, which confirms the high biological value of this product. Prospects for further studies are related to determining the effect of amino acid nutrition on the two main limiting amino acids (lysine and methionine) on the commercial qualities of trout and the biological value of its meat protein.

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# THE USE OF MUTTON IN SAUSAGE PRODUCTION

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## ABSTRACT

The work analyzes the quality of sausage with mutton. The proportion of individual commodities was as follows 40% sheep thigh, 40% pork shoulder, and belly 20%. The protein content in pork shoulder was 20.11 g.100g$^{-1}$ in sheep thigh 23.65 g.100g$^{-1}$ and sausage 19.89 g.100g$^{-1}$. Of the monitored amino acids, the highest content was in lysine, in the sausage was 1.9 g.100g$^{-1}$ and of the raw materials in the belly 2.1 g.100g$^{-1}$. We also found a higher proportion of leucine 1.7 g.100g$^{-1}$ in both sausage and sheep thighs. The arginine content in the sausage was also high 1.39 g.100g$^{-1}$. We found a high content of palmitic acid in the pork shoulder of 24.38 g.100g$^{-1}$ FAME. The content of palmitic acid in sheep meat was 24.32 g.100g$^{-1}$ FAME and in sausage 24.16 g.100g$^{-1}$ FAME. The content of stearic acid in the pork shoulder was 10.89g.100g$^{-1}$ FAME, in the sheep thigh 10.64g.100g$^{-1}$ FAME, in the belly 11.07 g.100g$^{-1}$ FAME, and the sausage 10.92 g.100g$^{-1}$ FAME. The MDA content in sheep meat was 0.185 mg.kg$^{-1}$, in pork shoulder 0.141 mg.kg$^{-1}$, in pork belly 0.22 mg.kg$^{-1}$ and in sausage on the day of production 0.45 mg.kg$^{-1}$. On the 30$^{th}$ day, the MDA content was in the sausage 0.78 mg.kg$^{-1}$. The high MDA content of the sausage was probably most influenced by the technological process, as all raw materials, because there was a lower MDA content.

**Keywords:** meat; sausage; fatty acids; malondialdehyde

## INTRODUCTION

According to Commission Delegated Regulation **(EU) 2017/1182**, sheep carcasses to 1 year old are classified as lambs and adult sheep over 1 year old. The meat of adult sheep has very good sensory and technological properties, but the consumer perceives it to a certain extent negatively. If the meat is used in meat products, the producer must declare his content in the final product.

In Slovakia, pork is the most preferred type of meat, whose chemical composition may vary depending on the topographic origin. In pork, depending on the cut parts, the protein content is 9% – 20% **(Poráčová et al., 2017)**. Meat quality is defined as a combination of basic meat characteristics **(Čuboň et al., 2017)**.

It is the first sign that the consumer notices and thanks to which he also makes a decision. The color of the meat is determined by the state of myoglobin, oxymyoglobin is bright red and metmyoglobin is brown **(Ning et al., 2019)**.

The color of the cut ripened pork is pale pink and its taste depends mainly on various factors such as the age of the animal and its method of feeding. The meat of younger animals is paler and suitable for cutting. Older individuals have dark red meat and are suitable for the production of mainly durable meat products **(Holland et al., 1991)**.

Pork has an optimal content of unsaturated fatty acids and also shows a good representation of essential substances and minerals. From a nutritional point of view, it is an important source of animal protein **(Fuastman and Suman, 2017)**.

In lean lamb meat contains about 20 – 25% of proteins, while in heat-treated meat their content is 28 – 36% because the water content in the meat is reduced and nutrients are concentrated during culinary processing. Protein digestibility is high, approximately 94% compared to beans (78%) or wheat (86%). Lamb meat contains all the essential amino acids **(Krishtafovich et al., 2016)**. According to **Williams (2007)**, the amino acids glutamine and glutamic acid are found in sheep meat in the largest amounts than in other types of meat. Other higher amino acids include arginine, alanine, and aspartic acid. The tryptophan content is highly variable depending on age and muscle area **Crăciun et al. (2012)**.

Sheepmeat contains saturated, monounsaturated, and polyunsaturated fatty acids, the content of which is optimal from a technological point of view and the point of view of a healthy diet **(Krishtafovich et al., 2016)**.

Sheep grazed on native pastures have a polyunsaturated fatty acid content of 200 – 500 mg.100g$^{-1}$ of fresh meat, such meat is considered an appropriate source of polyunsaturated fatty acids **(Cabrera and Saadoun, 2014)**.

Fat oxidation during meat processing and storage is influenced by lipid content and composition **(Tsikas, 2017)**. Fat oxidation increases with the amount of fat and mainly depending on the ratio between PUFA and SFA **(Bertolín**

**and Blanco, 2019)**. Lipid oxidation is a complex chain reaction process that results in the presence of reactive oxygen species, such as the superoxide radical anion. The peroxide non-radical anion can be produced by enzymes and non-enzymatic chemical reduction of molecular oxygen **(Cunha et al., 2018)**. Reactive oxygen species react with various biomolecules, e.g. PUFAs, forming aldehydes, ketones, acids, alcohols, and hydrocarbons that cause undesirable changes in structure, taste, and also color. Finally, they reduce the quality of the product until it becomes unfit for human consumption **(Jung et al., 2016)**.

Sensory and nutritional value are greatly affected by lipid oxidation, which is an undesirable manufacturing process. Oxidation causes the formation of aldehydes and ketones, which result in an unpleasant taste and smell of meat **(Bobko et al., 2015)**.

Lipid oxidation results in the formation of aldehydes. The most common aldehyde produced by damaging the polyunsaturated fatty acids is malondialdehyde (MDA). It is a simple alkandial derived from malonic acid **(Čuboň et al., 2019)**.

Currently, one of the markers used to determine the degree of lipid oxidation in meat and meat products is malondialdehyde, which is formed as a secondary product of lipid oxidation. The MDA content of meat is determined using thiobarbituric acid reactants (TBARS) **(Tsikas, 2017)**.

Meat and meat products are susceptible to quality deterioration due to their rich nutritional composition **(Bobko et al., 2017)**.

**Jung et al. (2016)** and **Bertolín and Blanco (2019)** report that TBARS is used to determine the degree of lipid oxidation in meat and meat products.

The excessive content of biogenic amines causes undesirable sensory properties. From a hygienic point of view, biogenic amines serve as indicators degree of food spoilage **(Čuboň et al., 2015)**.

### Scientific Hypothesis

The aim of the work was the analysis of the basic chemical composition, fatty acids, amino acid content, and fat oxidation in sausage with the proportion of mutton and the raw material.

## MATERIAL AND METHODOLOGY

### Samples

***Pork shoulder, pork belly, sheep thigh, sausage.***

The individual commodities were used in the product as follows: 40% lamb thigh, 40% pork shoulder, and belly 20%. The meat was cut into smaller pieces and then minced on a grinder with 3 mm holes. Subsequently, the ingredients were added and the work was mixed thoroughly. The meat work lasted several tens of minutes to 1 hour and then stuffed into pork intestines. The sausages were smoked in a traditional smokehouse with cold smoke.

Ingredients per 10 kg of sausage: salt 20 dkg, ground red pepper 100 g, 5 head garlic, ground cumin 25 g, cumin whole 25 g, ground black pepper 28 g.

### Laboratory Methods

Sausage samples were analyzed by FT-IR analysis (Nicolet 6700) of chemical composition, 100 g of sausages were taken. Subsequently, the samples were homogenized and analyzed. The method is based on the absorption of infrared radiation during the passage through the sample, during which changes in the rotational vibrational energy states of the molecule take place depending on changes in the dipole moment of the molecule. The resulting infrared spectrum is the functional dependence of energy, mostly expressed as a percentage of transmittance or units of absorbance at the wavelength of the incident radiation. The results of this analysis are given in $g.100g^{-1}$. Selected analyzed parameters of chemical composition were: content of proteins, water, lipids, omega 3 and 6 fatty acids, cholesterol, essential and selected non-essential amino acids, the content of selected monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA) and their total content in sausages.

Oxidative stability analysis by determining the concentration of malondialdehyde as the final oxidation product of the fat component of rabbit meat by the TBA (thiobarbiturate) method was performed on days 1 and 30 of sausage storage. The principle of the method is the spectrophotometric determination of the color complex, which is formed by the reaction of 2 molecules of TBA and the content of malondialdehyde (MDA) at a wavelength of 532 nm.

Approach:

- Weigh 1.5 g of the ground sample into a 50 mL centrifuge tube,
- addition of 1 mL EDTA (complexing agent) + mixing,
- add 5 mL of 0.8% BHT + mix,
- before homogenization, add 8 mL of 5% TCA,
- homogenization of 30 solutions 10,000 rpm,
- sample standing for 10 minutes followed by centrifugation for 5 minutes (3,500 x g, 4 °C),
- removal of the hexane layer after centrifugation and subsequent filtration of the samples,
- make up to 10 mL with 5% TCA,
- add 1 mL TBA to 4 mL sample,
- incubation in a water bath for 90 min at 70 °C,
- cooling and tempering to room temperature 45 min,
- extinction of samples for UV-VIS spectrophotometry at a wavelength of 532 nm,
- recalculation of the obtained data and determination of the concentration of malondialdehyde in $mg.kg^{-1}$ **(Marcinčák et al., 2005)**.

### Statistical Analysis

The measured results of the analyzes were varied and statistically processed by SAS (2008) 9.3 Enhanced Logging Facilities, Cary, NC: SAS Institute Inc., 2008. We also processed the data of the analyzed parameters in Microsoft Excel.

## RESULTS AND DISCUSSION

Table 1 shows the results of the basic chemical component of pork shoulder, sheep's thigh, pork belly, and sausage. The water content in the pork shoulder was 69.31 g.100g$^{-1}$, in the sheep's thigh 73.34 g.100g$^{-1}$, in the belly only 60.91 g.100g$^{-1}$ and in the sausage, the content was lower (62.34 100g$^{-1}$). The protein content in the pig's shoulder was 20.11 g.100g$^{-1}$, 23.65 g.100g$^{-1}$ in the sheep's thigh, and only 12.6 g.100g$^{-1}$ in the belly. We found in sausage a protein content of 19.89 g.100g$^{-1}$. However, the fat content was lowest in the sheep's thigh (2.11 g.100g$^{-1}$), higher in the sausage 14.04 g.100g$^{-1,}$ and highest in the pig belly 24.6 g.100g$^{-1}$. Consistent with our results **Nowak et al. (2016)** report a water content of 62.46 g.100g$^{-1}$, a fat content of 20.02 g.100g$^{-1}$, and lower protein content of 17.52 g.100g$^{-1}$. **(Rudy, 2009)** states in the mutton, under our results, in the first water content 72.80 g.100g$^{-1}$, fat content 3.60 g.100g$^{-1,}$ and protein 21.50 g.100g$^{-1}$. **Bertolín et al. (2018)** in contrast to our results report a higher cholesterol content of 0.69 g.100g$^{-1}$ in sheep meat.

Of all the monitored amino acids (Table 2), the highest proportion was the amino acid lysine, in sausage 1.9 g.100g$^{-1}$, of the raw materials the highest content in the belly was 2.1 g.100g$^{-1}$. We also found a higher proportion in the leucine content of 1.7 g.100g$^{-1}$ in both sausage and lamb thighs. The arginine content in the sausage was also high at 1.39 g.100g$^{-1}$.

**Cheng et al. (2017)** report a lower lysine content in pork *Longissimus thoracis* 0.98 g.100g$^{-1}$ compared to our results. **Marti-Quijal et al. (2019)** in turn report a lower lysine content of 1.36 g.100g$^{-1}$ in the sausage. Lower lysine content in mutton (0.79 g.100g$^{-1}$) is reported by **Huang et al. (2020)**.

**Cheng et al. (2017)** and **Huang et al. (2020)** compared to our results report a lower methionine content in pork 0.28 g.100g$^{-1}$ and mutton 0.33 g.100g$^{-1}$.

**Cheng et al. (2017)** found a lower threonine content of 0.70 g.100g$^{-1}$ in pork *Longissimus thoracis*. Also, **Huang et al. (2020)** also found a lower threonine content of 0.550 g.100g$^{-1}$, but **Marti-Quijal et al. (2019)** found a lower threonine content of (0.69 g.100g$^{-1}$) in pork sausage compared to our results.

**Jia et al. (2019)** found out a higher content of isoleucine in pork (0.91 g.100g$^{-1}$) compared to our results. On the other hand, **Huang et al. (2020)** report a lower isoleucine content (0.44 g.100g$^{-1}$). **Marti-Quijal et al. (2019)** report a lower isoleucine content of 0.73 g.100g$^{-1}$ in the sausage compared to our results.

Higher content of phenylalanine in pork than 1.37 g.100g$^{-1}$ was found by **Jia et al. (2019)**. **Marti-Quijal et al. (2019)** found in the sausage its lower phenylalanine content 0.662 g.100g$^{-1}$ compared to our results. Also, **Huang et al. (2020)** found a lower phenylalanine meat mutant content of 0.49 g.100g$^{-1}$ compared to our results.

A lower histidine content of 0.6 g.100g$^{-1}$ in pork was found by **Jia et al. (2019)**. Similarly, **Huang et al. (2020)** found a lower content of histidine (0.62g.100g$^{-1}$) compared to our results, but in mutton meat. **Marti-Quijal et al. (2019)** found a lower content of histidine in the sausage (0.852 g.100g$^{-1}$) compared to our results.

Table 3 shows the content of fatty acids in the raw material and sausage, the highest proportion of all fatty acids is oleic acid, of which 36.63 g.100g$^{-1}$ FAME in pork shoulder. **Aali et al. (2017)** report a higher oleic acid content in sheep *longissimus dorsi* (38.30 g.100g$^{-1}$ FAME) compared to our results. The content of oleic acid in the belly was up to 60.33 g.100g$^{-1}$ FAME and in the sausage 55.52 g.100g$^{-1}$ FAME. Compared to our results, **Cruxen et al. (2018)** found out lower oleic acid content in mutton sausage (41.25g.100g$^{-1}$ FAME).

We also found high content in the proportion of palmitic acid, in pork shoulder 24.38 g.100g$^{-1}$ FAME. Consistent with our results **Kim et al. (2009)** found out palmitic acid content in pork 24.17g.100g$^{-1}$ FAME. We found a palmitic acid content of 24.32 g.100g$^{-1}$ FAME in the sheep's thigh. Compared to our results **Chikwanha et al. (2018)** found out lower content of palmitic acid in sheep meat (22.2 g.100g$^{-1}$ FAME). The palmitic acid content of the sausage was 24.16 g.100g$^{-1}$ FAME, the lower content (23.03 g.100g$^{-1}$ FAME) is reported by **Cruxen et al. (2018)**.

**Table 1** Chemical composition of raw material and sausage (g.100g$^{-1}$ ±*SD*).

| Parameter | Pork shoulder | Sheep thigh | Pork belly | Sausage |
|---|---|---|---|---|
| Water | 69.31 ±1.21 | 73.34 ±1.03 | 60.91 ±1.53 | 62.34 ±2.14 |
| Protein | 20.11 ±0.47 | 23.65 ±1.14 | 12.60 ±0.54 | 19.89 ±2.19 |
| Fat | 8.93 ±1.01 | 2.11 ±0.4 | 24.60 ±1.85 | 14.04 ±4.67 |
| Cholesterol | 0.61 ±0.16 | 0.51 ±0.08 | 2.12 ±0.17 | 1.65 ±0.54 |

**Table 2** Amino acid content of raw material and sausage (g.100g$^{-1}$ ±*SD*).

| Parameter | Pork shoulder | Sheep thigh | Pork belly | Sausage |
|---|---|---|---|---|
| Protein | 20.11 ±0.47 | 23.65 ±0.14 | 12.60 ±0.54 | 19.89 ±2.19 |
| Lyzin | 1.71 ± 0.57 | 1.73 ±0.28 | 2.1 ±0.37 | 1.90 ±0.05 |
| Leucin | 1.57 ±0.09 | 1.7 ±0.25 | 1.71 ±0.33 | 1.7 ±0.05 |
| Metionin | 0.66 ±0.23 | 0.67 ±0.07 | 0.99 ±0.12 | 0.82 ±0.01 |
| Treonin | 0.81 ±0.24 | 0.79 ±0.12 | 0.61 ±0.14 | 0.89 ±0.04 |
| Valin | 0.82 ±0.19 | 0.82 ±0.09 | 0.82 ±0.11 | 0.79 ±0.05 |
| Izoleucin | 0.78 ±0.27 | 0.79 ±0.14 | 0.74 ±0.17 | 0.85 ±0.03 |
| Histidin | 0.86 ±0.31 | 0.82 ±0.11 | 0.81 ±0.14 | 0.85 ±0.06 |
| Fenylalanin | 0.81 ±0.25 | 0.82 ±0.12 | 0.77 ±0.6 | 0.88 ±0.03 |
| Cystein | 0.27 ±0.08 | 0.25 ±0.02 | 0.29 ±0.01 | 0.41 ±0.02 |
| Arginin | 1.273 ±0.42 | 1.29 ±0.12 | 1.20 ±0.18 | 1.39 ±0.04 |

**Table 3** Content of fat (g.100g$^{-1}$ ±*SD*) and fatty acids (g.100g$^{-1}$ ±*SD* FAME) in the raw material and sausage.

| Parameter | Pork shoulder | Sheep thigh | Pork belly | Sausage |
|---|---|---|---|---|
| Fat | 8.93 ±1.01 | 2.11 ±0.4 | 24.60 ± 1.85 | 14.04 ±4.67 |
| 3 omega FA | 0.53 ±0.01 | 0.54 ±0.26 | 0.85 ±0.11 | 0.7 ±0.04 |
| 6 omega FA | 9.5 ±1.02 | 11.4 ±0.81 | 5.27 ±1.9 | 1.17 ±0.41 |
| Esential FA | 8.43 ±0.9 | 9.13 ±1.12 | 6.38 ±1.91 | 1.08 ±0.75 |
| MUFA | 49.62 ±2.02 | 47.78 ±0.79 | 69.93 ±1.94 | 60.92 ±1.61 |
| PUFA | 12.1 ±1.07 | 14.98 ±1.03 | 3.02 ±1.36 | 8.51 ±0.98 |
| SAFA | 34.21 ±2.21 | 33.52 ±1.31 | 26.49 ±1.05 | 28.24 ±2.06 |
| C12:0 Lauric A. | 0.12 ±0.03 | 0.11 ±0.003 | 0.02 ±0.01 | 0.06 ±0.01 |
| C14:0 Myristic A. | 1.35 ±0.02 | 1.38 ±0.04 | 1.23 ±0.02 | 1.31 ±0.09 |
| C16:0 Palmit A. | 24.38 ±1.4 | 24.32 ±0.31 | 24.01 ±0.1 | 24.16 ±0.18 |
| C17:0 Heptadecanoid A. | 0.31 ±0.03 | 0.30 ±0.03 | 0.12 ±0.02 | 0.19 ±0.04 |
| C18:0 Stearic A. | 10.89 ±0.03 | 10.64 ±0.31 | 11.07 ±0.09 | 10.92 ±0.17 |
| 9c-C18:1 Oleic A. | 36.63 ±6.1 | 31.37 ±5.7 | 60.33 ±4.43 | 55.52 ±4.97 |
| 11c/15t-C18:1 Vakcen A. | 4.78 ±0.1 | 4.91 ±0.18 | 4.35 ±0.06 | 4.58 ±0.05 |
| C18:2 n-6 Linoleic A. | 6.94 ±1.02 | 8.66 ±0.66 | 1.64 ±1.29 | 3.24 ±0.51 |
| 9c,11t 18:2 Conjugated Linoleic A. | 0.13 ±0.02 | 0.13 ±0.01 | 0.06 ±0.01 | 0.09 ±0.02 |
| C18:3 n-3 Linolenic A. | 0.15 ±0.03 | 0.17 ±0.03 | 0.34 ±0.1 | 0.16 ±0.02 |
| C20:1 Eikozenoic A. | 0.51 ±0.17 | 0.54 ±0.11 | 1.26 ±0.15 | 0.99 ±0.12 |
| C20:4 n6 Arachidonic A. | 1.85 ±0.41 | 1.82 ±0.21 | 0.17 ±0.11 | 0.77 ±0.26 |
| C20:5 n3 Eikozapentaenoic A. | 0.097 ±0.02 | 0.1 ±0.02 | 0.01 ±0.00 | 0.04 ±0.01 |
| C22:5 n-3 Dokozapentaenoic A. | 0.13 ±0.01 | 0.14 ±0.02 | 0.11 ±0.00 | 0.12 ±0.01 |
| C22:6 n-3 Dokozahexaenoic A. | 0.03 ±0.03 | 0.04 ±0.01 | 0.04 ±0.00 | 0.04 ±0.003 |

**Table 4** MDA content in raw material and sausage on the first and thirtieth day after production (mg.kg$^{-1}$ ±*SD*).

| Parameter | Pork shoulder | Sheep thing | Pork belly | Sausage | |
|---|---|---|---|---|---|
| | | | | 1-st day storage | 30-th days storage |
| MDA | 0.141 ±0.03 | 0.185 ±0.053 | 0.22 ±0.03 | 0.45 ±0.022 | 0.78 ±0.08 |

The content of stearic acid in the pork shoulder was 10.89 g.100g$^{-1}$ FAME, in the sheep's thigh 10.64 g.100g$^{-1}$ FAME, in the belly 11.07 g.100g$^{-1}$ FAME, and in the sausage 10.92 g.100g$^{-1}$ FAME. **Huang et al. (2020)** report a higher content of stearic acid in pork (11.07 g.100g$^{-1}$ FAME) compared to our results. In contrast to our results, **Chikwanha et al. (2018)** found out higher content of stearic acid in mutton (18.1 g.100g$^{-1}$ FAME) and **Marti-Quijal et al. (2019)** slightly higher values in sausage 13.51 g.100g$^{-1}$ FAME.

**Cheng et al. (2017)** report an almost identical myristic acid content in pork of 1.44 g.100g$^{-1}$ FAME compared to our values of 1.35 g.100g$^{-1}$ FAME in pork shoulder and 1.31 g.100g$^{-1}$ FAME in sausage. Higher content of myristic acid in the mutton 3.3 g.100g$^{-1}$ FAME was found by **Chikwanha et al. (2018)** compared to our results 1.38 g.100g$^{-1}$ FAME.

The content of eicosenic acid in the sausage was 0.99 g.100g$^{-1}$ FAME, in the pork shoulder 0.51 g.100g$^{-1}$ FAME, in the belly 1.24 g.100g$^{-1}$ FAME and the sheep's thigh 0.54 g.100g$^{-1}$ FAME. **Kim et al. (2009)** found a lower content of eicosenic acid (0.35 g.100g$^{-1}$ FAME) in pork compared to our results and also **Chikwanha et al. (2018)** in mutton meat 0.45 g.100g$^{-1}$ FAME. **Feng et al. (2020)** found a lower content of eicosenic acid in sausage (2.0 g.100g$^{-1}$ FAME).

The content of docosahexaenoic acid in the pig's shoulder was 0.03 g.100g$^{-1}$ FAME, in the sheep's thigh, in the belly and sausage was equally 0.04 g.100g$^{-1}$ FAME. **Chikwanha et al. (2018)** reported up to 0.15 g.100g$^{-1}$ FAME in comparison with our results in mutton meat, in contrast to **Cruxen et al. (2018)** in sausage 0.09 g.100g$^{-1}$ FAME.

The highest MUFA content 69.93 g.100g$^{-1}$ FAME in the belly was then in pork shoulder 49.62 g.100g$^{-1}$ FAME, in mutton 47.78 g.100g$^{-1}$ FAME, and sausage 60, 92 g.100g$^{-1}$ FAME. SAFA content was 34.21 g.100g$^{-1}$ FAME in pork shoulder, 33.52 g.100g$^{-1}$ FAME in mutton, and only 28.24 g.100g$^{-1}$ FAME in sausage. The PUFA content in the mutton was 14.98 g.100g$^{-1}$ FAME, in the belly 3.02, and the sausage 8.51 g.100g$^{-1}$ FAME. **Abdallah et al. (2020)** found out similarly with our results the MUFA content in mutton 46.9 g.100g$^{-1}$ FAME, while **Cheng et al. (2017)** report only 41.60 g.100g$^{-1}$ FAME in pork and **Marti-Quijal et al. (2019)** in sausage 48.84 g.100g$^{-1}$ FAME. **Cheng et al. (2017)**, in contrast to our results, found a lower PUFA content of 15.14 g.100g$^{-1}$ FAME, and compared to our study higher SAFA content in pork 39.99 g.100g$^{-1}$ FAME identically also **Abdallah et al. (2020)** found out a higher SAFA content in mutton 36.7 g.100g$^{-1}$ FAME.

The content of omega 3 FA was highest in the belly 0.85, lowest in the pork shoulder 0.53, and the sausage 0.7 g.100g$^{-1}$ FAME. Consistent with our results, **Feng et al. (2020)** found in mutton sausage omega 3 FA content 0.75 g.100g$^{-1}$ FAME.

Table 4 shows the content of malondialdehyde (MDA) as an indicator of oxidative stability in pork shoulder, belly, and mutton on the day of production. In sausage on the first and thirtieth day after production. The MDA content in mutton was 0.185 mg.kg$^{-1}$, in pork shoulder 0.141 mg.kg$^{-1}$, in pork belly 0.22 mg.kg$^{-1}$ and in sausage on the day of production 0.45 mg.kg$^{-1}$. On day 30, the MDA content was in the sausage (0.78 mg.kg$^{-1}$). In comparison with our results **Marcinčák et al. (2005)** found out lower MDA content in pork 0.1166 mg.kg$^{-1}$, also **Reitznerová et al.**

**(2017)** found a lower content of MDA (0.055 mg.kg$^{-1}$). In sausage after ripening 1-month **Šojić et al. (2015)** found an MDA content of 0.520 mg.kg$^{-1}$. The high MDA content of the sausage was probably most influenced by the production process, as all raw materials had a lower MDA content.

## CONCLUSION

The basic composition of sausage representation of individual ingredients. The lower water content was influenced by its content in the belly but also by the reduction during drying and smoking. The content of individual monitored amino acids corresponding to the raw material used. The SAFA content of the raw materials ranged from 26.49 to 34.21 g.100g$^{-1}$ FAME and is generally considered sufficient for fat oxidation. Our results confirmed the increased content of MDA. The high MDA content of the sausage was probably most influenced by the production process, as all raw materials had a lower MDA content.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# ASPECTS OF THE INFLUENCE OF VEGETABLE-OIL-BASED MARINADE ON ORGANOLEPTIC AND PHYSICOCHEMICAL INDICATORS OF THE QUALITY OF SEMI-FINISHED NATURAL MARINATED MEAT PRODUCTS

***Oksana Shtonda, Kateryna Semeniuk***

## ABSTRACT

This work evidences the expediency of marinade application based on blends of refined vegetable oils in the technology of natural marinated semi-finished meat products. Formulations of natural marinated semi-finished meat products using blends of refined vegetable oils (rapeseed, olive, and sunflower) enriched with the enzyme bromelain were developed in this work. Such formulations can provide the human body with the necessary amount of protein, released by enzymatic hydrolysis of the connective tissue proteins collagen and elastin catalyzed by the plant-based enzyme bromelain, as well as the necessary ω-3 and ω-6 polyunsaturated fatty acids, which are not synthesized in the human body but sourced from food and are one of the main structural units for many vital processes. The article presents the results of a study of organoleptic and physicochemical parameters of natural marinated semi-finished meat products from beef and pork. It is confirmed that the use of rapeseed oil and rapeseed:sunflower oil blend in a ratio of 70:30 produce the best organoleptic characteristics in both semi-finished beef and semi-finished pork: a tender, juicy texture, a pleasant taste and aroma, and an attractive appearance. It has been proven that the use of marinades based on blends of refined vegetable oils reduces the free moisture content and moisture-retaining capacity of the product, due to the presence of refined vegetable oils in the marinade, which contributes to the binding of moisture. The use of marinades based on refined vegetable oils can increase product yield. Vegetable oil, when used in meat marinade, mitigates the ill effects of acid inclusion. It dissolves well the aromas of added spices, and as it seeps through the structure of the meat, the oil gently envelops it, sealing in moisture and preventing drying out during cooking.

**Keywords:** natural marinated semi-finished meat products; blends; vegetable oils; organoleptic parameters; physicochemical parameters

## INTRODUCTION

Improving the technology of natural marinated meat products is a very important issue because the pace of human life changes very quickly and increases every day. This leads to a deterioration of the diet, which in turn is harmful to human health.

Moreover, due to the insufficient quantity of raw meat produced in Ukraine, there is a need to create food that will have a higher yield and provide the human body with the necessary amount of protein and micronutrients **(Sukhenko et al., 2017)**. Therefore, it is necessary to create products that can be cooked rapidly, without losing their biological and nutritional value **(Koh, 2005)**.

For that reason, it is necessary to use meat with a high content of connective tissue for the production of marinated semi-finished meat products, as it is less expensive than traditional raw materials used for semi-finished products. In this manner, the cost of the product can be reduced.

Thus, there is an urgent need for meat products of high nutritional and biological value, which are balanced in fatty acid composition and rapid to cook. Once this problem is solved, the range of natural marinated meat products can also be expanded **(Mushtruk et al., 2020b)**.

Scientists from all over the world are turning their attention to creating products that are quick to prepare and have a positive effect on the human body without losing their biological and nutritional value. In particular, the following domestic and foreign scientists are working in this area of research: L. Vinnikova, V. Yevlash, L. Caprelyants, M. Klimenko, I. Radzievska, A. Belinska, B. Pasichny, G. Simakhina, N. Tkachenko, A. Nechaeva, L. Diaz, W. Schnackel, F. Jemenez-Colmenero, I. Yilmaz, T. Dzudie and others **(Kolyanovska et al., 2019)**. However, there is a need to continue research in this area, namely on the ingredients that increase the biological and nutritional value of products, as well as the effect of these ingredients on finished products, in particular the effect of blends of refined vegetable oils enriched with bromelain on the properties of natural marinated semi-finished meat products **(Mushtruk et al., 2020a)**.

Vegetable oils are an important component of the diet. It has been shown that reducing the proportion of fat in the diet from 37% to 30% of total calories can prevent approximately 2% of deaths from cardiovascular disease among people over 65 years of age. Nevertheless, avoiding total fat is not the best strategy for the human body. The best option is to balance the consumption of unsaturated (oils) and saturated fats. This can be achieved by introducing unsaturated fats into the product, balanced in fatty acid composition. In nature, oils with a balanced fatty acid composition do not exist, which raises the question of mixing (blending) **(Tyshchenko et al., 2019; Belinskaya, 2011)**.

World Health Organization **(WHO)** statistics show that 15% – 30% of the total energy required for the normal functioning of the human body should come from the consumption of dietary fats: 10% from saturated fatty acids, 6% – 10% from polyunsaturated fatty acids (5% – 8% ω-6, 1% – 2% ω-3), 10% – 15% from monounsaturated fatty acids and less than 1% from the consumption of trans-isomers of fatty acids. Scientists have proved that the most useful for the human body are two-chain polyunsaturated fatty acids, which are not synthesized in the body but can only be sourced from food. Especially important is the ω-6:ω-3 ratio, which for a healthy person is 10:1 **(Domaretskyy and Ostapchuk, 2003; Gubsky, 2000)**.

Many studies have confirmed the important role of a balanced ratio of ω-6: ω-3 fatty acids, which are involved in many biochemical processes in the human body, which reduces the risk of serious diseases, especially cardiovascular disease, cancer, osteoporosis, diabetes, and others. The amount of polyunsaturated fatty acids is greater in vegetable oils than in products of animal origin, so the inclusion of these products in the diet is necessary **(Brenna, 2002; Proust, Lucas and Deawailly, 2014; Tvrzická et al., 2009)**.

The creation of blends from different types of oils is already very popular in Europe, but the culture of consumption of such products is not yet sufficiently formed among Ukrainians. Nevertheless, products of this type are already appearing on the shelves of Ukrainian stores **(Sukhenko et al., 2020)**. The diet of Ukrainians is dominated by oils containing fatty acids of the ω-6 family, mostly sunflower oil, and almost excludes products rich in acids of the ω-3 family, such as flaxseed, rapeseed, pumpkin and walnut oils. However, to increase the biological and physiological value of oils by regulating the ω-6:ω-3 ratio within the PUFA content, oils can be mixed or blended **(Okara, Zemlyak and Kalenik, 2009; Krychkovska, Belinska and Zhulinska, 2010)**.

Adding marinades for natural marinated semi-finished meat products based on blends of refined vegetable oils gives the products the desired consistency; allows determination of the energy value; maintains product yield and ensures the formation of taste and aroma of the product. Moreover, enrichment of blends with the enzyme bromelain will accelerate the maturation and softening of meat by a factor of 2 – 2.5 **(Nechaev, Traubenberg and Kochetkova, 2007)**.

The goal of this research was to analyze the effect of marinade components based on blends of vegetable oils using the enzyme bromelain on the organoleptic and physicochemical parameters of natural marinated semi-finished meat products. The following specific aims were further defined: to develop recipes for pickled semi-finished products and to study the influence of marinade components on organoleptic and physicochemical parameters of marinated semi-finished products.

### Scientific hypothesis

The research hypothesis was based on the assumption that marinades based on blends of vegetable oils enriched with the enzyme bromelain can have a positive effect on the organoleptic and physicochemical parameters of semi-finished products. The objects of the study were marinades based on vegetable oils: rapeseed, blend, sunflower:rapeseed (70:30) and blend, sunflower:olive (80:20). According to the results, it is stated that semi-finished products using a marinade based on pure rapeseed oil and a blend of sunflower (70%): rapeseed (30%) have the best organoleptic characteristics. It is proved that the use of marinades based on blends of vegetable oils reduces the amount of moisture in the product and increases the fat content, which affects the shelf life of the product.

## MATERIAL AND METHODOLOGY

### Samples

In this article, 8 samples were examined: 4 samples using pork and 4 samples from beef. Sample 1 – pork without marinade. Sample 2 - pork with a marinade based on rapeseed oil. Sample 3 – pork with a marinade based on a blend of oils (sunflower: rapeseed = 70:30). Sample 4 – pork with a marinade based on a blend of oils (sunflower: olive = 80:20). Sample 5 – beef without marinade. Sample 6 – beef with a marinade based on rapeseed oil. Sample 7 – beef with a marinade based on a blend of oils (sunflower: rapeseed = 70:30). Sample 8 – beef with a marinade based on a blend of oils (sunflower: olive = 80:20).

### Chemicals

To determine the protein content in the prepared samples using the following chemical reagents:

Sulfuric acid $H_2SO_4$, (Inter-Synthesis Limited, Liability Company, Ukraine, chemically pure for analysis).

Copper sulfate catalyst $CuSO_4$, (Inter-Synthesis, Limited Liability Company, Ukraine, chemically pure for analysis).

Inorganic sulfuric acid salt $Na_2SO_4$, (Inter-Synthesis, Limited Liability Company, Ukraine, chemically pure for analysis).

Sodium hydroxide NaOH, (Inter-Synthesis, Limited Liability Company, Ukraine, chemically pure for analysis).

Boric acid $H_3BO_3$, (Inter-Synthesis, Limited Liability Company, Ukraine, chemically pure for analysis).

Petroleum ether was used to determine the fat content in the prepared samples.

### Animals and Biological Material:

We used beef grade II and pork grade II, with a high content of connective tissue.

### Instruments

The following tool was used to determine the moisture content in the prepared samples:

Analytical balance (Radwag AS 220/C, (Inter-Synthesis, Limited Liability Company, Ukraine).

Drying cabinet SNOL 58/350, (Inter-Synthesis, Limited Liability Company, Ukraine).

Boxes aluminum laboratory, (Inter-Synthesis, Limited Liability Company, Ukraine).

Desiccator without tap 2-180, (Inter-Synthesis, Limited Liability Company, Ukraine).

To determine the protein content in the prepared samples used the following equipment:

Analytical balance Radwag AS 220/C, (Inter-Synthesis, Limited Liability Company, Ukraine).

Drying cabinet SNOL 58/350, (Inter-Synthesis, Limited Liability Company, Ukraine).

Boxes (aluminum laboratory, (Inter-Synthesis, Limited Liability Company, Ukraine).

Desiccator without tap 2-180, (Inter-Synthesis, Limited Liability Company, Ukraine).

Velp mineralizer DKL8, (Inter-Synthesis, Limited Liability Company, Ukraine).

Distillation apparatus UDK 129, (Inter-Synthesis, Limited Liability Company, Ukraine).

Folded burette with tap Class A 25 mL-0.1, (Inter-Synthesis, Limited Liability Company, Ukraine).

The fat content in the prepared samples was determined on a Soxhlet apparatus, as well as a drying cabinet, boxes, desiccator, paper bags made of filter paper.

To determine the ash in the samples, the following equipment was used:

Analytical balance Radwag AS 220/C, (Inter-Synthesis, Limited Liability Company, Ukraine).

Porcelain crucible tongs No2, (Inter-Synthesis, Limited Liability Company, Ukraine).

Desiccator without tap 2-180, (Inter-Synthesis, Limited Liability Company, Ukraine).

**Laboratory Methods**

The authors used the following laboratory research methods:

**DSTU 4823.2 (2007)** – Meat products. Organoleptic evaluation of quality indicators.

**DSTU ISO 1442 (2005)** – Meat and meat products. Moisture content method (control method).

**DSTU ISO 937 (2005)** – Meat and meat products. Determination of nitrogen content (control method).

**DSTU ISO 1443 (2005) –** Meat and meat products. Method for determination of total fat content.

**DSTU ISO 936 (2008) –** Meat and meat products. Method for determining the mass fraction of total ash.

**Description of the Experiment**

The main purpose of the presented experiment is to determine the effect of marinades based on blends of vegetable oils enriched with the enzyme bromelain on the physicochemical and organoleptic characteristics of natural marinated meat semi-finished products. The content of moisture, protein, fat, and ash, moisture-holding, capacity, as well as organoleptic parameters were determined: appearance, sectional view, color, taste, aroma, consistency, and juiciness.

**Number of samples analyzed:** For research, 8 samples were prepared, 4 samples using pork, and 4 samples from beef.

**Number of repeated analyses:** All measurements of instrument readings were performed 10 times.

**Number of experiment replication:** The number of repetitions of each experiment to determine one value was also 10 times.

**Statistical 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 2016 values were estimated using mean and standard deviations and subsequently evaluated in the statistical program XL Stat. In hypothesis testing, if the *p*-value is lower than the 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

Traditionally, marinated semi-finished products are made from the tender parts of the carcass, which have a significant amount of muscle protein and a small amount of fat. The scapular part of the carcass, which has a high content of connective tissue, was selected for the study. Similar scientific researches are described in works **(Benzik et al., 2020; Zhumanova et al., 2018; Keniiz et al., 2019)**.

To reduce the toughness of the meat, we add the enzyme bromelain to the marinade, which can hydrolyze connective tissue proteins such as collagen and elastin. Scientific studies **(Yang et al., 2018; Doneva et al., 2018; Cai et al., 2018)** describe similar studies using other enzymes, which in our opinion are not rational to use due to their high cost. As a result, we can obtain a tender, juicy product from tough meat that is easily digested by the body. Moreover, the use of vegetable blends will retain moisture in the product during heat treatment, as well as increase product yield. In scientific works **(Park et al., 2019; Shin et al., 2019; Łopacka, Półtorak and Wierzbicka, 2016; Erdaw et al., 2016)** conducted more in-depth research on the use of various enzymes and their effects on the physicochemical properties of raw meat.

Recipes for natural marinated meat products differ based on marinades. In scientific works **(Nesterenko et al., 2017; Jinap et al., 2018; Wang et al., 2018)** research and recipes of various marinades, both for meat raw materials and for various kinds of seafood are carried out. It should be noted that when using different types of marinades there was a change in the organoleptic properties of the finished product. Due to the addition of marinade, there is a decrease in the amount of basic raw materials compared with the control sample, which does not include marinade. This will save significant funds in the manufacturing of the product (Table 1, Table 2). Recipes for marinades are given in the patents of Ukraine for the utility model "Marinade based on vegetable oils" No 134474, No 134475, No 134476 **(Shtonda and Semeniuk, 2019a; Shtonda and Semeniuk, 2019b; Shtonda and Semeniuk, 2019c)**.

Scientific research **(Semeniuk and Shtonda, 2021)** describe influence of fatty acid structure of vegetable oils on physical and chemical indicators of quality of blends vegetable oils.

Preparation of the marinade based on blends of vegetable oils and the introduction of the enzyme bromelain increased the product yield and improved the digestibility compared with control samples.

Organoleptic evaluation of natural marinated semi-finished meat products was conducted in the form of a tasting, which was attended by teachers and students of the Department of Meat, Fish and Seafood Technology of the National University of Life and Environmental Sciences of Ukraine. The following indicators were determined: appearance, sectional view, color, aroma, taste, consistency, and juiciness. The organoleptic evaluation was performed and scored on a five-point scale (Figure 1, Figure 2).

As a result of the organoleptic evaluation of marinated semi-finished pork products, it was found that the best indicators were found in samples treated with marinades based on pure rapeseed oil (sample No1) and a mixture of rapeseed (30%) and sunflower (70%) oil (sample No2). These samples possessed a delicate, juicy consistency, a clear appearance in the cut, and a pleasant taste and aroma. Scientific articles **(Suárez et al., 2021; Trabelsi et al., 2018; Obajuluwa et al., 2020; Jung et al., 2018)** presents several experimental studies concerning the use of marinades based on olive, corn, soybean, and peanut oils in various combinations and ratios. We performed several experiments and found that some samples did not have a juicy consistency and an unpleasant taste and aroma (a mixture of corn and soybean oils).

The organoleptic evaluation of marinated semi-finished beef products showed that the best base for the marinade was a mixture of rapeseed (30%) and sunflower (70%) oils (sample No6).

Chemical composition is one of the main indicators that affects the quality of the product. Chemical composition studies were performed on the first day of storage of natural marinated semi-finished meat products (Table 3). Scientific papers **(Ivankin et al., 2020; Lorenzo et al., 2018; Reis et al., 2018; Smetanska et al., 2021)** describe a number of experimental studies on the chemical composition of natural marinated meat semi-finished products, but they were conducted on the third, fifth and tenth days of storage.

The results of the study of the chemical composition show that for marinated semi-finished products in comparison with the control sample there is a decrease in the amount of moisture and an increase in the amount of fat in the product due to the presence of oil in the marinade. This helps to increase the shelf life of the product.

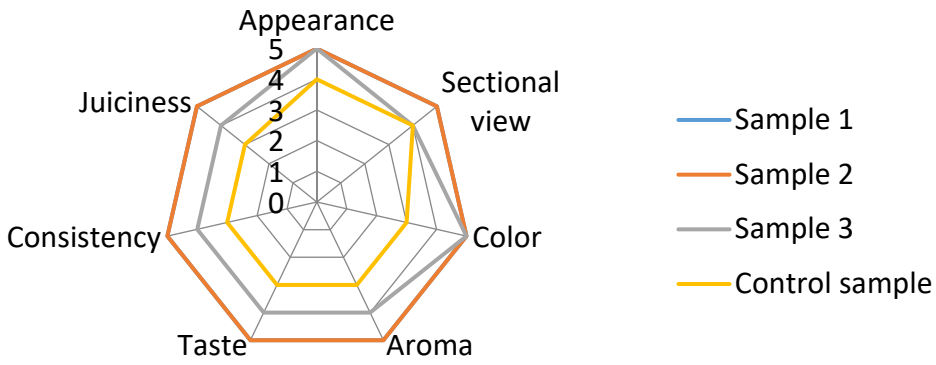


**Figure 1** Organoleptic evaluation of marinated semi-finished pork products.

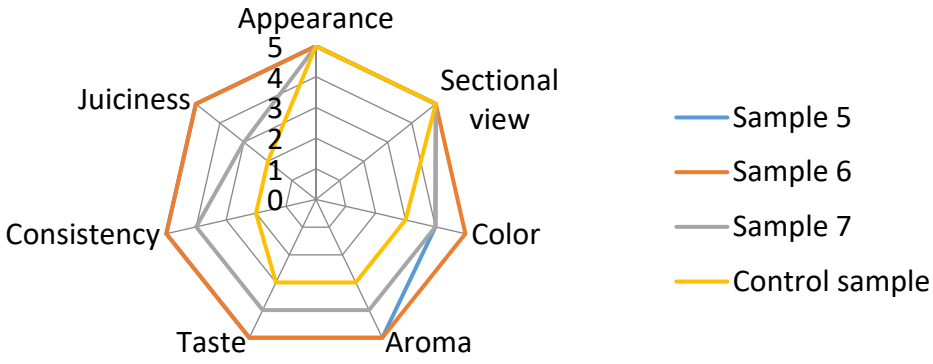


**Figure 2** Organoleptic evaluation of marinated semi-finished beef.

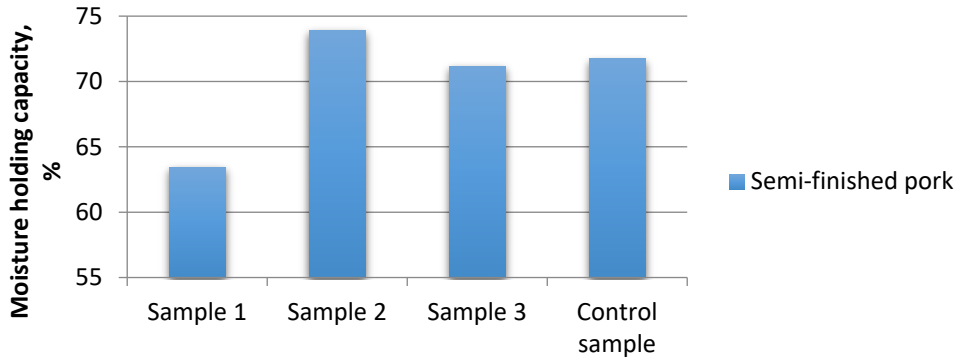


**Figure 3** Moisture-holding capacity of marinated semi-finished pork products.

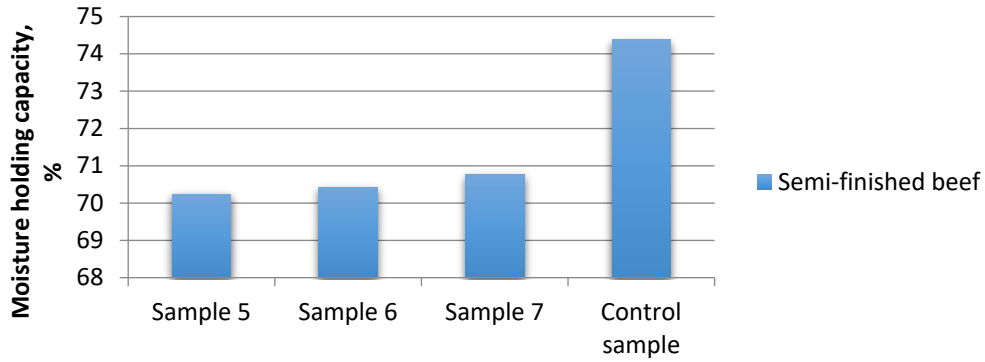


**Figure 4** Moisture-holding capacity of marinated semi-finished beef products.

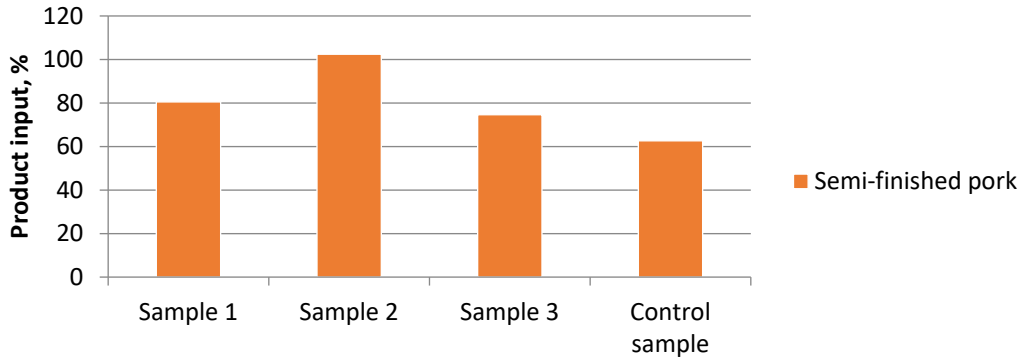


**Figure 5** Yield of marinated semi-finished pork.

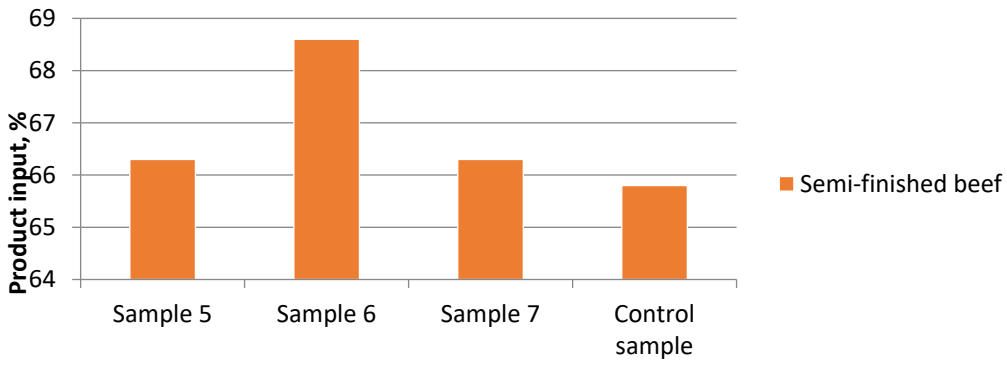


**Figure 6** Yield of marinated semi-finished products from beef.

A decrease in the amount of free moisture in the product leads to an increase in the moisture-binding capacity **(Shtonda and Semeniuk, 2018; Makarenko et al., 2021)** and, accordingly, to a decrease in the moisture-holding capacity (Figure 3, Figure 4).

The use of vegetable oil blends in the marinade not only ensures the balance of the product in terms of fatty acid composition but also increases the yield of the product (Figure 5, Figure 6).

It was found that the product yield after heat treatment increased by 19.17% – 63.58% for semi-finished pork products relative to the control sample and by 0.76% – 4.25% for beef semi-finished products, compared with the control sample.

## CONCLUSION

Six recipes for marinated semi-finished products have been developed: three recipes using pork and three recipes using beef. As a result of the research, it was found that semi-finished products using a marinade based on a pure rapeseed oil and sunflower blend (70%): rapeseed (30%) have the best organoleptic characteristics. We have shown that the use of marinades based on blends of vegetable oils leads to a decrease in the amount of moisture in the product and an increase in fat content, which affects the shelf life of the product. The possibility of reducing the duration of production of natural marinated semi-finished meat products by a factor of 5 due to the introduction of enzyme preparations into the marinade has been established experimentally.

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# THE CARCASS AND MEAT QUALITY CHARACTERISTICS OF JAPANESE QUAIL FED A DIET SUPPLEMENTED WITH POWDERED *LACTUCA SERIOLA* LEAVES

***Azad Sabow, Nazim Abdulla, Bestoon Ahmed***

## ABSTRACT

The present study was aimed to evaluate the effect of dietary supplementation with *Lactuca serriola* leaves on growth performance, carcass characteristics, meat quality, and its antioxidant stability of Japanese quails. A total of eighty growing quails (1-week old) were distributed into 2 equal groups consisting of 40 birds (5 replicates of 8 birds each). The first group was fed a basal diet without *Lactuca serriola* leaves (0 $g.kg^{-1}$ diet) and the second group received diets containing 20 $g.kg^{-1}$ *Lactuca serriola* leaves. At age of 6 weeks, quails were slaughtered for meat and carcass examinations. The growth performance for the quails fed with *Lactuca serriola* leaves diet was similar to that of a control group. Carcass measurements, physical properties, and chemical composition of quail breast meat did not differ between the control and experimental group. However, the inclusion of *Lactuca serriola* leaves significantly increased the omega-3 polyunsaturated fatty acid content and improved breast meat lipid stability during postmortem refrigerated storage compared to the control diet. In conclusion, dietary supplementation of *Lactuca serriola* leaves can improve the performance of Japanese quail enrich its meat with an omega-3 polyunsaturated fatty acid, and reduce lipid oxidation during storage.

**Keywords:** growth; *Lactuca serriola*; meat traits; polyunsaturated fatty acid; quail

## INTRODUCTION

In recent years, products of different species of poultry are marketed including quail products. Japanese quail is the main species of poultry whose meat is commonly demanded everywhere in the world, especially in America, Europe, and Asian nations **(Partovi and Seifi, 2018)**. Nowadays, a major concern of poultry producers is to optimize the produced meat both qualitatively and quantitatively **(Abdulla et al., 2017)**. Consequently, many recent studies have been represented herbs and spices as efficient and safe feed additives to improve poultry health as well as the rate of growth **(Adegoke et al., 2018; Rasul, 2020; Reda et al., 2020)**. Additionally, the active molecules in herbs and spices or their oils can prevent meat oxidation that negatively affects the meat's organoleptic properties and also abates its nutritional value **(Reda et al., 2020)**. Lipid oxidation is a limiting factor, which determines the safety and shelf-life of meat. It is considered a major cause of non-microbial meat spoilage, specifically under pro-oxidative conditions such as storage during refrigeration and frozen **(Sabow et al., 2016)**.

*Lactuca serriola* L. (Compositae) is an herbaceous species widely grown in Europe, India, Pakistan, India, and Middle Eastern countries. The plant is used in a medicinal application that is the old practice of human history **(Vargas-Sánchez et al., 2019)**. Research has demonstrated that *Lactuca serriola* contains lactic acids and β- carotene. It is also a rich source of almost all vitamins and contains a high amount of calcium, zinc, and iron **(Janbaz et al., 2013)**. *Lactuca serriola* contains phenolic compounds that help to enhance its antioxidants capacity. The antioxidant effect of *Lactuca serriola* is explained by its phenolic compounds including cichoric acid, alkamides, caffeic acid, and chlorogenic acid **(Nabavi et al., 2012)**.

Early investigations studied the effects of *Lactuca serriola* as pharmacological properties in humans, rabbits, and mice such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive, and antioxidant **(Al-Marzoqi, Hussein and Al-Khafaji, 2015; Ahangarpour, Oroojan and Radan, 2014; Janbaz et al., 2013)**. Investigations regarding the inclusion of powdered *Lactuca serriola* leave in Japanese quail diets and its effects on carcass quality were not found. It is hypothesized that the dietary addition of *Lactuca serriola* is expected to exert beneficial effects on the performance quality of carcass and meat of quail. Therefore, the present study was designed to assess the impacts of dietary inclusion of *Lactuca serriola*

leaves on performances, carcass quality, and oxidative stability of Japanese quail meat.

## Scientific hypothesis

The results of the current study will provide new knowledge and useful information about the *Lactuca serriola* plant and its antioxidant activity, which will give a wide range of possibilities for employing this plant as a source to improve the quality of carcass and oxidation stability of poultry meat.

## MATERIAL AND METHODOLOGY

We assessed meat quality characteristics of Japanese quail fed a diet supplemented with or without *Lactuca seriola* leaves. A total of 80 male growing Japanese quails (one-week-old) with an average weight of 11.667 ±0.21 g were obtained from a commercial farm (Quail Farming, Qushtapa, Erbil-Kurdistan Region of Iraq) and randomly distributed into 2 groups, each of which included 5 replicates of 8 birds. Birds were housed in conventional type cages (90 × 40 × 40 cm) with feed and fresh water provided *ad libitum*. This study lasted until the 6$^{th}$ week of age. The dietary treatments were as follows: (1) basal diet without supplementation (0 g.kg$^{-1}$ diet) and (2) basal diet + 20 g *Lactuca serriola* per 1 kg. The aerial parts of *Lactuca serriola* were collected from the botanical garden of Erbil city (Kurdistan Region, Iraq) in the year 2019. The plant was identified based on taxonomy from the College of agriculture engineering and science. The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven (CS101-beb ventilation oven, Chonping Sida instrument, China) at 40 °C for 48 h, ground into a fine powder and stored at 4 °C in dark containers until using as feed additive. On the day of manufacture, *Lactuca serriola* leaves powder was added to a 1 kg sample of basal feed-in a Univex Mixer (SRM20 Counter Mixer, USA) and mixed for 5 minutes before being remixed with the allotted feed. The chemical compositions of fresh leaves were as following: 93.7% moisture, 1.4% protein, 0.4% fat, 1.1% fiber, 2.2% carbohydrate and 1.2% ash.

The composition of the basal diet (Table 1) was formulated to meet the quails' requirements based on the guidelines provided by the **National Research Council (1994)**. Birds were exposed to 24 h of lighting with the temperature kept at 20 to 24 °C throughout the experiment.

### Chemical

All the chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Growth performance

At six weeks of age, individual body weight was recorded. Also, feed intake was recorded during the experimental periods on a replicate basis to estimate feed conversion ratio (FCR = g feed per g gain).

### Slaughtering procedures and carcass measurements

At the end of the experiment at 6 weeks of age, ten quails from each treatment were randomly selected, weighed, and slaughtered following the halal slaughter procedure. The halal slaughtering method was carried out by cutting the neck, severing both carotid arteries and jugular veins without decapitating the head during the process. Upon slaughter, carcasses were immediately taken to the laboratory for carcass examinations. Dressed carcasses were chilled at 4 °C for 24 h and reweighed (cold carcass weight; CCW). The dressing out the percentage of the carcass was determined as the proportion of CCW to the slaughter weight.

### Physical analyses of quail breast meat

For meat quality characteristics, the breast muscle was separated into two parts. The first part was properly labeled, vacuum packaged, and stored at 4 °C for determination of drip loss. The second breast portion was used for other meat quality measures including pH, cooking loss, and tenderness. The pH of meat was recorded immediately after 24 h postmortem on the breast muscle using a portable pH meter (HANNA® instruments, Woonsocket, USA) after calibration using two buffers of pH 4.0 and 7.0. Cooking loss and tenderness of the breast samples were measured using the procedure described by **Sabow (2020)**. To measure cooking loss, the meat samples were cooked at 80 °C in a pre-heated water bath (HAAKE ® instruments, Woonsocket, USA) for another 10 min once the internal temperature of the samples has reached 78 °C. After cooking loss determination, the cooked meat samples were collected and used to determine tenderness using the Volodkovitch bite jaw attached to a Brookfield Texture Analyzers (CT3™, Middleborough, USA). For each sample, a block of 1 cm (height) × 1 cm (width) × 2 cm (length) was cut parallel to the direction of the muscle fibers and sheared vertically in the middle to the longitudinal direction of the muscle fibers. The sheer force values were represented as the average peak positive peak force (kg).

### Chemical analysis of quail breast meat

The proximate composition of the breast meat samples was analyzed using the procedure provided by the Association of Analytical Chemists according to AOAC **(Horwitz, 2000)**. Moisture was determined by drying 1 g of meat in an oven (CS101-beb ventilation oven, Chonping Sida instrument, China) at 75 °C until a constant weight was obtained. Crude protein was measured by the Kjeldahl method (VAPODEST® 500, Gerhardt, Germany). The crude protein was obtained as 6.25 × nitrogen %. The fat content of the meat was determined by the Soxhlet extraction method (Classic Soxhlet Apparatus, Gerhardt, Germany) using petroleum ether. The Ash content of the meat was estimated by burning the sample in a muffle furnace (Heraeus KR 170E, Germany) at 550 °C for 3 h.

### Oxidative stability of quail breast meat

The Thiobarbituric acid reactive substances (TBARS) value of the breast meat after 1 and 7 days of refrigerated storage was determined in duplicate according to the method described by **Aminzade Karami and Lotfi (2012)**. Briefly, 5 g of meat was homogenized with 48 mL of distilled water and 1.25 mL of 4N HCl for 2 min. The mixture was distilled until 25 mL was obtained. After filtration, 2.5 mL of the distillate was mixed with 2.5 mL of Thiobarbituric acid reagent (15% trichloroacetic acid,

0.375% thiobarbituric acid). The samples were incubated in a water bath at 100 °C for 35 min and then cooled under running tap water for 10 min. The absorbance was measured at 538 nm against a blank with a spectronic®20 GENESYSTM spectrophotometer (Spectronic Instruments, USA). The TBARS values were obtained by multiplying optical density by 7.843 and expressed as malondialdehyde (MDA) per kg meat.

### Statistical Analysis

The obtained data were subjected to one-way analysis of variance (ANOVA) in the general linear model using Statistical Analysis System package (SAS) version 9.1 software (SAS Institute Inc., Cary, NC, USA). For comparison of TBARS values in day 1 and 7 postmortems, the parametric repeated measures ANOVA test were performed, and interaction between treatment groups and sampling times are calculated but were not statistically different. Differences between groups were tested with an independent sample T-test and the significance was established at a level of 0.05 ($p$ <0.05).

## RESULTS AND DISCUSSION

### Quail growth performance and carcass measurements

The effect of diet supplementation with *Lactuca serriola* leaves on the total final body, feed intake weight, feed conversion ratio, carcass weight, dressing percentage of Japanese quail at 6 weeks of age are given in Table 2. The inclusion of *Lactuca serriola* leaves in the quail diet led
to improve final body weight, feed intake, and feed conversion ratio compared to the control treatment, although the values were not statistically significant. *Lactuca serriola* plant through its content of antioxidants plays a significant role in increase the activities of a digestive enzyme such as amylases, proteases, and lipases. Generally, stimulation of the function of pancreatic enzymes leads to promote the synthesis of bile acids in the liver and their secretion in the bile, which has a beneficial effect on digestion and absorption as well as growth stimulation. **Adegoke et al. (2018)** reported that growth performance is affected by active compounds of plant-derived supplements used in poultry nutrition. The present findings are in agreement with the findings of **Partovi and Seifi (2018)** who found no significant difference in better growth performance results ($p$ >0.05) of Japanese quail fed medicinal herb as feed additives compared with the control group.

The quail carcass traits such as carcass weight and carcass yield are an indicator of their significant efficiency and the ability for meat production **(Nasr, Ali and Hussein, 2017; Sabow, 2020)** which could be influenced by the inclusion of natural plants used in poultry nutrition **(Vargas-Sánchez et al., 2019)**. Some investigations have demonstrated the positive effect of adding natural herbs to the nutrition on poultry carcass measurements **(Ghazaghi Mehri and Bagherzadeh-Kasmani, 2014; Nosrati et al., 2017)**, while others have not emphasized such effects **(Abd El-Hack, Alagawany and Abdelnour, 2019)**. In the present study, dietary supplementation of *Lactuca serriola* leaves did not affect ($p$ >0.05) on the carcass weight and carcass yield (Table 2). In agreement with the current findings, **Reda et al. (2020)** found no significant effect of plant supplements feeding on the carcass traits of quails. In growing rabbits, **Abd El-Hack, Alagawany and Abdelnour (2019)** also reported that there was no significant difference in carcass measurements between animals fed diets containing herbal natural feed additives and those in the control animals.

### Physical properties and chemical composition of quail breast meat

As shown in Table 3, the values of drip loss, cooking loss, and shear force for both experimental and control diets were not significantly different. This observation may be because of the similarity in the ultimate pH of breast meat which was 6.26 and 6.12 for quails fed control and experimental diet, respectively. The ultimate pH is a significant factor that influences the physicochemical attributes of meat **(Chan, Omana and Betti, 2011; Sabow, Haddad and Nakyinsige, 2020)**. The results of the current study agreed with **Partovi and Seifi (2018)** who detected no statistical influence of adding a natural plant to the quail diet on meat physical traits.

The inclusion of natural ingredients such as medicinal herbs and plants in the poultry diet effects on the nutritional composition of meat is unclear. The present results of proximate compositions of breast meat are not significantly different ($p$ >0.05) between the experimental and control group. This finding is consistent with that of **Partovi and Seifi (2018)** who clarified those herbal dietary supplements for Japanese quail diets had not affirmative effects in protein and fat values. Also, **Shirzadegan and Falahpour (2014)** found that meat samples of broiler chickens supplemented with dietary mixture medicinal herb has similar moisture, protein, and fat as compared to meat samples of the control group.

### Polyunsaturated fatty acids of quail breast meat

Polyunsaturated fatty acids are widely taken into account to have a more beneficial biological effect **(Givens, Kliem and Gibbs, 2006; Gecgel et al., 2015)**. The effects of dietary treatments on polyunsaturated fatty acids (PUFA) of quail breast meat are given in Table 4. Although n-6 PUFA concentration did not differ between dietary treatments, feeding *Lactuca serriola* leaves diet improved significantly the total n-3 fatty acids of quail breast meat. The ratio of n-6 to n-3 PUFA was significantly lower in the breast muscles of quails fed on *Lactuca serriola* leaves as a result of their relatively high total n-3 PUFA concentrations. The n-6 to n-3 ratio is generally used as an index to assess the nutritional value of dietary fat that has particular relevance to human health **(Young, 2009; Valencak et al., 2015)**. The recent ratio of n-6 to n-3 fatty acids in the diets of North American adults should be in a range of 10:1 to 50:1, thus the ratios detected in the current study fall within this range.

**Table 1** Composition of the experimental diet.

| Ingredient | g.kg$^{-1}$ diet |
|---|---|
| Wheat | 175.0 |
| Corn | 200.0 |
| Wheat flour | 250.0 |
| Soya bean meal | 290.0 |
| Protein concentrate (fish meal concentrate) | 50.0 |
| di – calcium phosphate | 6.0 |
| Methionine | 0.9 |
| Lysine | 0.5 |
| Choline chloride | 0.5 |
| Salt | 0.9 |
| Soya bean oil | 13.0 |
| Feed toxic | 1.0 |
| Limestone | 10.0 |
| Vitamins premix[1] | 0.7 |
| Feed sterilizes | 1.0 |
| Anticoccidial | 0.5 |
| Analyzed feed composition | |
| Crud protein (g.kg$^{-1}$) | 230.0 |
| Energy metabolism (MJ.kg$^{-1}$) | 12.6 |

Note: [1]Multi vitamin contained retinol 2 mg, cholicalciferol 0.03 mg, α-tocopherol 0.02 mg, menadione 1.33 mg, cobalamin 0.03 mg, thiamine 0.83 mg, riboflavin 2 mg, folic acid 0.33 mg, biotin 0.03 mg, panthothenic acid 3.75 mg, niacin 23.3 mg, pyridoxine.

**Table 2** Growth performance and carcass traits of growing Japanese quail as affected by graded levels *Lactuca serriola* leaves.

| Items | Lactuca serriola leaves level (g.kg$^{-1}$ diet) | | SEM | *p* value |
|---|---|---|---|---|
| | 0 | 20 | | |
| Live body weight (g) | 141.168 | 153.792 | 0.24 | 0.198 |
| Total feed intake (g per day) | 13.966 | 14.608 | 0.77 | 0.332 |
| Feed conversion ratio (g feed per g gain) | 3.463 | 3.325 | 0.15 | 0.851 |
| Slaughter body weight (g) | 145.801 | 160.798 | 5.96 | 0.063 |
| Carcass weight (g) | 91.457 | 100.412 | 3.47 | 0.087 |
| Dressing (%) | 62.717 | 62.446 | 1.27 | 0.772 |

Note: SEM = Standard Error of the Means.

**Table 3** Physical characteristics and chemical composition of breast meat of growing Japanese quail as affected by graded levels of Lactuca serriola leaves.

| Items | *Lactuca serriola* leaves level (g.kg$^{-1}$ diet) | | SEM | *p* value |
|---|---|---|---|---|
| | 0 | 20 | | |
| pH | 6.260 | 6.120 | 0.07 | 0.288 |
| Drip loss (%) | 1.471 | 1.261 | 0.13 | 0.352 |
| Cooking loss (%) | 17.302 | 16.619 | 0.91 | 0.503 |
| Shear force (kg) | 0.916 | 0.872 | 0.07 | 0.604 |
| Moisture (%) | 72.453 | 71.712 | 0.71 | 0.476 |
| Protein (%) | 20.539 | 21.110 | 0.59 | 0.516 |
| Fat (%) | 5.926 | 6.059 | 0.48 | 0.853 |
| Ash (%) | 1.081 | 1.117 | 0.05 | 0.651 |

Note: SEM = Standard Error of the Means.




**Figure 1** Lipid stability of breast meat obtained from growing Japanese quail fed dietary *Lactuca serriola* leaves supplementation.
Note: [a,b] Means with different letters are significantly different at $p < 0.05$.

**Table 4** Polyunsaturated fatty acids of breast meat of growing Japanese quail as affected by graded levels of *Lactuca serriola* leaves.

| Items | *Lactuca serriola* leaves level (g.kg$^{-1}$ diet) | | SEM | *p* value |
|---|---|---|---|---|
| | 0 | 20 | | |
| Total n-3 PUFA (%) | 0.629[b] | 0.874[b] | 0.03 | 0.019 |
| Total n-6 PUFA (%) | 14.882 | 15.341 | 0.96 | 0.308 |
| n6/n3 ratio | 24.031[a] | 18.457[b] | 2.11 | 0. 028 |

Note: [a,b] Means within the same row with different letters are significantly different at $p < 0.05$.
Total n-3 PUFA = Total omega-3 poly unsaturated fatty acids.
Total n-6 PUFA = Total omega-6 poly unsaturated fatty acids.
SEM = Standard Error of the Means.

## Lipid oxidation of quail breast meat

Lipid oxidation is the main evidence of the quality deterioration of meat by reducing shelf life during refrigeration storage **(Estévez, 2015; Sabow, 2020)**. Therefore, the shelf-life of fresh meat is limited to a few days during storage at refrigeration temperature unless preservation methods are used. Natural ingredients such as medicinal herbs and plants which have been used in the poultry, diet have been thought to provide beneficial compounds with antioxidant activity, which might be beneficial for shelf-life extension or meat quality maintenance **(Krishnan et al., 2014; Alagawany, Ashour and Reda, 2016; Aziz and Karboune, 2018)**. The results of the lipid stability of breast meat during the first five days postmortem are given in Figure 1. At 1st day postmortem, no significant difference was observed in lipid oxidation of breast meat between experimental and control diet. However, the MDA values of groups fed with *Lactuca serriola* leaves were significantly lower than those in the control, which indicated a good antioxidant capable of removing free radicals. Furthermore, the ability of *Lactuca serriola* leaves to reduce breast muscle lipid oxidation could be due to its total n-3 polyunsaturated fatty acids content as reported by **Sabow, Haddad and Nakyinsige (2020)** who attributed that lipid oxidation is significantly influenced by muscle n-3 polyunsaturated fatty acids and muscle with high n-3 polyunsaturated fatty acids gives lower malondialdehyde values than those with low n-3 polyunsaturated fatty acids. The current data are following those of **Ghazaghi Mehri and Bagherzadeh-Kasmani (2014)** who observed that dietary inclusion of medicinal herbs decreased the lipid oxidation of quail

meat. Regardless of dietary groups, the MDA concentration of all breast meat samples kept at refrigerator temperature for 5 days increased ($p$ <0.05) compared to that of refrigerated storage samples on day 1. These observations are consistent with those of **Sabow, Haddad and Nakyinsige (2020)** and **Partovi and Seifi (2018)** who observed that lipid oxidation values of quail breast meat significantly increased with storage time at refrigerator temperature.

## CONCLUSION

The results of the current study indicated that dietary supplementation of *Lactuca serriola* increased the omega-3 polyunsaturated fatty acids content and improved lipid stability during postmortem aging of growing Japanese quails. Further research is required to investigate the impact of dietary *Lactuca serriola* leaves on performance, some blood parameters, and morphology with microbial populations of the small intestine in quail (*Coturnix coturnix japonica*).

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

All birds received humane care according to the standard local guidelines. The experimental protocol was approved by the local animal care and use committee of the college of agriculture engineering and science, Salahaddin University-Erbil, Kurdistan Region, Iraq.

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# THE MICRONUTRIENT PROFILE OF MEDICINAL PLANT EXTRACTS

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## ABSTRACT

Medicinal plants contain biologically active substances that have a physiological effect on the human body. In the territory of Ukraine, 15 of the most important medicinal plants grow from a medical point of view, among which are *Melissa officinalis* L and *Calendula officinalis*. Micronutrients are necessary for the body in small quantities, not being a source of energy, they take part in their assimilation, as well as in the regulation of various functions and the implementation of the processes of growth and development of the human body. The study aims to establish the micronutrient profile of extracts and infusions from medicinal raw materials – *Calendula officinalis* and *Melissa officinalis*. The established micronutrient profile includes data on the content of such macro- and microelements as calcium, potassium, sodium, iron, zinc, and copper in extracts and infusions from *Melissa officinalis* L officinalis L and *Calendula officinalis*. Sodium predominates from certain macronutrients, the superiority of which is manifested in *Calendula officinalis* when infused. A large amount of calcium also passes into the aqueous-alcoholic infusion from *Calendula officinalis*. Copper and zinc prevail among the determined microelements in water extracts of *Calendula officinalis*. Comparing the results obtained, we can say in the affirmative about the micro- and macro elements that have passed into extracts that *Calendula officinalis* is richer in these substances. Because infusions and extracts are recommended to be added as an additional ingredient to vegetable and fruit juices, their positive infusion on the human body will increase the recommended daily requirement of potassium and sodium. Based on the results of this study, extracts and infusions of *Melissa officinalis* L and *Calendula officinalis* can be considered as an essential source of micronutrients for enriching fruit and vegetable juices in canned food for health purposes.

**Keywords:** micronutrients; biologically active substances; extract; *Calendula officinalis; Melissa officinalis* L

## INTRODUCTION

The body is not able to synthesize trace elements and makes up for their deficiency only from food, therefore trace elements are irreplaceable, and their absence or excess causes the development of many diseases **(Ebid, 2015)**. For thousands of years, people have received mineral elements only with food in an organically bound form. The entire system of digestion, active transport, and assimilation of our body is focused precisely on the consumption of organically bound macro- and microelements. This system not only controls the assimilation process, depending on the needs of the body at a given moment but also has a mechanism for the accumulation of vital organically "connected elements in special" cellular depots **(Liaqat et al., 2016)**.

Unfortunately, our system of consumption of elements is not at all adapted to the assimilation of inorganic compounds and salts of various elements that enter our body with water, food, air as a product of technogenic pollution **(Mushtruk et al., 2020a)**. For the same reason, due to the inadequacy of our assimilation system and the absence of special mechanisms for assimilation, transport, and accumulation, in most cases inorganic forms of macro- and microelement compounds have an extremely low toxicity threshold and, at the slightest excess of the dosage, cause severe intoxication of all vital organs and systems. That is why, for example, preparations of inorganic iodine and selenium are prescribed only under medical supervision **(Park and Kim, 2016)**.

Preparations containing inorganic microelements are prescription pharmaceuticals and are taken only under medical supervision. Extracts and infusions of biologically active substances from medicinal raw materials, compensate for the deficiency of macro- and microelements, contain, as a rule, only organically bound elements, are not able to harm the body even with thoughtless and incorrect use **(Waheed et al., 2018)**.

The peculiarity of extracts and infusions from medicinal plants is that their biologically active substances are in a certain ratio, contributing to the optimal effect on the human body. Some components of plant extracts are similar in chemical structure to physiologically active substances of the body (hormones, vitamins, enzymes, etc.). Therefore, such natural medicines are more actively involved in the biochemical processes of the human body together with juices **(Sheiko et al., 2019)**.

The need to fortify food with micro- and microelements is dictated by objective changes in the lifestyle, set, and nutritional value of the food used in food **(Mushtruk et al., 2020b)**. To provide the human body with the necessary micronutrients, food should be varied, and products are rich in biologically active substances, therefore, the addition of extracts and infusions to any juice will allow the use of beverages in food that significantly restore human health and reduce various diseases.

## Scientific hypothesis

Is to determine some micro- and macroelements in medicinal plants, data for which are not available in the literature, to create tables of the chemical composition of medicinal plants. There is an assumption that *Calendula officinalis* is richer in substances that were determined by tests, due to their intense coloration. Studies of the number of micronutrients of extracts and infusions from *Melissa officinalis* L and *Calendula officinalis* should be carried out using flame photometry and spectrometry methods for their exact amounts. Surely the transition of micronutrients into infusions and extracts will also be different due to the different

## MATERIAL AND METHODOLOGY

For research, we took medicinal raw materials – *Calendula officinalis* and *Melissa officinalis L*. Calendula flowers and melissa leaves were used for research. According to the manufacturer, the plants were collected at the initial stage of flowering. Dried and ready for research raw materials were purchased in a pharmacy network. For experimental studies, the raw material was ground on a laboratory crusher. Then added aqueous and aqueous-alcoholic solutions at hydromodule 1, ie the same amount of raw material and extractant. Extraction of micronutrients or their infusion was carried out according to the methods presented in the research results.

**Chemicals**

Distilled water ($H_2O$) for extraction, it was pre-purified in an aqueous distiller. The electrical conductivity of manufactured distilled water meets the national standard of laboratory distilled water of the first level and can be used in medicine and everyday life.

Ethanol ($C_2H_5OH$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Sodium chloride (NaCl, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Potassium chloride (KCl, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Calcium carbonate ($CaCO_3$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

The above anhydrous salts were used to make reference solutions and to construct calibration graphs of macronutrient content.

**Animals and Biological Material**

Medicinal raw materials (*Melissa officinalis* L and *Calendula officinalis*) for research (provided by Natalia farm, Khmelnytsky region, Shepetivka district).

**Instruments**

Automated refractometer (Anton Paar, producer «Inter-Synthesis» Limited Liability Company, Ukraine). By which the dry matter content was determined.

The thermometer is laboratory (TLS-200, producer «Inter-Synthesis» Limited Liability Company, Ukraine).

Surfactometer (Saturn-4, producer «Inter-Synthesis» Limited Liability Company, Ukraine). By which the content of trace elements was determined.

Flame photometer (BWB Technologies, producer «Inter-Synthesis» Limited Liability Company, Ukraine).

**Laboratory Methods**

Determination of dry matter content was carried out according to **ISO 2173:2003 (2003)**. The method of flame photometry is based on the construction of a calibration graph. It is used when the composition of the sample and the approximate amount of interfering ions are known. This makes it possible to bring the composition of the reference solutions as close as possible to the composition of the determined samples. Quantitative determination of elements by flame, photometry is possible only in the presence of reference solutions.

For the determination of macroelements, the flame photometry method was used, which is an arbitration method for the determination of metals in food products.

Emission photometry is based on the method of constructing a calibration graph. It is used when the composition of the sample is known, and the approximate amount of ion interferes. This makes it possible to maximally approximate the composition of the standard solutions to the composition of the samples to be determined. Standard solutions were prepared from standard solutions by dilution. Water was used as an extract, and a 20% aqueous-alcoholic solution was used for infusion.

Reference solutions are solutions (samples) in which the content of the element to be determined is known with sufficient accuracy. Reference solutions were prepared from standard solutions by diluting them. The starting materials for the preparation of reference solutions are double-distilled water and anhydrous chemically pure salts of the brand "HC" or NaCl, KCl, and $CaCO_3$. The calculated amount of salt was weighed on an analytical balance to the nearest 0.0001 g and dissolved in a volumetric flask. For each solution (both reference and determined) took 5 readings of the device and found the average value.

Calibration graphs were constructed in the coordinates:

$$I = f(C) \quad (1)$$

where: I is the current on the microammeter (instrument readings), μA;

C is the concentration of the solution, $\mu g.mL^{-1}$.

The number of the element in the sample was determined according to the schedule. Determination of trace elements using the atomic absorption method was carried out according to **GOST 30178 (1996)** (Table 1).

**Description of the Experiment**

Sample preparation: Two samples of medicinal raw materials were used for research – *Melissa officinalis* L and

*Calendula officinalis*, which were purchased at the pharmacy.

Number of samples analyzed: During the experimental research, 20 different samples (*Melissa officinalis* L and *Calendula officinalis*) of one manufacturer purchased in different pharmacy chains were examined.

Number of repeated analyses: All measurements of instrument, readings were performed 5 times.

Number of experiment replication: The number of repetitions of each experiment to determine one value was also 5 times.

### Statistical 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 2016 values were estimated using mean and standard deviations and subsequently evaluated in the statistical program XL Stat. In hypothesis testing, if the *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 alternativehypothesis was confirmed.

## RESULTS AND DISCUSSION

The established micronutrient profile includes data on the content of such macro- and microelements as calcium, potassium, sodium, iron, zinc, and copper in extracts and infusions from *Melissa officinalis* L and *Calendula officinalis*. Sodium predominates from certain macronutrients, the superiority of which is manifested in *Calendula officinalis* when infused. A large amount of calcium also passes into the aqueous-alcoholic infusion from *Calendula officinalis*. Copper and zinc prevail among the determined microelements in water extracts of *Calendula officinalis*. The following scientists were engaged in similar researches in works **(Spiegler, Liebau and Hensel, 2017; Teanpaisan et al., 2017)** investigated the process of extraction of *Matricaria recutita*, and in works **(Tiwary et al., 2017; Veni, Pushpanathan and Mohanraj, 2017**) similar process of *Plantago lanceolata* was investigated.

Comparing the results obtained, we can say in the affirmative about the micro- and macro elements that have passed into extracts that *Calendula officinalis* is richer in these substances. Because infusions and extracts are recommended to be added as an additional ingredient to vegetable and fruit juices, their positive infusion on the human body will increase the recommended daily requirement for potassium and sodium by 4 – 5%, calcium by 2 – 3%, iron by 15 – 17%, zinc, and copper up to 20% with a portion of the drink in the amount of 0.00025 $m^3$.

Before starting the experiments, the raw material was preliminarily crushed for better transfer of the raw material substances into the extract or infusion. When using water, the crushed raw material was subjected to an increase in temperature from 20 – 70 ºC, determining the dry matter content every 10 minutes (Figure 1). In scientific works **(Bazarnova et al., 2017; Bezerra et al., 2018)**, temperature regimes above 70 ºC were used, and the dry matter content was recognized after the completion of the process, which in our opinion can lead to mixing of dry matter content. After 40 minutes from the beginning of the experiments, the dry matter content did not increase, which indicates swollen raw materials. In this case, the hydronic modulus was 1. Therefore, it was decided to carry out the extraction process by maintaining the mixture for some time and at a certain temperature. These two factors will be the main ones in the process of the conducted research.

For the transfer of extractives from medicinal raw materials to the extract, the temperature range was taken from 40 – 60 ºC. The authors of scientific works **(Konovalenko and Polovko, 2019; Florea et al., 2020)** took the temperature range from 30 – 40 ºC, applying the settling process for 24 hours, which in our opinion can lead to mixing of dry matter content.

As seen in Figure 2 at a temperature of 60 ºC and keeping such an extract at 60 minutes, the value of the dry matter content is of the greatest importance, and the continuation of experiments does not make sense. The authors of scientific works **(Makasana et al., 2017; Ranjith, 2018; Mamadalieva et al., 2019; D'Auria, Mecca and Todaro, 2020)** performed similar experiments at a temperature of 75 ºC and for 2 hours but the maximum dry matter yield is in the range of 6.5 – 7.2%.

For another extractant of a water-alcohol solution, thanks to which fat-soluble substances can be extracted, a concentration of 20 wt % was taken. When using a water-alcohol solution, the temperature of the mixture was not increased, as it was done for water, since alcohol is a volatile substance. Measurements were carried out every 30 minutes and the dry matter content was determined over 2.5 hours. Based on the research results, curves were constructed for both medicinal plants (Figure 3). The authors of the following scientific papers **(Ulusoy, Acıdereli and Tutar, 2017; Chronopoulou et al., 2019)** investigated the extraction of fat-soluble substances at different concentrations of water-alcohol solution using the following ratios of water: alcohol 1: 3, 1: 6 and 1: 9. **Melfi et al. (2018)** and **Rathi et al. (2017)** investigated the extraction of fat-soluble substances using liquid and gas, such a process and its technological aspects will be covered in future research.

It is visible on the graph the transition of dry substances into infusions, and for *Calendula officinalis*, it took 2 hours for the dry matter content to increase to 9%, and for *Melissa officinalis* L – 1.5 hours, but the maximum amount of dry matter was 8.8%. Therefore, the best time regimen for infusion in a water-alcohol solution with a concentration of 20% wt. at hydro module 1 and a process temperature of 60 ºC for *Melissa officinalis* L it is 1.5 hours, and for *Calendula officinalis* – 2 hours. But the authors of scientific works **(Gullón et al., 2017; Al-Snafi, 2020; Jadhav et al., 2020; Zhang et al., 2020)** argue that the best time regimen for infusion in a water-alcohol solution with a concentration in the range of 5 – 15% wt. with a hydro module of 0.2 – 0.75 and a process temperature of 45 – 55 ºC for *Melissa officinalis* L and *Calendula officinalis* is 1 – 3 hours but the maximum dry matter yield is in the range of 4.5 – 6.2%.

**Table 1** Conditions for atomic absorption determination of elements.

| Element | Resonance line length, nm | Spectral slit width, nm | Concentration of solutions for graduation, μg.mL$^{-1}$ |
|---|---|---|---|
| Cu (II) (copper) | 324.7 | 0.1 | 0.5 – 4.0 |
| Zn (II) (zinc) | 213.9 | 0.5 | 0.1 – 2.0 |
| Fe (III) (iron) | 248.3 | 0.1 | 1 – 10 |

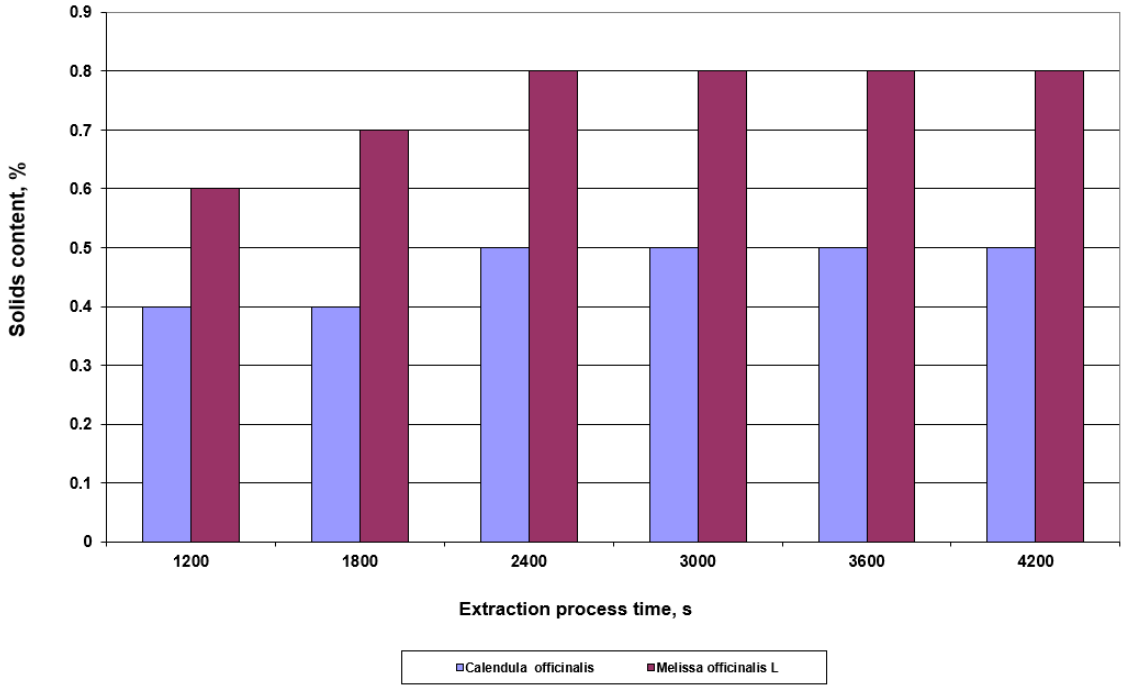


**Figure 1** Change in the number of dry substances in medicinal raw materials with an increase in the duration of extraction.

**Table 2** The content of macronutrients in extrtacts and infusions, μg.mL$^{-1}$.

| Element | Water extract | | Water-alcohol infusion | |
|---|---|---|---|---|
| | *Calendula officinalis* | *Melissa officinalis L* | *Calendula officinalis* | *Melissa officinalis L* |
| K (potassium) | 1.5 ±0.01 | 1.27 ±0.01 | 1.2 ±0.01 | 1.05 ±0.01 |
| Ca (calcium) | 20 ±0.1 | 16 ±0.05 | 36 ±0.15 | 23.5 ±0.1 |
| Na (sodium) | 280 ±5 | 148 ±3 | 275 ±3 | 12 ±1 |

**Table 3** Content of trace elements in extracts and infusions, μg.mL$^{-1}$.

| Trace element | Water extract | | Water-alcohol infusion | |
|---|---|---|---|---|
| | *Calendula officinalis* | *Melissa officinalis L* | *Calendula officinalis* | *Melissa officinalis L* |
| Zn (zinc) | 1.03 ±0.01 | 0.3 ±0.01 | 0.48 ±0.01 | 0.23 ±0.01 |
| Cu (copper) | 0.73 ±0.01 | 0.18 ±0.01 | 0.6 ±0.01 | 0.2 ±0.01 |
| Fe(III) (iron) | 3.8 ±0.015 | 0.32 ±0.015 | 0.2±0.01 | 0.03 ±0.015 |

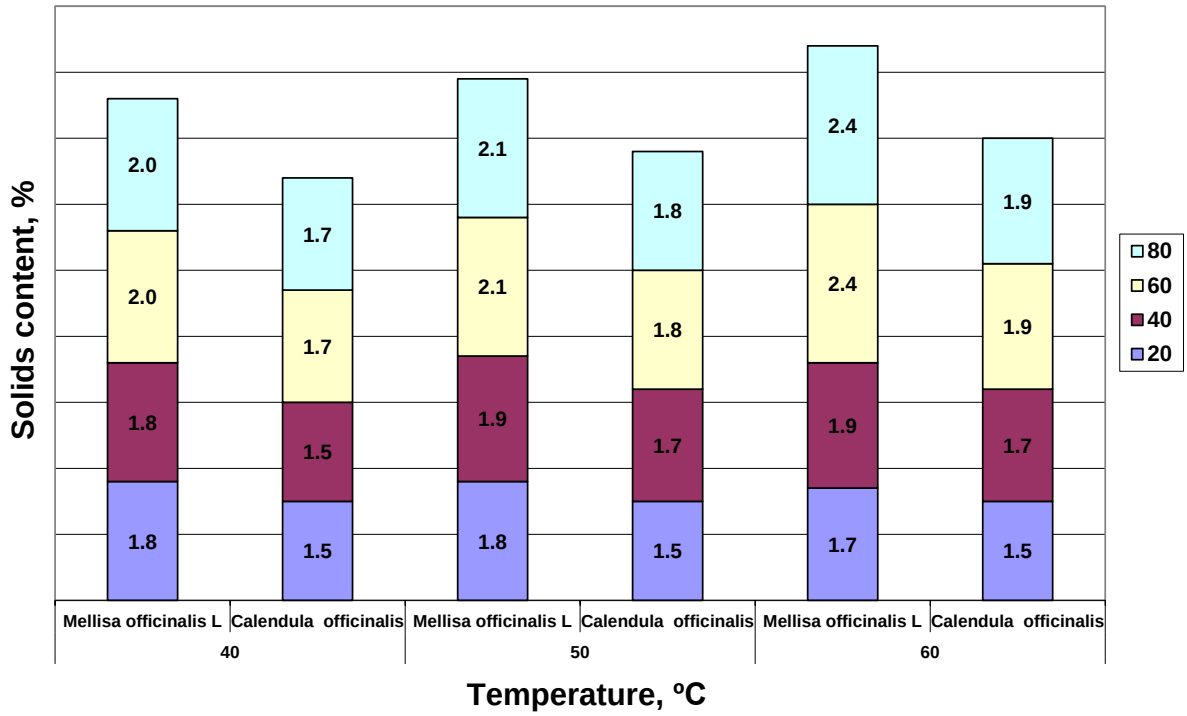


**Figure 2** Change in the content of dry substances in medicinal raw materials with an increase in the duration of time at different temperatures.

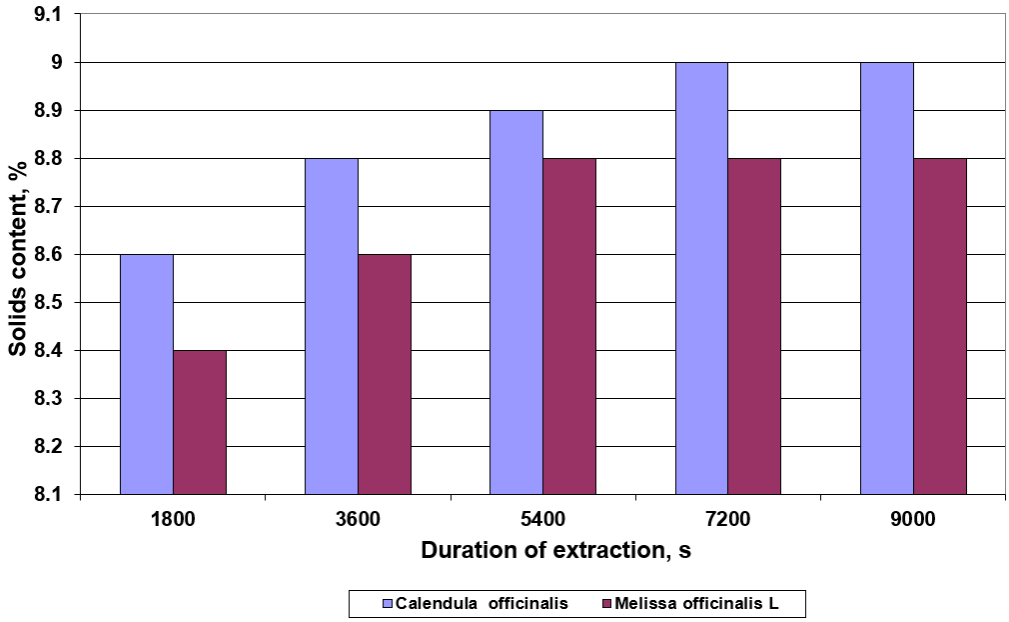


**Figure 3** Influence of the duration of infusion of medicinal raw materials in a water-alcohol solution on the dry matter content.

As shown by the results of our research (Table 2), at optimal parameters of extraction and infusion, the content of such macroelements as potassium, calcium, and sodium in aqueous and aqueous-alcoholic solutions slightly fluctuates, but the order remains the same. Except for the amount of sodium in *Melissa officinalis* L, more than 12 times more of this element passes into the aqueous extract, while calcium, on the contrary, is less for both *Melissa officinalis* L and *Calendula officinalis*.

Data analysis Table 3 indicates a higher content of the transferred zinc, copper, and iron in the aqueous extract than their amount in the aqueous-alcoholic solution. Especially noteworthy is a large amount of iron, which, after extraction, passes into an aqueous solution (19 times more). Considering that the necessary dose of all these substances is greater for the normal life of the human body, we can confidently talk about the benefits of using such extracts from *Melissa officinalis* L and *Calendula officinalis*, which will have a beneficial effect on the development and growth of the human body.

These data are consistent with the results of studies of the composition of macro- and microelements for medicinal raw materials and, in particular, lie within the data obtained at different extraction temperatures **(Gupta et al., 2018;**

**Bober et al., 2020)**. Among the micronutrients, the amounts of ascorbic acid were also determined, which passed into extracts and infusions from *Calendula officinalis* and *Melissa officinalis L*. Based on the results obtained, it can be argued that the maximum amount of this substance passes into the aqueous extract of *Melissa officinalis*, which corresponds to 6.2 mg.100g$^{-1}$ after 1 hour of extraction. An increase in the amount of ascorbic acid in an aqueous-alcoholic infusion of *Melissa officinalis* L up to 4.2 mg.100g$^{-1}$ is also less important for 1 hour of infusion, but this amount is more than for the extract and infusion from *Calendula officinalis* by 56 and 55%, respectively. The extraction time of ascorbic acid from *Calendula officinalis* was 1 hour, and the infusion time was 2 hours.

The composition of micronutrients in extracts and infusions from medicinal raw materials corresponds to the standards of consumption for adults recommended by the Food and Agriculture Organization of the United Nations and the World Health Organization **(Zheplinska et al., 2020)**. The information provided in the micronutrient profile can be used for non-commercial communications and cannot be used for other purposes, including product labeling. The data obtained can be useful for creating tables of the chemical composition of extracts and infusions of such medicinal raw materials as *Calendula officinalis* and *Melissa officinalis L*. There is a need for further scientific research on this topic to determine other biologically active substances and generalize the results on the amount of these substances in extracts and infusions from medicinal raw materials. The relevance and novelty of the research are confirmed by obtaining patents of Ukraine for a useful model for vegetable drinks containing extracts and infusions from these types of medicinal raw materials **(Palamarchuk et al., 2019; Zheplinska et al., 2019)**. Because infusions and extracts are recommended to be added as an additional ingredient to vegetable and fruit juices, their positive infusion on the human body will increase the recommended daily requirement of potassium and sodium by 4 – 5%, calcium by 2 – 3%, iron by 15 – 17%, zinc, and copper up to 20% with a portion of the drink in the amount of 0.00025 m$^3$.

## CONCLUSION

The amounts of micronutrients of extracts and infusions from *Melissa officinalis* L and *Calendula officinalis* were investigated using flame photometry and spectrometry methods. The established micronutrient profile includes data on the content of such macro- and microelements as calcium, potassium, sodium, iron, zinc, and copper in extracts and infusions from *Melissa officinalis* L and *Calendula officinalis*. Sodium predominates from certain macronutrients, the superiority of which is manifested in *Calendula officinalis* when infused. A large amount of calcium also passes into the aqueous-alcoholic infusion from *Calendula officinalis*. Copper and zinc prevail among the determined microelements in water extracts of *Calendula officinalis*. Comparing the results obtained, we can say in the affirmative about the micro- and macro elements that have passed into extracts that *Calendula officinalis* is richer in these substances.

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# INCREASING THE RETURN ON EQUITY BY FINANCIAL LEVERAGE IN SUGAR BEET FARMS IN SLOVAKIA

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## ABSTRACT

The paper evaluates and analyses selected financial ratios of a set of farms in Slovakia in 2017. The farms are divided into two groups (sugar beet producers and sugar beet non-producers). The data used for the analysis were obtained from the National Bank of Slovakia (NBS) over the period 2009 – 2017 and the database of the Ministry of Agriculture and Rural Development of the Slovak Republic (MARD SR). Farmers generally achieve a low level of return on equity. In the paper, we analyze the development of interest rates and measure the potential effect of financial leverage, which can increase the profitability (return on equity) of the farms. Moreover, we test the statistically significant difference of selected ratios between the group of producers and non-producers of sugar beet in Slovakia. In 2017, the return on equity of sugar beet producers in Slovakia reached lower results in comparison to sugar beet non-producers. The differences in return on equity are explained by the lower level of equity per ha and thus higher indebtedness of non-producers of sugar beet.

**Keywords:** farm; sugar beet; profitability; indebtedness; financial leverage

## INTRODUCTION

Before September 30, 2017, there existed quotas for sugar beet production in the EU member states. They represented restrictions for sugar beet production in individual EU member states (MS) and reduced competition between the countries. Nowadays, neither sugar beet producers nor sugar beet growers are constrained by quotas. It increases the risk of a potential bankruptcy of sugar companies in less competitive areas, and continually affects the cultivation of sugar beet, which has not been a marketable commodity for a long–term period **(Tóth and Pokrivčák, 2019)**. Sugar prices have been developing very specifically and dynamically in recent years. In the EU member states, due to the deregulation of the sugar market, the production increased approximately by 25% between 2016/2017 and 2017/2018. This situation, combined with the increase in isoglucose production, growing imports from the ACP countries, and changes in consumer preferences, puts significant pressure on the price, which stays at the low level **(Řezbová et al., 2013)**.

To sustain competitiveness, it is necessary to look for effective ways to increase the profitability of the sugar beet business, not only to produce it. One option is to optimize the capital structure of a company, to achieve an optimal ratio between the financial sources of a company, to increase the profitability of a business. Moreover, it increases the market value of a company.

In the paper, the basic financial ratios (profitability ratios, debt ratios) of sugar beet producers in Slovakia are assessed and compared to other businesses (sugar beet non-producers). Moreover, the practical application of decision – making process whether to increase the indebtedness of the company is assessed. The outcome of managers´ decisions may have a positive impact on the return on equity. The increase in the return on equity and the growth of the market value of a company is the primary goal of each business in the market economy.

In the theory, there are several approaches how to optimize the financial structure of a company. We consider the authors **Modigliani and Miller (1958)** to be pioneers in capital structure optimization. In the Compromise theory **(Brealey and Myers, 1992)**, the authors combined the M – M model with real market conditions and defined the optimal indebtedness as a compromise between the interest tax shield and the cost of financial distress. Other theories of optimal capital structure are the Theory of hierarchical order of authors **Robischek and Myers (1966)** or the Signalling model from **Ross (1977)**. The traditional approach, the Theory of the U Curve, is also popular; according to **Bartošová (2005)**, it accepts the validity of individual differences between companies in the level of indebtedness and claims, that the companies should have as much debt as they can handle themselves.

The theory of the U – curve, in comparison to the classical approach, admits that the growth of a company's indebtedness increases the risk of creditors, who compensate it by raising interest rates. Shareholders also compensate for the increase in risk by requiring a higher return on their capital. Therefore, a company can borrow at the normal interest rate until creditors start to consider the

debt ratio as an indicator of increasing their risk. Subsequently, the weighted average costs of capital (WACC) start to increase as creditors provide additional loans only at a higher interest rate. Shareholders will also demand a higher return on invested capital due to growing debt risk. It means that till a certain moment, the total cost of capital decreases as the indebtedness increases, and then it begins to increase, which graphically reminds the shape of the "U" curve. The optimal capital structure is the point, where the cost of capital WACC reaches the minimum value. At this point, the highest market value of the company is achieved, too **(Tóth et al., 2019)**. However, the theory deals with the problem of determining the cost of equity. Similarly, the other theories face several different problems in practical application.

An interesting approach to capital structure optimization is the theory of authors **Neumaierová and Neumaier (1996)**. The advantage of their approach is the ability to replace the data on the cost of equity with the data from a company´s financial statements. The main objective is to provide managers with a model on how to decide about the appropriate level of indebtedness in a company.

However, it is important to realize that the negative aspect of increased debts in a company is the increased financial risk, and consequently, the threat of financial distress, which may result in the bankruptcy of the company **(Hrdý and Krechovská, 2013)**.

**Practical implications**

The increased share of debt can have a positive effect on the return on equity. However, on the other hand, the increased share of borrowings is connected with increased interests. Interests reduce the share of profits for the investors and cause the decrease of the interest burden ratio and thus cause the decrease of return on equity (ROE).

In the literature, Slovak authors sometimes use the ratio ROI (return on investments) for expressing the return on total capital. However, in foreign literature, it is rather used for measuring the profitability of a particular investment. Therefore, it is preferable to use the abbreviation BEP (basic earning power), since this indicator represents the ratio of EBIT to total capital or assets (total assets = total capital) **(Tóth et al., 2019)**. The indicator is suitable especially in spatial comparison of companies, but also for comparison over time. The ratio does not take into account different taxation and different capital structure of companies.

The problem of the capital structure of agricultural companies in Slovakia, as well as the effect of financial leverage in agricultural financing, has been analyzed in various scientific papers. The main findings have been that only a few companies could increase their indebtedness, mainly due to the low level of return on total capital **(Čierna and Rábek, 2012)**. Conversely, companies that achieved higher values of the ROE, have been able to take the advantage of the favorable leverage effect **(Rábek et al., 2013a)**. The period since 2008 has been characterized by a decline in interest rates in Slovakia, driven by a general decline in central bank interest rates in the EU. The reduction of costs of debt increases its attractiveness and leads to an increase in the share of debt in financing the needs of a company **(Rábek et al., 2013b)**. The return on equity of agricultural companies has been generally at a low level. In almost half of the agricultural companies, the level of ROE did not exceed 3%. However, there were 25% of companies on the market with a return on equity of over 10% **(Rábek et al., 2018)**.

## Scientific hypothesis

Sugar beet is one of the most efficient crops from the perspective of production and energy potential **(Janíček, 2019; Holúbek, 2011)**. Sugar beet farms achieve higher average ROA (return on assets), but the result has not been statistically proved in each year of authors´ analysis **(Tóth et al., 2017)**.

However, sugar beet producers can be expected to achieve higher ROE than non-producers. Sugar beet cultivation is technically demanding and requires initial financial investment. As stated before, debt is considered a cheaper source of financing. Therefore, it is assumed that sugar beet producers will use more borrowed capital and will be more indebted.

In the paper, we test the following hypotheses:

H0: There is no statistically significant difference in the return on equity between the group of sugar beet producers and the group of sugar beet non-producers.

H1: There is a statistically significant difference in the return on equity between the group of sugar beet producers and the group of sugar beet non-producers.

H0: There is no statistically significant difference in indebtedness between the group of sugar beet producers and the group of sugar beet non-producers.

H1: There is a statistically significant difference in the indebtedness between the group of sugar beet producers and the group of sugar beet non-producers.

## MATERIAL AND METHODOLOGY

To assess the level of return on equity and indebtedness of agricultural companies in Slovakia in 2017, individual data about sugar beet producers were used. Data for evaluation of selected ratios were obtained from information letters of MARD SR (Ministry of Agricultural and Rural Development of the Slovak Republic). Agricultural companies were classified into 2 groups, sugar beet producers and non-producers. The company was considered as a producer of sugar beet if the ratio of the sugar beet harvesting area to the total agricultural area LPIS (Land Parcel Identification System) was non – zero. Authors **Tóth et al. (2019)** used a similar classification criterion based on the share of sugar beet harvesting area to the LPIS area in their analysis, classifying farms into 4 groups.

Companies with a negative value of equity were excluded from the analysis. The final data set consisted of 1417 agricultural companies in the primary sector in 2017. The companies were legal entities operating on 1.298 thous. hectares (ha) according to LPIS. From the overall data set, 132 companies belonged to the sugar beet producers with a sugar beet harvesting area of 18.5 thous. ha out of the total area 215.785 thous. ha. The sugar beet production thus covered 8.6 % of the harvesting area on average.

The parallel, matrix, and pyramidal systems of financial and economic ratios are used not only in business practices but also in scientific research, as confirmed by several scientific studies. One of the most widely used decompositions of ratios is the Du Pont's decomposition of return.



**Figure 1** Sugar beet fields in Slovakia. Source: **Pol'noinfo (2021)**.



**Figure 2** Sugar beet cultivation in Slovakia. Source: **Pol'noinfo (2021)**.



**Figure 3** Collecting sugar beet yield on the field. Source: **Pol'noinfo (2021)**.

Du Pont's return decomposition provides two theoretical variants, the decomposition of return on assets (ROA) and the decomposition of return on equity (ROE). In company practice, the exclusive use of equity capital is relatively rare. External sources in the capital structure affect the final performance of the company and cause the distinguished ROA and ROE values. The share of debt on the total capital is important, determining the intensity of leverage. Therefore, the basic Du Pont equation is further expanded, allowing the second form of decomposition **(Kotulič et al., 2010)**:

$$ROE = \frac{EAT}{Equity} * \frac{Assets}{Assets} = \frac{EAT}{Assets} * \frac{Assets}{Equity} \quad (1)$$

Where:
EAT – earnings after taxes; EAT/Asset – return on assets; Assets/Equity – leverage.

The equation for return on equity can be transformed using different categories of profit – earnings before interest and taxes (EBIT), earnings before taxes (EBT), and earnings after taxes (EAT). The following calculation can be expressed as **(Holečková and Grunwald, 2001):**

$$ROE = \frac{EAT}{Equity} * \frac{Assets}{Assets} * \frac{EBIT}{EBIT} * \frac{EBT}{EBT} = \frac{EBIT}{Assets} * \frac{EBT}{EBIT} * \frac{Assets}{Equity} * \frac{EAT}{EBT} \quad (2)$$

From that relationship, it follows that the return on equity may be broken down into:
EBIT/Assets – BEP ratio (basic earning power) or return on total capital; EBT/EBIT – interest burden ratio (interest rate reduction of profit), Assets/Equity – leverage, EAT/EBT – tax burden ratio (tax reduction of profit).

From these relations, it is clear that the ROE is affected by the return on total assets, taxation, and the level of debt in a company. The impact of indebtedness on the return on equity is expressed by the two above-mentioned factors: interest rate reduction of profits and financial leverage, which are acting in a contradictory way. The ratios used in the analysis are the following:

$$Return\ on\ equity\ (ROE) = \frac{EAT}{Equity} * 100 \quad (3)$$

$$EAT\ on\ ha\ of\ area\ LPIS = \frac{EAT}{Agricultural\ area\ in\ ha} \quad (4)$$

$$Equity\ on\ ha\ of\ area\ LPIS = \frac{Equity}{Agricultural\ area\ in\ ha} \quad (5)$$

$$Indebtedness = \frac{Total\ debt\ (including\ accruals)}{Total\ capital} * 100 \quad (6)$$

$$Financial\ leverage = \frac{Total\ capital}{Equity} \quad (7)$$

$$BEP = \frac{EBIT}{Total\ assets} * 100 \quad (8)$$

To be able to evaluate the companies, the basic descriptive statistics are used:

- lower quartile – includes the lower 25% ascending values of the statistical character,
- median – the mean value, includes the lower 50% ascending values of the statistical character,
- upper quartile – includes the upper 25% of ascending values of the statistical character.

In the case of examining 1000 companies, if the value of ROE in a given year reached 5% in the lower quartile, 25% of the companies with the lowest results would reach ROE up to 5%. The remaining 750 enterprises reached ROE more than 5%. Analogically, it can be applied to the median and the upper quartile. The distribution of the results into quartiles was similarly used in the comparison of financial ratios of Slovak sugar factories **(Lukáč, 2020)**.

For evaluation of whether the company can increase its profitability by increasing the level of debt, we use the Neumaiers model. The model is based on the decomposition of the ROA, with the EBIT in the nominator and the total assets in the denominator. In the paper, we adjusted the terminology used by Neumaier to present the currently used abbreviations (e. g. profit replaced by EBT – earnings before taxes). Neumaier's expressed the ROE as a function of:

$$ROE = \frac{EAT}{Equity} = \frac{EAT}{EBT} * \frac{Assets}{Equity} * \left[\frac{EBIT}{Assets} - \frac{Interests}{\text{Borrowings}} * (1 - \frac{Equity}{Assets} - \frac{Debt}{Assets})\right] \quad (9)$$

$$\frac{EBIT}{Assets} > \frac{Interests}{\text{Borrowings}} * \left[1 - \frac{Debt}{Assets}\right] \quad (10)$$

In the comparison of the BEP ratio with the interest rate, the company paid for borrowed capital (debt), while the interest rate was reduced aliquotly. If the relationship is valid **(Kotulič et al., 2010)**, then the company can be in debt. If the sign is opposite, it is not worthy for the company to be indebted. If the sign is equal, then ROE does not depend on indebtedness. For the cost of debt in the analysis, the agreed average annual interest rate reported by the NBS in 2017 for total loans to non – financial corporations was used. Its value of 2.4070% was used as a constant, based on the Neumairs methodology.

## Statistical Analysis

We used the Statgraphics Centurion 19 software (produced by Statgraphics Technologies, Inc., Virginia).

In the first step, we tested the normality of data by the Shapiro-Wilk test and the goodness of fit test. The normality was tested in both groups (producers and non-producers of sugar beet). The Shapiro-Wilk test **(Shapiro and Wilk, 1965)** is based on comparing the quantiles of the fitted normal distribution to the quantiles of the data. The goodness of fit test **(Stephens, 1974)** is a special case of the Kolmogorov-Smirnov test for testing the normality of the distribution. Data are standardized and compared with the normal distribution. If the *p*-value of the Shapiro-Wilk test and the goodness of fit test is less than 0.05, we reject that data have the normal distribution with 95% confidence. The nonparametric test, the Mann – Whitney W test, is further used for testing hypotheses.

The Mann – Whitney W **(Mann and Whitney, 1947)** test is used to compare the medians of selected groups, the sugar beet producers, and the non-producers. This test is constructed by combining the two groups, sorting the data from smallest to largest, and comparing the average ranks of the two groups in the combined data. If the *p*-value is less than 0.05, there is a statistically significant difference between the medians at the 95.0% confidence level. The test is used for analyzing the statistically significant differences of ratios ROE and indebtedness between the selected groups.

## RESULTS AND DISCUSSION

Sugar beet is an important crop in Europe, with the production of around 120 million tones in 2018, representing almost 45% of the world's production **(FAOSTAT, 2019)**. Sugar beet is grown in our conditions only to obtain sucrose, while its cultivation contributes to the by-products of its processing (slices, sweetened cuttings, molasses) as well as to certain payments in feed balances. **(Bojňanská et al., 2011; Camara-Salim et al., 2021; Mujumdar, 2014)**. The year 2017/18 was the first year in the sugar sector after the abolition of quotas. This has been reflected in record-breaking results in the amount of sugar produced in the EU. The authors **Buysse et al. (2007)** dealt with farm-based modeling of the EU sugar reform. Simulation results from a sample of Belgian sugar beet farms show that the sugar CMO (common market organization) reform includes different supply and income effects across farms depending on their share of out-of-quota sugar beet relative to their total beet supply and their quota rent. In Slovakia, the abolition of quotas has not significantly affected sugar beet cultivation. According to the data of the Statistical Office (SO) of SR, the total sugar beet harvested area in 2017 was increased, but only by 4.2% (22.38 thous ha). With an average yield of 55.00 t per ha, sugar beet production achieved 1 230.79 tones, from which 179.5 thous. tons of sugar were produced. In 2017, the year–on–year price of sugar beet decreased by 9.5% to 24.61 EUR/t (including subsidies) **(Meravá, 2019)**.

Theoretical studies suggest that subsidies may have a positive impact on farm production and at the same time a negative impact on farm productivity **(Kozáková et al., 2014)**. Inefficiency in sugarbeet production results in a shortfall of potential revenues not only for producers but also for landlords and processors **(Wu et al., 2003)**. Support of business can bring along the risk of government failure in the form of misuse of such help by its beneficiaries for their enrichment **(Prochazkova et al., 2015)**.

Not only the production quotas but also the system of export licenses outside the European market has been terminated. The minimum price for sugar beet and the reference price for sugar has been abolished **(Zdráhal et al., 2020)**. In the analysis of the relationship between sugar prices according to the International Sugar Organization (ISO) and sugar prices in selected stock exchanges (USA, UK, India, Brazil, Mexico, China, Russia), the impact of the world price of sugar on prices in individual markets (except for Russia) has been pointed out **(Smutka et al., 2019)**.

The abolition of the sugar quota in 2017 constitutes a turning point for the sugar sector in the European Union. Domestic sugar prices are increasingly linked to world market prices, which used to be far below the EU´s in the past. Therefore, questions arise about the EU sugar sector´s response to the new market situation without quota and whether sugar beet farming will remain profitable in the future **(Wimmer and Sauer, 2020)**.

The price of sugar beet affects the profitability of its cultivation, as well as the overall profitability of a company. Business owners are primarily interested in the results of return on equity. The owners and investors usually demand the profit on deposited capital to be higher in comparison to other potential investment abilities (e.g. buying bonds, depositing money in financial institutions) **(Tóth et al., 2019; Klieštik and Valášková, 2013)**. The cost of capital is the minimum rate of recoverability which a company must generate to satisfy its creditors, owners, and other providers of capital because otherwise, they will invest elsewhere **(Pratt and Grabowski, 2010)**. In the case of loans, the company must be able to meet the requirements of debt providers by paying interest. According to the **Van Binsbergen et al. (2010)**, the cost of foreign capital is a complex variable representing the cost of debt capital, which is a tax-deductible expense, and it corresponds to the required yield of a creditor taking into account risks and costs of lost opportunities. Therefore, it is desirable for the owners that the return on total capital (BEP) is greater than the cost of debt (represented by the interest rate).

Models of quantification of capital costs and capital structure are solved by many authors **(Klieštik and Valášková, 2013; Grabowski, 2009; Porras, 2011; Vo, 2021)**.

**Kotulič et al. (2010)** explain the positive and negative leverage as follows:

- positive leverage – if the cost of debt (interest rate on invested debt sources) is lower than the return on total capital, ROE increases as the company's indebtedness increases.
- negative leverage – if the cost of debt (interest rate on invested debt sources) is higher than the return on total capital, with the company's increasing debt, the ROE decreases.

Although strong companies with prospects for prosperity are gradually emerging in the sugar industry, most economic and financial indicators have recorded average negative trends. The problems are caused by the high price of the raw material and the low possibility of selling sugar **(Suhr and Schulze, 2003)**.

### Return on equity (ROE)

Return on cost and return on equity are important factors from the financial and economic point of view according to **Kozáková et al. (2014)** and **Greer et al. (2008)**. The authors compared selected managerial and financial indicators between organic and conventional production in Slovakia. They state that according to the theory, organic farms are less profitable and therefore the product should be more subsidized when compared to conventional farms. Higher subsidies for organic farms successfully compensate lower revenues and therefore the motivation for the owner to focus on organic farming is not lower than to focus on conventional farming. In 2017, sugar beet producers in Slovakia achieved a lower return on equity (ROE) than non-producers of sugar beet (Table 1). The data were tested by the Shapiro-Wilk test **(Shapiro and Wilk, 1965)**, the goodness of fit test **(Stephens, 1974)**, and the Mann – Whitney W test **(Mann and Whitney, 1947)** in both selected groups. The results of P – values of the Shapiro – Wilk test and the goodness of fit test were lower than 0.05 in each selected group for the ratio ROE. As the normality of data was not approved with 95% confidence, we used the Mann – Whitney W test in further analysis. The *p*-value of the Mann – Whitney W – test is 0.00180413, which was lower than 0.05.

**Table 1** Return on equity of agricultural companies in 2017.

| Ratio | Companies | Count | Lower Quartile | Median | Upper Quartile |
|---|---|---|---|---|---|
| ROE | SB producers | 132 | -1.72% | 1.13% | 5.67% |
| ROE | SB non-producers | 1285 | -0.51% | 3.42% | 15.19% |

Note: SB – sugar beet. Source: Information letters of the MARD SR; own processing.

**Table 2** Values of EAT, equity in EUR per ha p.p. for and indebtedness in % in 2017.

| Ratio | Companies | Count | Lower Quartile | Median | Upper Quartile |
|---|---|---|---|---|---|
| EAT/ha | SB producers | 132 | -33.59 | 16.05 | 71.31 |
| | SB non-producers | 1285 | -2.34 | 24.60 | 86.81 |
| Equity/ha | SB producers | 132 | 819.32 | 1490.92 | 2155.60 |
| | SB non-producers | 1285 | 289.69 | 700.48 | 1471.86 |
| Indebtedness | SB producers | 132 | 23.85% | 44.97% | 67.24% |
| | SB non-producers | 1285 | 32.43% | 57.26% | 80.39% |

Note: SB – sugar beet. Source: Information letters of the MARD SR; own processing.

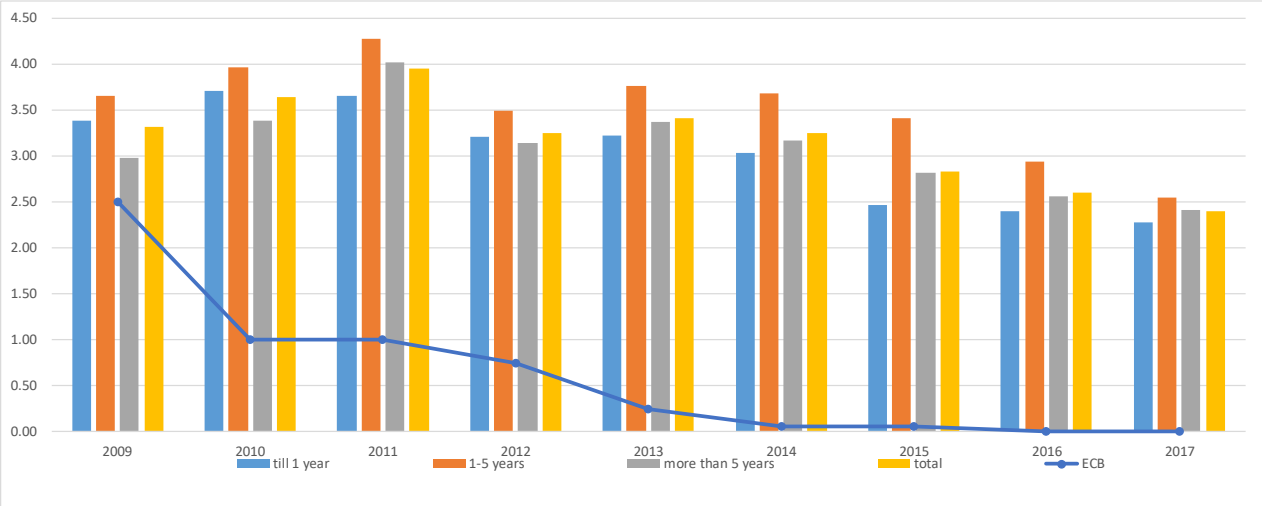


**Figure 4** Development of interest rates (%) on all types of loans in non-financial companies (December 31, 2020). Source: **NBS (2020)**, own processing.

It means there were statistically significant differences between the ratio ROE in our selected groups (sugar beet producers and non-producers) at the 95.0% confidence level. It means we rejected the H0 hypothesis. It can be explained by the lower profit per ha, but also by the lower indebtedness of sugar beet producers. In general, the profitability of all agricultural companies is low. The exception is 25% of companies in the upper quartile of non-producers of sugar beet. These companies achieve a three times higher return on equity than the sugar beet producers.

There are more reasons why sugar beet producers achieved lower ROE values:

1. The differences in ROE are not only due to the higher profit (EAT) per ha of non-producers of sugar beet. The median value (EAT) is 16.05 EUR/ha for sugar beet producers and 24.6 EUR/ha for non-producers of sugar beet. The reason is also the fact that sugar beet producers have a higher need for equity per hectare. In the median value, it is only 700 EUR/ha for non-producers of sugar beet and more than twice as much, 1490 EUR/ha, for sugar beet producers.
2. Sugar beet producers show lower values of the degree of indebtedness in all quartiles, which means they use more equity. The results of our analysis are presented in Table 2.

Each type of capital has a price that a company must pay for its use. For loans, the price is the interest rate. Interest is paid from earnings before taxes, in the case of a company´s bankruptcy, the creditors are paid off earlier than the owners. It causes a lower risk of debt, and therefore a lower required return **(Tóth et al., 2019)**. The advantage of financing the needs of a company through debt is the use of a cheaper type of business capital. Interest on borrowed capital is tax-deductible and the debt is less risky than equity, and therefore, it is considered to be cheaper.

The problem of capital taxation efficiency of agricultural business in the Slovak Republic was assessed by **Andrejovská et al. (2019)**. The authors state that to keep costs as low as possible, the companies try to optimize their capital structure. Corporate tax is the cost of equity financing, and this cost is often higher than other costs, such as in form of interests, which are a tax-deductible item, thereby causing a reduction in the tax base, so-called interest tax shield. A more complex overview of taxation-related aspects of dividends and their development is provided in **Krajčírová et. al (2019)**.

**Evolution of interest rates**

However, the growth of the agriculture sector and agricultural firms is highly constrained by their access to finance **(O'Toole et al., 2014)**.

**Table 3** Financial leverage and BEP in 2017.

| Ratio | Companies | Count | Lower Quartile | Median | Upper Quartile |
|---|---|---|---|---|---|
| Financial leverage | SB producers | 132 | 1.31 | 1.82 | 3.05 |
| | SB non-producers | 1285 | 1.48 | 2.34 | 5.10 |
| BEP | SB producers | 132 | -0.57% | 1.33% | 4.09% |
| | SB non-producers | 1285 | 0.32% | 2.42% | 6.49% |

Note: SB – sugar beet. Source: Information letters of the MARD SR; own processing.

Global trends in interest rates were also addressed by **Del Negro et al. (2019)** and **Borio et al. (2017)**.

The trend in the world's real interest rate fluctuated close to 2% for more than a century but has dropped significantly over the past three decades. This decline has been common among advanced economies, as trends in real interest rates across countries have converged over this period. It was driven by an increase in the convenience yield for liquidity and by lower global economic growth **(Del Negro et al., 2019)**.

The current trend of low-interest rates of the ECB has been reflected in lowered interest rates of commercial banks. Low interest rates may encourage companies to use a higher share of debt for business financing, and thus increase the return on equity through the financial leverage for a certain period. Interest rates for businesses have fallen significantly.

According to NBS data (Figure 4), since 2009, the interest rates on all types of loans have fallen. At the beginning of 2009, the European Central Bank (ECB) started lowering the rate of main refinancing operations from the original 2.5% (1.1.2009) to 0.00% till March 16, 2016, and nowadays (February 2021).

These measures of the ECB have been objected to supporting the growth of the Euro Area economy while avoiding a fall into a deflationary spiral, as inflation oscillated around zero **(Rábek et al., 2013b)**. Low interest rates should motivate businesses, but also the population, to take out more loans. In the period from 2009 to 2017, the interest rates decreased, with the lowest rates of commercial banks for loans maturing within 1 year (Figure 4). The average interest rate on loans in 2017 was 2.4070% and we considered it in our calculations.

**Lin and He (2020)** investigated the effect of a targeted easing (TE) monetary policy in boosting lending to the agriculture sector. Using longitudinal data of listed Chinese firms between 2012 and 2017, they documented that the TE implementation is effective in increasing loan levels of agriculture businesses relative to non-agricultural firms.

**Financial leverage**

The problem of the different impact of the leverage effect on the default risk of small and large firms was solved by **Cathcart et al. (2020)**.

Companies can increase the return on equity by increasing the debt ratio (indebtedness), which means using financial leverage. For proper use, the condition that the return on total capital (BEP) is greater than the cost of borrowed capital (debt) has to be met. The results of the analysis of financial leverage effect on agricultural companies are presented in Table 3.

In 2017, 25% of sugar beet producers with the lowest value of financial leverage (lower quartile) reached a value of up to 1.31, and non-producers of sugar beet of up to 1.48. The median values for sugar beet producers are at 1.82, and non-producers at 2.34 level.

However, 25% of sugar beet non-producers achieved a result of financial leverage of up to 5.10, compared to 3.05 in the case of sugar beet producers. These companies can better use the effect of debt financing. The result also referred to the higher level of indebtedness of sugar beet non-producers´ companies (see Table 2). The data were tested by the Shapiro-Wilk test, the goodness of fit test, and the Mann – Whitney W test in both selected groups. The result of P – values of the Shapiro-Wilk test and the goodness of fit test was lower than 0.05 in each selected group, with 95% confidence. Therefore, we rejected the assumption that data of ratio indebtedness had a normal distribution with 95% confidence. As the distribution was not normal, we used the Mann – Whitney W test for testing hypotheses. The *p*-value of the Mann – Whitney W test for the ratio of indebtedness is 0.000564923, which means less than 0.05. There was a statistically significant difference between the ratio of Indebtedness in our two groups (sugar beet producers and non-producers) at the 95.0% confidence level. It means we rejected the H0 hypothesis. To use the leverage effect, it is necessary to assess the level of return on total capital. Similarly, as in the case of ROE, sugar beet non-producers achieved higher values of BEP ratios in all quartiles (Table 3).

An increase in the return on equity can be reached if the return on total capital is higher than the cost of debt. We considered the cost of debt to be the average interest rate in 2017, 2.4070%. According to the Neumair model, we multiplied it with [1-(liabilities/assets)].

In 2017, 60 companies from the group of sugar beet producers met the requirement, which represents 53.1% of companies. In the group of non-producers of sugar beet, 788 companies met the requirement, which represents 63.3% of companies. The companies which meet the requirement may increase the share of debt in total capital and would increase their return on equity. **Beck and Demirguc-Kunt (2008)** found that small firms used less external finance, especially bank loans when analyzing firm-level data of small and large firms across 48 countries. They also showed that large credit-constrained firms could more easily increase external sources of finance than small firms.

## CONCLUSION

In 2017, the CAP production quotas for sugar were abolished, resulting in the increased sugar production in the EU. The increase in production leads to pressure on the price decrease and may threaten the competitiveness of sugar beet producers and growers. Therefore, the farmers not only have to pay attention to the production levels but also to finding ways to increase the profitability of the business.

One of the options is the use of the leverage effect, which results in the potential increase in the return on equity. The

leverage effect can be applied if the return on total capital is higher than the cost of debt. Then, by increasing the indebtedness, the company will increase the return on equity. Firms that do not achieve a sufficiently high return on total capital should try to reduce the level of indebtedness, which would also have a positive impact on the return on equity, through the effect of financial leverage. Almost 53.1% of sugar beet producers in Slovakia can positively influence their return on equity by increasing the level of indebtedness.

In 2017, the return on equity of sugar beet producers in Slovakia reached lower results in comparison to sugar beet non-producers. In general, the return on equity for all agricultural companies is at a low level, except for 25% of non-producers of sugar beet in the upper quartile (with the highest values). These companies achieved a return on equity of 15.19%, which is three times higher in comparison to sugar beet producers in this quartile. The differences in return on equity are explained by the lower level of equity per ha and thus higher indebtedness of non-producers of sugar beet.

We recommend the sugar beet producers increase the level of indebtedness for two reasons.

Firstly, interest rates are at a low level and the indebtedness of sugar beet producers is lower in comparison to non-producers of sugar beet.

Secondly, if the cost of debt in the company is below the return on total capital (BEP), an increase in indebtedness increases return on equity.

The leverage effect is a mathematical matter that can be applied in any company. However, it should be noted that the managers have to be careful when increasing the level of indebtedness. The negative aspect of debt financing is the fact that from a certain level of debt, there is a higher risk of financial distress, which can lead to a company´s bankruptcy.

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# THE USE OF SECONDARY FISH RAW MATERIALS FROM SILVER CARP IN THE TECHNOLOGY OF STRUCTURING AGENTS

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## ABSTRACT

The expediency and prospects of using secondary fish raw materials from silver carp in the structuring agents technology have been substantiated. The combination of secondary fish raw materials and seaweed with different mechanisms of gelation has a targeted effect on the organoleptic, structural-mechanical, and physicochemical properties of food products. Accordingly, the expediency of adding the Black Sea alga cystoseira (2%) to optimize the mineral composition of the structuring agents and increase their jelly-making properties was established. Using experimental studies and multicriteria optimization, the optimal ratio of secondary fish raw materials – 40%; water – 60% and rational heat treatment – 150 min, at a temperature of 85 – 100 °C has been determined. An analysis and comparison of various preservation methods have confirmed the expediency of using the freeze-drying method of drying fish broths for the production of structuring agents, which allows to preserve of the original properties of the product, biologically active substances, reduce the mass of the dried product and increase the shelf life of the structuring agents. The optimal storage conditions have been determined – the ready-made structuring agents were packed in three-layer paper kraft bags and stored in a cool dry place at a temperature of 22 ±2 °C and relative humidity of no more than 70% for 12 months. It was found that the physical and chemical indicators of the structuring agents depend on the features of the structure, amino acid composition of fish collagen. The presence of the overwhelming number of high-molecular-weight molecules in the composition of the structuring agents and the preservation of the native structure determine high indicators of dynamic viscosity, dissolution time, and strength. As a result of the research, it was revealed that the high protein content largely depends on the amount of collagen in the secondary fish raw material, which has a positive effect on the gelling properties of the product being created.

**Keywords**: secondary raw material; silver carp; structuring agent; physical indicator; chemical indicator; biological value

## INTRODUCTION

The natural and climatic conditions and significant resource potential of Ukraine contribute to the development of fisheries in their freshwater reservoirs. The production of commercial fish in domestic aquaculture farms is 23 thousand tons, of which 60% is silver carp **(State Statistics Service of Ukraine, 2020)**.

Accordingly, an important task of fish processing enterprises in Ukraine is to ensure and increase the efficiency of using the resource potential of the domestic fishery complex, possibly under the condition of rational use of fish from own water reservoirs of the country **(Makarenko et al., 2021)**.

One of the main ways of efficient use of fish resources is the processing of secondary fish raw materials left after their processing. About a third of secondary fish raw materials from silver carp are heads, bones, and fins, which are a valuable source of proteins, primarily collagen, and are widely used in the food industry **(Mushtruk et al., 2020)**.

A promising direction in the processing of collagen-containing secondary fish raw materials from silver carp is the production of structuring agents. Monitoring of modern trends in the market for structuring agents testifies to their limited assortment based on domestic raw materials, the most common of which is gelatin. However, due to massive cases of cattle diseases, the use of collagen of animal origin is dangerous **(Suchenko et al., 2017)**.

A great contribution to the solution of the problem of complex fish processing and production of structuring agents were made by scientists **Kaklyugin and Belousova (2017)**, **Nikiforova (2020)**, **Antipova et al. (2019)**,

**Andreev and Morozov (2020)**, **Kao et al. (2014)**, **Makarov et al. (2019)** and **Postnov et al. (2018)**.

However, there are no conceptual developments in the direction of the study of consumer properties of structuring agents based on secondary fish raw materials from the most common object of aquaculture in Ukraine – silver carp, which determines the relevance and practical importance of scientific research.

## Scientific Hypothesis

The hypothesis of the research work was based on the assumption that the combination of protein (heads, bones, fins of silver carp) and polysaccharide (cystoseira alga) structuring agents improves the organoleptic characteristics of food products, increases the water-holding capacity, and allows to create products with a predictable complex of consumer properties. According to the results obtained, it was confirmed that the combination of structuring agents of various natures makes it possible to more accurately regulate the consistency of the product, purposefully affects the structural-mechanical and physicochemical properties of food products through the use of raw materials with various mechanisms of gelation.

## MATERIAL AND METHODOLOGY

### Samples

Research of structuring agents for control and experimental samples (packed edible gelatin, grade P-7, manufactured by the Lysychansk Gelatin Plant; structuring agents based on secondary fish raw materials; structuring agents based on secondary fish raw materials with the addition of the Black Sea algae cystoseira) were carried out using the following raw materials: heads, bones, and fins of freshwater herbivorous fish – silver carp (*Arictichthys nobolis Rich*, two-year-old, autumn catch, weight 2 – 2,5 kg ), grown in the Irkliivskyi herbivorous fish hatchery according to **DSTU 2284 (2010) (**Figure 1); algae cystoseira, which is sold in the form of a dry powder following **GOST 31413 (2010)**; drinking water following **DSTU 7525 (2014)**.

### Chemicals

Calcium chloride, $CaCl_2$. (AGROZONE Ukraine).

Hydrochloric acid, HCl (grade A, analytical grade, LLC "Khimlaborreaktyv" Ukraine).

Sodium hydroxide, NaOH (grade A, analytical grade, LLC "Khimlaborreaktyv" Ukraine).

Sulfuric acid, $H_2SO_4$ (grade A, chemically pure, LLC "Khimlaborreaktyv" Ukraine).

### Animals and Biological Material

The studies used variegated silver carp weighing 2 – 2.5 kg, 50 – 60 cm long, autumn catch, which was grown at the Irkliivskyi fish farm of herbivorous fish. Conducted studies of the structural and morphological composition of experimental groups (heads, internal organs, carcasses, fillets, scales, fins, bones), calculated the proportion of edible and inedible parts.

### Instruments

Drying cabinet (SNOL, LLC "Labimpex LTD" Ukraine).

Muffle furnace (SNOL, LLC "Labimpex LTD" Ukraine).

Automatic amino acid analyzer (T 339 produced by "Mikrotekhna" Czech Republic).

Atomic absorption spectrophotometer (Shimadzu AA-6200, LLC "SPEKTROLAB" Ukraine).

Fat analyzer (SOX 406, LLC "Khimlaborreaktyv" China).

Gas chromatograph ("Kupol-55", "Shimadzu Corporation" Japan).

Program viscometer (Brookfield LVDV-II+PRO USA), pH meter HI8314 HANNA ("SPECTRO LAB" Ukraine).

### Laboratory Methods

The study of the chemical composition of the structuring agents was carried out according to the following methods: mass fraction of moisture by drying a sample of the product to constant mass at a temperature of 100 – 105 °C according to **DSTU 8029 (2015)**; mass fraction of ash – by the gravimetric method, after mineralization of the sample weight in a muffle furnace at a temperature of 500 – 600 °C following **DSTU 8718 (2017)**; mass fraction of lipids by the Soxhlet method, which consists in the fact that fat is weighed after its extraction with a solvent from a dry sample weight in a Soxhlet apparatus, based on the determination of the change in the mass of the sample after extraction of fat with a solvent following **DSTU 8718 (2017)**; mass fraction of protein by determination of total nitrogen by the Kjeldahl method, based on the ability of the organic material of the product sample to be oxidized with concentrated sulfuric acid in the presence of a catalyst following **DSTU 8030 (2015)**.



**Figure 1** Silver carp.



**Figure 2** Structuring agents.

The amino acid composition of proteins was determined by ion-exchange liquid column chromatography on an automatic amino acid analyzer T 339 using an LG ANB cation exchange resin **(Sorochan and Shtemenko, 2005)**.

The mineral composition was determined by inductive plasma atomic emission spectrometry according to **DSTU ISO 11885 (2005)**.

The study of physical and chemical indicators was carried out according to the following methods: transparency – according to own patented method **(Patent No. 73282, 2012)** on a universal computing device; strength – by the method of establishing the maximum load required for the destruction of the gelatin surface following **GOST 11293 (2017)**; melting temperature by defining the temperature at which the gelatin turns into a liquid state following **GOST 11293 (2017)**; dissolution time by the method of establishing the duration of complete dissolution according to **GOST 25183.3 (1982)**; dynamic viscosity by the method of determining the viscosity of a solution at certain temperatures and a decrease in viscosity after determining the holding time on a capillary viscometer according to **GOST 25183.4 (1982)**; active acidity by determining the pH of a solution of a certain concentration at a certain temperature with using a pH meter according to **GOST 11293 (2017)**.

**Description of the Experiment**

**Sample preparation:** Fish raw materials (heads, bones, fins) of silver carp were washed in clean running water at a temperature not exceeding 15 – 20 °C; while mucus, blood, and other residues were removed simultaneously with washing. Washing was carried out until the water was clear. The quality of washing was monitored visually. The washed fish raw material was crushed and subjected to heat treatment. Next, the fish raw material is fed to the shredder "Volgar 5 U", where it is ground to a homogeneous mass. In the process of mixing, all the raw materials are thoroughly mixed for further loading into the boiler cooker of the required portion. The experimentally established optimal ratio of fish waste and water was 1:1.5; heat treatment was 2.5 hours at a temperature of 85 – 100 °C. At the initial stage of heat treatment, cystoseira was added in the form of a dry powder (2%). To reduce the mass fraction of moisture, the fish broth was subjected to freeze-drying at a temperature of (50 – 70 °C). The ready-structuring agents were packed in three-layer paper kraft bags and stored in a cool dry place at a temperature of 22 ±2 °C and relative humidity of no more than 70% for 12 months. A control sample, structuring agents based on secondary fish raw materials were sent for analysis; structuring agents based on secondary fish raw materials and cystoseira. Then, an average sample was taken from each unit, which was characterized by its general chemical composition, mineral content, the amino acid composition of proteins, and physicochemical parameters.

**Number of samples analyzed:** Three types of structuring agents were used in the study of the samples: control – gelatin of animal origin and two samples based on secondary fish raw materials of silver carp and addition of cystoseira.

**Number of repeated analyses:** The study was repeated 5 times, and the experimental data were processed using the methods of mathematical statistics.

**Number of experiment replication:** Each study was carried out five times, the number of samples was three, resulting in fifteen repeated analyzes (Figure 2).

## Statistical Analysis

The statistical analysis data were produced by Microsoft excel and Statistica 15. The accuracy of the obtained experimental data was determined using the Student's test for a confidence probability of $\leq 0.05$ based on the number of parallel determinations at least 5. Linear programming problems were solved using the MS Excel spreadsheet processor "Search for a solution" setting (Excel Solver).

## RESULTS AND DISCUSSION

Structuring agents experimental samples were evaluated according to the following physicochemical parameters: dissolution time, active acidity, dynamic viscosity, gelatin strength, gelatin melting point, solution transparency (Table 1). Previous studies have established the effectiveness of the use of fish collagen in comparison with collagen of animal origin in terms of the main physicochemical parameters **(Mezenova, 2017; Hanani, 2016)**. Research shows that dissolution time is one of the important quality criteria for structuring agents **(Andreev and Morozov, 2020; Kao, 2020)**. It is known that the degree of solubility of the structuring agents depends on many factors: the size of the granules, the type of raw materials used, temperature, etc.

**Table 1** Physical and chemical quality indicators of structuring agents.

| Indicators | Control | Structuring agents based on | |
|---|---|---|---|
| | | secondary fish raw materials | secondary fish raw materials and cystoseira |
| Dissolution time, min. | 12 ±0.6 | 5 ±0.2 | 6 ±0.3 |
| Dynamic viscosity, mPa·s$^{-1}$ | 16 ±0.8 | 17 ±0.7 | 18 ±0.9 |
| Gelatine strength, H | 10 ±0.5 | 10 ±0.5 | 11 ±0.5 |
| Solution transparency,% | 28 ±1.4 | 32 ±1.6 | 30 ±1.5 |
| Melting temperature of gelatine, ºC | 27 ±1.3 | 30 ±1.5 | 31 ±1.5 |
| Active acidity, pH | 5.5 ±0.2 | 6 ±0.3 | 6 ±0.3 |

Note: (n = 5, $p \leq 0.05$).

However, first of all, dissolution is associated with the hydration of proteins, that is, the binding of water molecules to proteins. Scientists associate this phenomenon with the structural features of collagen in various fish species **(Shokina et al., 2020; Bredikhina and Zarubin, 2019)**. Protein, like any hydrophilic high molecular weight compound, swells, and then the protein molecules begin to gradually transfer into solution.

When swelling, water molecules penetrate the protein and bind to its polar groups. A dense network of polypeptide chains is delimited. Further absorption of water leads to the separation of protein molecules from the total mass and dissolution. This is the process of electrostatic binding of water molecules to the polar groups of side radicals of acidic amino acids with a negative charge and basic amino acids with a positive charge. However, swelling does not always lead to rapid dissolution, for example, animal collagen, unlike fish collagen, can remain in a swollen form, having absorbed a large amount of water. Similar statements were highlighted in the works of scientists **(Mezenova et al., 2018; Zarubin, 2018; Palamarchuk et al., 2020)**. This can explain almost twice as fast the dissolution time of research structuring agents based on secondary fish raw materials – 5 minutes and cystoseira – 6 minutes, compared to the control – 12 minutes. The solubility of animal collagen is hampered by structural features – cross-links between polypeptide chains. A paradoxical phenomenon is observed: the protein contains many anionic or cationic groups, and its solubility in water is relatively low **(Vorobiev and Nizhnikova, 2017; Dzyuba and Zemlyakova, 2019)**. It was noted that the melting point of the control sample is the lowest (27 °C), of the structuring agents based on secondary fish raw materials (30 °C), and structuring agents based on secondary fish raw materials and cystoseira (31 °C). Scientists have proven that the lowered melting temperature of fish gelatin-based on scales of various fish species (by 4 °C compared to the minimum normalized value for gelatin of animal origin) is due to the peculiarities of the amino acid composition of the raw material **(Yakubova and Bekesheva, 2018a; Abdelhedi et al., 2019; Palamarchuk et al., 2019)**.

The transparency of the structuring agent solution characterizes the degree of their contamination with mechanical impurities. It was found that the value of the transparency index of new structuring agents is higher than that of control. During storage, the transparency of all samples ranged from 27 – 30%. Accordingly, high transparency indicates their high quality. The predecessors **(Yakubova and Bekesheva, 2018b)** noted that the transparency index of a 5% solution of fish gelatin exceeds the traditional standard values by more than 17.0%, which confirms its high organoleptic properties as a food consistency regulator.

Dynamic viscosity is one of the main characteristics of structuring agents. According to this indicator, all product samples are within acceptable limits (16 – 18 mPa.s$^{-1}$). Scientists noted the higher values of viscosity indicators of fish structuring agents (by more than 18%), compared with animal gelatin **(Bekesheva, 2019)**. However, it is worth noting that the results of the prototypes of Structuring agents based on secondary fish raw materials are the most optimal compared to the control and Structuring agents based on secondary fish raw materials and cystoseira. It has been proven that in the manufacture of any product with a gelatin structure, it is advisable to use structuring agents with medium viscosity, since structure formers with high viscosity can render the created product too rigid and elastic consistency, respectively, structuring agents with low viscosity, on the contrary, do not provide the required strength of the product.

In works **(Nikolaev et al., 2016; Zarubin and Bredikhina, 2016)**, the effectiveness of combining protein-polysaccharide structuring agents to obtain the required structure of a food product is confirmed.

An indicator of the quality of the structuring agents is the strength of the gelatin, which directly depends on the pH of the medium. The greatest strength is observed in the range from 5 to 7 pH.

The nutritional value of the structuring agents was examined by their chemical composition, namely, the content of proteins, fats, and minerals (Table 2).

So, the high content of proteins (82%) and minerals characterizes the degree of the biological value of natural structuring agents. It should be noted that the structuring agents based on secondary fish raw materials and cystoseira contain a greater amount of minerals (1.6%) compared to the structuring agents without cystoseira (1.3%). Accordingly, the addition of cystoseira made it possible to rationalize the mineral composition of the product, since cystoseira contains a complete complex of mineral elements.

**Table 2** Chemical composition of structuring agents.

| Content, % | Control (gelatine) | Structuring agents based on | |
|---|---|---|---|
| | | secondary fish raw materials | secondary fish raw materials and cystoseira |
| Moisture | 14.0 ±0.7 | 15.0 ±0.7 | 15.0 ±0.7 |
| Protein | 83.0 ±4.1 | 82.0 ±4.1 | 82.0 ±4.1 |
| Fat | 1.5 ±0.07 | 1.4 ±0.07 | 1.1 ±0.05 |
| Minerals | 1.4 ±0.07 | 1.3 ±0.06 | 1.6 ±0.09 |

Note: (n = 5, $p \leq 0.05$).

**Table 3** Amino acid rate of protein of structuring agents.

| Amino acid, % | Amino acid content according to the FAO/WHO scale | Control | Structuring agents based on | |
|---|---|---|---|---|
| | | | secondary fish raw materials | secondary fish raw materials and cystoseira |
| Valine | 50 | 45 | 47 | 49 |
| Isoleucine | 40 | 29 | 32 | 34 |
| Leucine | 70 | 50 | 71 | 74 |
| Lysine | 55 | 72 | 101 | 105 |
| Methionine + Cystine | 35 | 34 | 78 | 83 |
| Threonine | 40 | 50 | 93 | 87 |
| Phenylalanine + Tyrosine | 60 | 38 | 70 | 71 |

**Table 4** Mineral composition of structuring agents, mg.100g$^{-1}$.

| Mineral element | Control | Structuring agents based on | |
|---|---|---|---|
| | | secondary fish raw materials | secondary fish raw materials and cystoseira |
| Potassium | 7.94 ±0.31 | 98.0 ±4.1 | 90.45 ±4.2 |
| Calcium | 343.0 ±10.61 | 68.1 ±3.32 | 69.3 ±3.46 |
| Iron | 0.91 ±0.04 | 1.09 ±0.05 | 1.02 ±0.05 |
| Chlorine | 454.0 ±22.49 | 545.7 ±27.16 | 475.6 ±23.70 |
| Selenium | - | - | 6.24 ± 0.28 |
| Bromine | 2.78 ±0.13 | 3.37 ±0.16 | 5.01 ±0.24 |
| Phosphorus | 300 ±10.12 | 120 ±5.6 | 170 ±8.1 |
| Zirconium | 0.10 ±0.004 | 0.05 ±0.002 | 0.16 ±0.008 |
| Sulfur | 2.1 ±0.10 | 2.8 ±0.14 | 4.98 ±0.24 |

Note: (n = 5, $p \leq 0.05$).

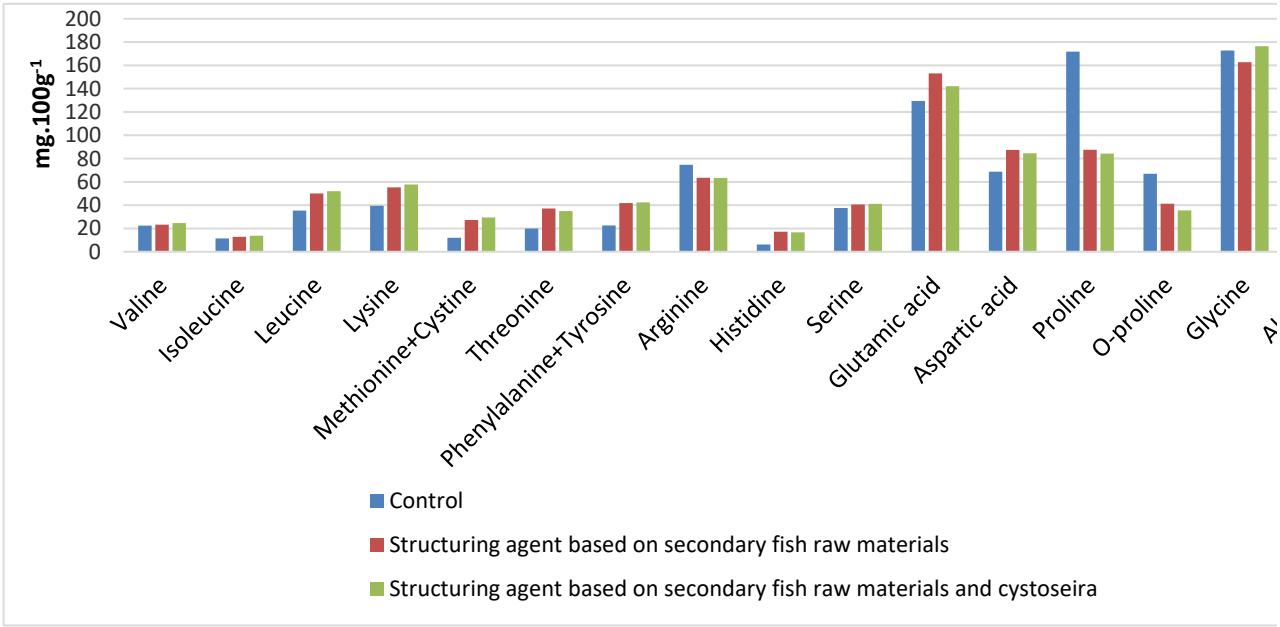


**Figure 3** Analysis of the amino acid composition of protein structuring agents.

In the works **(Kao, 2017; Baidalinova and Lyapustina, 2018; Zheplinska et al., 2019)** it is indicated that the amount of protein in the research fish gelatin is within the range (87.36 – 91%), which confirms the rather high biological value of the product.

One of the important quality criteria for structuring agents is their biological value, which is largely determined by the content and balance of amino acids, especially essential ones **(Holembovska et al., 2021; Nesterenko et al., 2020)**. Characteristics of the amino acid composition are shown in Figure 3.

During studies of the amino acid composition of proteins, the structuring agents, the highest content of such amino acids was noted: glycine, alanine, proline, aspartic and glutamic acids. These amino acids form a repetitive sequence in the polypeptide chain, resulting in the helical structure of collagen. Accordingly, the high content of glycine 162.7 – 176.4 mg.100g$^{-1}$ and glutamic acid 142.1 – 153.1 mg.100g$^{-1}$ in research structuring agents is a characteristic feature of the amino acid composition of fish collagen. Amino acids such as histidine, isoleucine, valine are found in a smaller amount, which is due to the smaller number of cross-links. Experimental studies of the amino acid composition of fish gelatin **(Antipova et al., 2019)** showed that it is almost identical to the animal. However, animal gelatin lacks tyrosine and cystine.

It was also found that fish gelatin has a lower content of hydroxyproline, but almost 2.5 times more proline **(Antipova and Storublevtsev, 2016)**. An objective assessment of the biological value of proteins was determined by a set of indicators: amino acid rate, KRAS, utilitarian coefficient. Amino acid rate of structuring agent proteins was determined following the FAO/WHO scale (Table 3). The biological value of protein compositions depends on the content of essential amino acids in them and their ratio in the product and the idealized model **(Karpenko, 2019)**. So, the dominant amino acids are lysine, threonine, phenylalanine, and tyrosine, the limiting ones are valine and isoleucine.

To assess the degree of protein utilization, the coefficient of difference of the amino acid rate was calculated. KRAS of the control sample is 16.4%, of the research ones is 37.8 – 38.2%.

The biological value of the reference protein is 100%. In experimental samples, biological value is 61.8% (structuring agents based on secondary fish raw materials) and 62.2% (structuring agents based on secondary fish raw materials and cystoseira), control ones – 83.6%. The value of the utilitarian coefficient of the amino acid composition of the experimental samples (0.618 – 0.836) indicates a high balance of amino acids relative to the standard.

Thus, the qualitative composition of structuring agent proteins based on secondary fish raw materials from silver carp indicates the expediency of their effective use in the food industry.

One of the important indicators of the nutritional value of structuring agents is the content of the main macro- and microelements in their composition (Table 4).

Analyzing the quantitative composition of macro- and microelements of research structuring agents in comparison with the control, an increase in the level of potassium, iron, sulfur, bromine, chlorine should be noted. Experimental samples of structuring agents, in comparison with the control, contain a significant amount of potassium necessary for the human body, which ensures the normal activity of the cardiovascular system. The potassium content in structuring agents based on secondary fish raw materials is 98.0 mg.100g$^{-1}$, in the sample from cystoseira – 90.45 mg.100g$^{-1}$, which exceeds the value of the control sample by 113.94%.

An increased amount of iron 1.2 times in experimental samples compared to the control will contribute to a positive effect on the protective functions of the body, since iron is involved in respiration, hematopoiesis, immunobiological and redox reactions, and a sufficient amount of calcium (68.1 – 69.3 mg.100g$^{-1}$) will improve its assimilation by the human body.

In terms of sulfur content, the dominant is structuring agents sample based on secondary fish raw materials and cystoseira – 4.98 mg.100g$^{-1}$, because sulfur is used in the human body to neutralize many toxic products formed during metabolism.

A distinctive feature of the studied samples of the structuring agents with cystoseira is a significant bromine content, which is 5.01 mg.100g$^{-1}$ which is almost twice as much as in the control – 2.78 mg.100g$^{-1}$, and selenium – 6.24 mg.100g$^{-1}$. The enrichment of the mineral composition of the structuring agents with bromine and selenium can be explained by their sufficient content in cystoseira.

The results of the study show that the addition of seaweed – cystoseira to the structuring agents contributes to the optimization and enrichment of their mineral composition in comparison with the structuring agents without cystoseira and the control sample.

## CONCLUSIONS

It was found that structuring agents based on secondary fish raw materials are characterized by a high protein content (82%), which has a positive effect on the jelly-making properties of the structuring agents. The dominant amino acids are glycine, proline, alanine, aspartic and glutamic acids. The value of the utilitarian coefficient of the amino acid composition of the new structure-formers is within the range (0.61 – 0.83), which indicates a high degree of assimilation of their proteins by the human body.

It has been proven that the addition of cystoseira to the structuring agents based on secondary fish raw materials increases the content of Bromine (5.01 mg.100g$^{-1}$) compared to the control (2.78 mg.100g$^{-1}$) and Selenium (6.24 mg.100g$^{-1}$).

Confirmed a high degree of jelly-making properties of structuring agents, characterized by a complex of indicators of viscosity (16 – 18 mPa.s$^{-1}$), strength (10 – 11 H), melting point (27 – 31 °C), and solubility (5 – 6 minutes).

It was found that the addition of cystoseira to the structure-forming agents promotes optimization and enrichment of their mineral composition in comparison with the structure-forming agents without cystoseira and the control sample.

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# THE BIOCHEMICAL CHANGES IN LEGUMES DURING HIGH-TEMPERATURE MICRONIZATION

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## ABSTRACT

The article considers the change in chemical and biological characteristics in some legumes grains, under conditions of high-temperature micronization with different moisture contents during heat treatment with infrared rays. The heat treatment of grains was carried out on a laboratory apparatus with a quartz radiant infrared panel. The temperature variation in the heat treatment zone occurred due to changing the distance between the panel and the surface of grains. The grain temperature was determined using a laser thermometer, and with a timer. To determine chemical and biological characteristics, we used a special optical density metering device. We have studied: 1. The dependence of starch content on the temperature in the changing initial moisture content. We found that after 30 seconds of high-temperature micronization of, "Tsanava" beans at a grain moisture content of 12.7%, the starch content in the grain increases from 39.65% to 40.12%, then gradually decreases, and at 18.3% moisture content, it increases from 38.71% to 41.2%, with a moisture content of 28.6% it increases from 37.36% to 42.42%. Similar processes are also observed for the beans "field red" and "white lupine"; 2. The dependence of glucose content on the temperature in the changing initial moisture content. As the mass fraction of starch decreases, the percentage of sugar (in terms of the equivalent amount of glucose) at a moisture content of 12.7% at the initial stage increases from 1.36% to 1.46%, and then the percentage of sugar increases relatively quickly to 1.64%, at a moisture content of 18.3% it increases from 1.3% to 1.38%, and then increases to 1.51, with a moisture content of 28.6%, it increases from 1.28% to 1.35% and then increases to 1.54. Similar processes are also observed for the beans "field red" and "white lupine".

**Keywords:** bean; lupine; starch; glucose, high-temperature micronization; heat treatment.

## INTRODUCTION

The bean ranks second after pea among legumes in popularity and spread. In earlier centuries, legumes such as lupine proved to be very popular in Georgia, but now it is almost forgotten. Various food products are produced from beans. The dried beans are extensively used in dietetic nutrition for the treatment of atherosclerosis and preventing heart rhythm disorders. The beans are part of the Arfazetin collection and are used in the treatment of diabetes. In folk medicine, the dried bean tincture is used in the treatment of kidney disease, rheumatism, hypertension, and diabetes **(Markov, Markov and Vodenicharova, 2016)**.

The authors **(Ntatsi et al., 2018)** point out that legumes are considered to be important plant sources. They contain protein, essential minerals, carbohydrates, vitamins, and biologically active compounds, which are very important for humans and also contain phytochemicals such as phenolic acids, anthocyanins, proanthocyanidins, and flavonols **(Murphy et al., 2018)**. These phytochemicals have been identified in different legume species **(Moreno-Valdespino et al., 2019)**. Bean contains about 23% proteins, most of which are water-soluble **(Recommendations for healthy, 2006)**; up to 55% carbohydrates (starch); small amounts of oligosaccharides, including 4.7% cellulose; up to 1.5% fatty acids; carotene; macronutrients, such as potassium (1160 mg%), calcium (220 mg%), phosphorus, copper, zinc; nitrogen-containing substances, including amino acids; flavonoids, organic acids, as well as B and PP group vitamins, pyridoxine, thiamine, pantothenic and ascorbic acids **(Markov, Markov and Vodenicharova, 2016)**. Raw beans, especially red beans contain significant amounts of alkaloids that have toxic effects. To neutralize the latter, the heat treatment is used by boiling water (for less than 30 minutes) **(Markov, Markov and Vodenicharova, 2016)**. Grain Legumes are very important for human nutrition and contain high amounts of carbohydrates that are slowly released **(Afzal et al., 2020)**, where starch is the major carbohydrate in legumes. The share of starch is 40 – 50% of their total weight. In their chemical compositions, beans and



A B C

**Figure 1** General view of the test legumes. Note: A – Beans of the „Tsanava“ variety; B – Beans of the „Field red“ variety; C – Beans „White Lupine“.

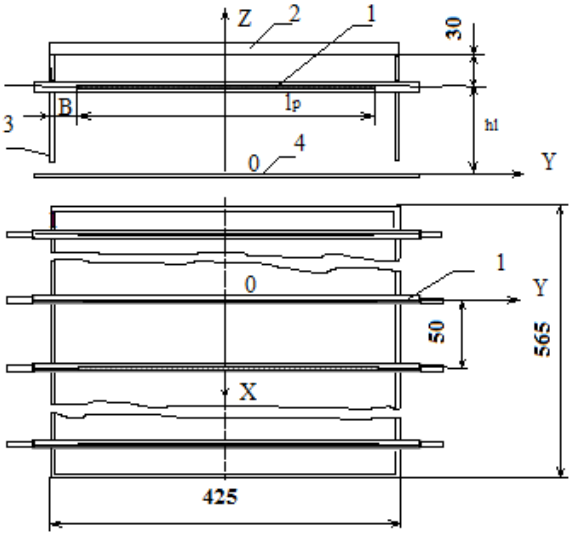


**Figure 2** Diagram of the experimental-industrial block of the emitters: 1 – QP1-type infrared source; 2 – Top reflector; 3 – Side reflector; 4 – A working surface of the processing zone.

lupine are close to meat, which is well assimilated into the human body **(Samchenko, 2014)**. In a variety of metabolic syndromes, legumes have several health benefits **(Jeong et al., 2018)**. These crops could be valuable functional and/or medicinal food **(Mainali et al., 2014)**.

During thermal treatment of grain with infrared rays, chemical processes are activated, however, infrared rays do not appear to have a direct impact on biological objects, as in the case of ultraviolet rays, at least, there are few or no results achieved in this area available in the existing literature. Various biochemical processes occur in the grain resulting from thermal action. At this time, there can take place protein denaturation, starch dextrinization, inactivation of biologically active substances and microorganisms, and so on.

The starch contained in legumes swells up in the presence of moisture at a temperature of 50 – 90 °C the swollen granules lose the double refraction ability, crystallites are melting and the polymers are opening. When the temperature is about 65 °C, under conditions of excess moisture, after the completion of the first stage of pregelatinization of starch, starch can retain about 85% of water **(Khosny, 2006)**. During further heating, the pregelatinization process continues **(Fast and Kolduell, 2007)**.

Individual components and microflora of a heterogeneous structure of legume grains have different absorption coefficients, and therefore, in processing them with infrared rays, they have different local temperatures, especially in the non-stationary period, which indicates the singularity of radiation heating **(Fast and Kolduell, 2007)**.

Based on the review of the literature and the analysis of the problem to be solved, we developed the direction of the research and defined the purpose of the research.

The research described in this article aims to study the dependence of the starch and glucose content on temperature during high-temperature micronization, with a change in the initial hygroscopic humidity. Based on the obtained experimental data, the compilation of a mathematical model will allow us to investigate the process of other legumes.

## Scientific Hypothesis

In the process of high-temperature micronization, in a short time, under the high heat impact, carbohydrates in the legume grains are partially inactivated and split into simple carbohydrates with the formation of dextrins, biologically active substances are inactivated, and proteins are partially denatured. Activation of this process in the grain, by creating proper optimal conditions, will eventually lead to an improvement in the nutritional value of legumes.

## MATERIAL AND METHODOLOGY

The research covered the varieties of bean such as „Tsanava“ variety **(**Gori, Gori Region, Georgia), „field red“ bean (Tskhaltubo, Tskhaltubo Region, Georgia), and the so-called „white lupine“ (Khoni, Khoni Region, Georgia), Figure 1.

The heating of legume grain was carried out on the laboratory equipment with the quartz halogen linear infrared emitters panel type QP1 (Elcer, Odesa, Ukraine). The diagram of this equipment is shown in Figure 2.

The irradiance on the working surface in the processing zone was determined through a calculation using a specially developed program. Variation by height installation of IR panel above the monolayer of grain product and the type of pallet allowed changing independently, under a limited range, the irradiance and ambient temperature in the treatment zone.

The authors **(Tóth et al., 2018)** note that many factors affect the content of useful compounds in legumes, such as agrochemical processing, climatic conditions, as well as varieties and conditions of their storage.

The initial moisture content of beans was determined using an electronic digital meter of grain and seed moisture (moisture meter) VSP-100 (PAtools, Kharkiv, Ukraine). Additionally, the moisture loss during IR heating was estimated as the difference between the initial sample weight and its weight after heat treatment. The sample weight of the grain before and after heat treatment was determined using an SF-400C model (Toms, Qilin, China) electronic digital analytical balance, with a weighing accuracy of 0.01 g. The moisture content (W) after heat treatment was calculated based on the initial moisture content ($W_0$) and mass loss（$\Delta m$）according to the formula based on the standards **GOST 13586.5 (2015)** and **ISO 3166 (2015)**

$$W = 100\,[W_0/100 - \Delta m/m_0]/[1 - \Delta m/m_0] \qquad (1)$$

Where:
$W_0$ – initial moisture, %; $m_0$ – initial sample weight, g; $\Delta m$ – sample weight loss, g.

To determine the percentage of starch in the samples, we prepared 5 samples of calibrating with different starch contents from pure starch and determined their optical density. After treatment with potassium iodide (Potassium iodide for analysis, KI, Merck, ≥99.5%) and iodine solution (Crystalline iodine, $I_2$, Merck, ≥99.9%), data obtained by using the optical density metering device KFK-2 (PP ZOMZ, Zagorsk, Russia), we constructed a calibration curve at coordinates: starch content mg·$(10\ mL)^{-1}$ – optical density.

Three samples of beans and lupine for each moisture content were crushed in a laboratory grain crushing machine (Retsch, Kyiv, Ukraine) and passed through a laboratory sieve No. 08 (Retsch, Kyiv, Ukraine)**.** The obtained bean flour in the amount of 0.5 g was put in a porcelain cup and crushed well, then it was placed in a flask and fill it with 300 mL of distilled water. The flask was placed in a water bath and the temperature of the mixture was brought up to 95 °C, then it was quickly cooled with running water and the flask was filled with distilled water up to the 500 mL mark. The solution was filtered through a laboratory filter paper. After treatment with potassium iodide and iodine solution, we determined the optical density of solution obtained on the machine KFK-2 (PP ZOMZ, Zagorsk, Russia), and with the help of a calibrating curve, we determined the percentage of starch in the sample in a g-mg·$(10\ mL)^{-1}$ solution. The analysis of the samples was repeated three times and the experiments were repeated three times. The mass content of starch in the sample was calculated by a formula based on the standards **GOST 10845 (1998)** and **ISO 6493 (2000)**

$$X_s = g \cdot 100 \cdot V/m \cdot 1000 \cdot 10 \qquad (2)$$

Where:
g – starch content which is determined in accordance with the optical density from a calibrating curve, mg·$(10\ mL)^{-1}$; V – the total volume of solution, mL; m – the mass of the analysis sample, g.

To determine the amount of reducing sugars in the samples, we prepared 6 samples of calibrating solution from pure glucose with different glucose contents, to which we added potassium hexacyanoferrate (III) solution (Potassium hexacyanoferrate (III), $K_3Fe(CN)_6$, Merck, ≥99.0%) and then we determined their optical density using the optical density metering device KFK-2 (PP ZOMZ, Zagorsk, Russia), then according to data obtained, we constructed a calibration curve at coordinates: glucose content mg·$(35\ mL)^{-1}$ – optical density.

Samples for the determination of reduced sugars were prepared as a previous experiment with three samples of beans and lupine for each moisture content. We placed in a flask 25 mL of potassium hexacyanoferrate (III) solution, 8 mL of a clear filtrate of the analysis sample, and 2 mL of distilled water. Then we boiled it for 1 minute and cooled it quickly with running water. Then we determined the optical density of the solution using the optical density metering device KFK-2 (PP ZOMZ, Zagorsk, Russia), and with the help of a calibrating curve, we determined the percentage of reduced sugars in the sample in a g-mg solution. The analysis of the samples was repeated three times and the experiments were repeated three times.

The percentage of reducing sugar content in the sample was calculated by a formula based on the standards **GOST 29177 (2004)** and **ISO 3946 (1982)**

$$X_{gl} = g \cdot 100 \cdot V_1/m \cdot V_2 \cdot 1000 \qquad (3)$$

Where:
g – glucose content which is determined following the optical density from a calibrating curve, mg; $V_1$ – the volume of an aqueous solution of the prepared sample, mL; $V_2$ – the volume of an aqueous solution of the sample taken for the reaction with potassium hexacyanoferrate (III) solution, mL; m – the mass of the sample, g.

The author **(Samchenko, 2014)** has studied the possibility of using legumes (peas, beans, and lentils) in the production

**Table 1** A comparative analysis of the chemical composition of legumes and beef.

| Parameter | Chemical composition, % | | | |
|---|---|---|---|---|
| | **Peas** | **Beans** | **Lentils** | **Beef** |
| Moisture content | 14.0 | 14.0 | 14.0 | 64.5 |
| **Macronutrients** | | | | |
| Protein | 20.5 | 21.0 | 24.0 | 18.6 |
| Fat | 2.0 | 2.0 | 1.5 | 16.0 |
| Carbohydrates including: | 49.5 | 47.0 | 46.3 | 0.9 |
| Starch | 44.9 | 43.8 | 43.4 | - |
| Mono- and disaccharides | 4.6 | 2.9 | 3.2 | - |
| Dietary fibers | 11.2 | 11.5 | 12.4 | - |

of minced meat semi-finished products. A comparative analysis of the chemical composition of legumes and raw meat was carried out, the results of which are given in Table 1.

It has been shown that legumes contain significantly less water than meat, which represented their high nutritional value, and they also contain significantly less fat, i.e. are dietary products. Peas, beans, and lentils surpass meat in the percentage of protein and carbohydrates, including dietary fibers that are absent in meat, which allows for better binding and retaining moisture in the minced meat system.

For the process of heat treatment with infrared rays, mathematical models of temperature and humidity change in the grain are compiled, which are described by nonlinear differential equations:

$$C \cdot M \cdot d(\Delta T)/dt = S_1 \cdot \{K_s\, A \cdot E(t) + K_a\, [\Delta T_c\,(t) - -K_g \cdot \Delta T] - K_d(\lambda + \varepsilon/\mu) \cdot (dm_B)/dt\} \quad (4)$$

$$(dm_B)/dt = -f_w\, [T(t), W_c\,],$$

Where:

$m_B$ – Is the mass of the body, kg; $\lambda$ = (2.503 - 2.255) $10^6$ - Specific heat of free water evaporation, J.mol$^{-1}$; $\varepsilon$ – adsorption potential; $\mu$ = 0.018 – vapor molar mass; $K_d$- coefficient of proportionality.

The obtained differential equations cannot be integrated analytically and even numerical methods cannot be used. So we use different assumptions. To study the change in temperature we use the model **(Lykov and Mikhailov, 1972)**

$$\Delta T(t) = K_0\, [1 - \exp(-K_T\, t)], \quad (5)$$

Where:

We consider the empirical coefficients $k_0$ and $k_1$ as constants.

To identify the coefficients $k_0$ and $k_1$ we use the "linfit" function of the Mathcad-mathematical package **(Makarov, 2011)**.

To study the change in humidity we see the model:

$$U/U_0 = (1 - a \cdot \Delta T)^{b \cdot U_0^c} \quad (6)$$

To identify the coefficients a, b, and c we use the "genfit" function of the Mathcad-mathematical package **(Makarov, 2011)**.

## Statistical Analysis

For statistical processing of the data obtained, we determined the mean root square deviation and the arithmetic-mean dispersion, at which point, we determined the mean root square deviation of the arithmetic mean. We conducted each experiment at least three times and determined the arithmetic mean of the computed value. We have obtained the value of the reliability coefficient which is $p$ <0.05.

Statistical data are processed using the mathematical package- MathCad (Mathsoft, Cambridge, Massachusetts, USA), in particular, MathCad 15 **(Makarov, 2011)**.

## RESULTS AND DISCUSSION

The author **(Lizenko et al., 1985; Ba et al., 2013)** notes that starch undergoes hydrolysis on heating in presence of sulfuric acid, during which it produces glucose. Depending on the reaction conditions, hydrolysis can be carried out in successive phases, with the formation of intermediate products – dextrins.

(C6H10O5)n (starch) → (C6H10O5)m (dextrins (m<n) → xC12H22O11 (maltose) → nC6H12O6 (glucose)

Differential thermal analysis data obtained on a derivatograph show that starch has several endothermic peaks: 150 °C and 225 °C, corresponding to amylose, and 140 °C and 275 °C, corresponding to amylopectin **(Lizenko et al., 1985)**. Exothermic peaks were observed at temperatures above 350 °C.

To determine biochemical changes during the germination of rice grains and to increase the germination rate, the authors **(Veluppillai et al., 2009)** used gibberellic acid and surfactants (sodium dodecyl sulfate (SDS) (1.0 g.L$^{-1}$) and Triton-X-100 (1.0 mL.L$^{-1}$), whole rice grains soaked in distilled water for 12 h at 30 °C were germinated in the dark at 30 °C for five days. The highest germination (77.1%) was obtained on the 5th day. An increase in the content of reducing sugars from 7.3 to 58.1 mg.g$^{-1}$ dry matter was observed from the 1st day of germination.

Complex carbohydrates undergo several transformations as a result of heating, which changes their structure and properties **(Perez-Maldonado, 2002)**, and under the high heat impact (especially in the presence of a catalyst) can be split into relatively simple carbohydrates with a small molecular weight. During heating, the gelatinization process occurs, which is attributed to endothermic reactions with a specific heat capacity of 10 – 19 kJ·kg$^{-1}$. The

temperature range of gelatinization depends on the moisture content **(Fast and Kolduell, 2007; Khosny, 2006)**. Our research agrees with this view.

With an increase in the irradiation and the time of IR heat treatment, the degree of gelatinization and the content of dextrins in the grain is increasing **(Gunkin, 1992)**.

The study looked at some of the useful and available ingredients, making instant porridge. The data showed that the formula B2 which contained soaked mung beans, had the highest calorie content, total essential amino acids, biological value, and water absorption index (WAI). it was found that soaked mung beans were a good source of essential amino acids and calories to improve nutrition and the technological quality of the resulting porridge **(Mahgoub, Mohammed and Mobarak, 2020)**.

In grain processing with infrared rays, activation of chemical processes occurs, which can substantially change its chemical composition and the influence of microorganisms **(Deepa and Hebbar, 2016; Deepa and Hebbar, 2017)**.

The authors of **(Teixeira et al., 2013)** note that the seeds of some legumes (raw, steeped, or heated) came back with high levels of protein, which exceeds the levels found in other seeds of the same genus (from 16.8% to 29.3%). The levels of protein *B. cheilantha* in the seeds are comparable or much higher than those indicated for some important cultivated legume seeds such as soybeans (from 39.5% to 44.5%) and cowpea (from 19.5% to 26.1%). The seeds also found significant levels of dietary fibers, which were higher than those widely reported for the seeds of legumes such as soybeans (17.4%) and black beans (22.6%) **(Trugo et al., 2000)**.

The authors **(Klug et al., 2020)** have developed an innovative cowpea puree has been developed, containing 78.8% fresh cowpea seeds. Microwave (8 kW/35 s), high hydrostatic pressure (550 MPa/10 min/23 ℃) or sous-vide (80 ℃, 3 min) were analyzed as treatment methods. However, the physicochemical properties, mainly the color, were greatly influenced by heat treatment, although the treatment under high hydrostatic pressure remained greenish.

The work is aimed at evaluating the ability of spelled wheat (Triticum aestivum spp. Spelta) to cook porridge compared to oats. The studied cereals are instant products based on extruded spelled or oat flour. The approximate composition, hydration properties (water absorption index-WAI and water solubility index-WSI), consistency, and thermal properties were determined to compare the characteristics of the porridge. In general, spelled porridge was higher in total and insoluble fiber, protein, minerals, and less available carbohydrates than oatmeal. **(Šimurina et al., 2018)**.

The authors **(Lund and Ray, 2017)** take a critical look at recent advances in strategies for controlling the Maillard reaction and subsequent reaction products in food systems. Presents the main mechanisms involved, discusses the strengths and weaknesses of each strategy, and proposes reasonable response mechanisms to underpin the assessments. As a result, trapping or modification of Maillard targets, reactive intermediates, and glycation end products (AGEs) is manifested with their potential unwanted side effects.

The authors **(Xu et al., 2020)** note that there has been an increased interest in the application of Pulsed Electric Fields (PEF) technology as a pretreatment of plant-based foods before deep-frying to improve quality (e.g., lower browning tendency and oil uptake) and reduce production costs (e.g., better water and energy efficiencies). We have chosen high-temperature micronization for the pretreatment of legumes.

The authors **(Jeong, Kim and Lee, 2020)** investigated the effect of sequential pulsed electric field (PEF) processing, sous-vide cooking, and reheating on beef meat properties was investigated. Fresh meat was treated with PEF at various electric field strengths of 1.0, 1.5, and 2.0 $kV.cm^{-1}$, and then control beef samples and beef samples pretreated with PEF were cooked sous vide at 60 °C for up to 24 h. PEF pretreatment resulted in fresh meat tenderizing in proportion to the increase in electric field strength.

After studying the grain quality and processing characteristics of durum wheat (*Triticum turgidum* subsp. *Durum*), heat stress during grain filling caused grain shrinkage with a decrease in weight and ultrastructural changes in the aleuron layer and in the endosperm cells, it is also suggested that, during grain filling, high temperatures may affect the strength of the gluten, reducing the quality of the wheat flour **(Dias, Bagulho and Lidon, 2008)**.

The authors of **(Vadivel, Nandety and Biesalski, 2011)** note that some seeds of legumes are associated with the prevention of cardiovascular diseases, diabetes, and gastrointestinal diseases, given that they lower the glycemic index of food as well as cholesterol. The detected levels of moisture, ash, lipids, and digestible carbohydrates were lower or similar to those reported for legumes **(Carvalho et al., 2011)**.

The authors **(Sivakanthan et al., 2009)** to determine biochemical changes used Gibberellic acid and surfactants (sodium dodecyl sulfate (SDS) (1.0 $g.L^{-1}$) and Triton -X−100 (1.0 $mL.L^{-1}$)), whole rice grains soaked in distilled water for 12 hours at 30 °C, germinated in the dark at 30 °C for five days. The highest germination (77.1%) was obtained on the 5th day. An increase in the content of reducing sugars from 7.3 to 58.1 $mg.g^{-1}$ of dry matter was observed from the 1st day of germination. The content of free amino acids and soluble protein increased to 3.69 and 5.29 $mg.g^{-1}$ DM, respectively, on the $5^{th}$ day of germination.

The authors of **(Kupkanchanakul et al., 2018)** note the molecular weight distribution of pre-germinated brown rice starch (PGBRS) indicates partial hydrolysis of starch molecules, especially amylopectin, resulting in a decrease in the proportion of high molecular weight molecules after pre-germination.

The authors **(Olaleye**, **Oresanya and Ogundipe, 2020)** investigated the effect of fermentation time and temperature on the approximate composition and content of anti-nutrients in purified and unshelled *Mucuna cochinchinensis* flour. The results showed that fermentation time affected the approximate bean composition, causing a marked increase in crude protein (from 22.19 to 36.41%) and fat (from 4.94 to 10.79%) in the fermented bean samples. Mucuna. compared to the results of the

unfermented samples (22.19 and 4.94%), while the fermented beans showed a decrease in carbohydrates (from 57.34 to 40.85%).

This study showed that fermentation for 72 h at 45 °C was most effective in increasing the nutritional value of the beans as well as reducing the antinutrient content to a minimum.

The authors in their articles do not mention foods such as starch and legume monosaccharides. In the view of many authors, they are the necessary products for the human body.

Standardization of data to 100 g samples of unpolished rice (dry matter basis), showed intra-varietal ranges of; 9 g protein, 5.65 mg iron, 3.34 mg zinc, 1.6 mg thiamin, 0.392 mg riboflavin, and 7.2 mg niacin. Currently, several research institutions are working toward improving the nutrient content of rice **(Kennedy and Burlingame, 2003)**.

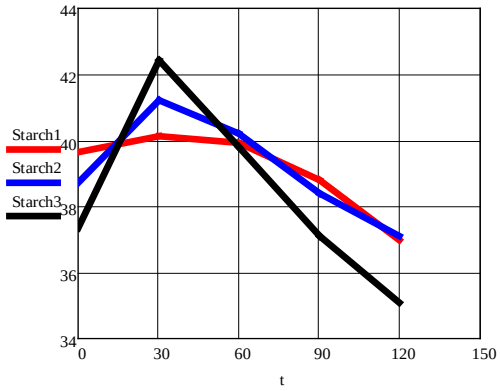


**Figure 3** The dependence of the change in starch content on the length of exposure, „Tsanava“ bean variety. Note: The distance between the product and IR panel H = 100 mm; Moisture content: 1 – $W_0$ = 12.7%; 2 – $W_0$ = 18.3%; 3 – $W_0$ = 28.6%. t : = (0, 30, 60, 90, 120)$^T$, Starch1= (39.65, 40.12, 39.92, 38.8, 37.0)$^T$, Starch2 = (38.71, 41.2, 40.19, 38.4, 37.1)$^T$, Starch3 = (37.36, 42.42, 39.8, 37.1, 35.08)$^T$.

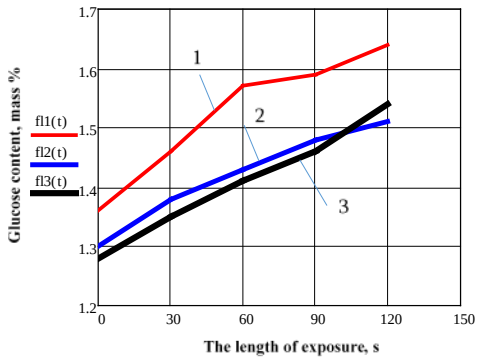


**Figure 4** The dependence of the change in glucose content on the length of exposure, „Tsanava“ bean variety. Note: The distance between the product and IR panel H = 100 mm; Moisture content: 1 – $W_0$ = 12.7%; 2 - $W_0$ = 18.3%; 3 – $W_0$ = 28.6%. t = (0, 30, 60, 90, 120)$^T$, Sugar 1 = (1.36, 1.46, 1.57, 1.59, 1.64) $^T$, Sugar 2 = (1.3, 1.38, 1.43, 1.48, 1.51) $^T$, Sugar 3 = (1.28, 1.35, 1.41, 1.46, 1.54)$^T$.

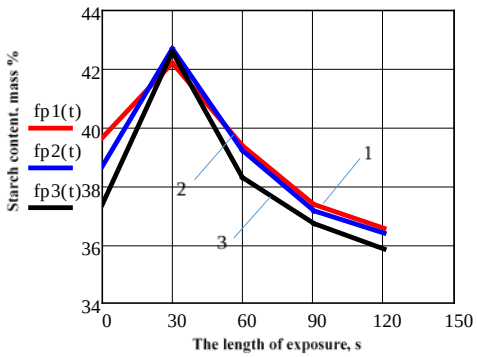


**Figure 5** The dependence of the change in starch content on the length of exposure, „Tsanava“ bean variety. Note: The distance between the product and IR panel H = 75 mm; Moisture content: 1 – $W_0$ = 12.7%; 2 – $W_0$ = 18.3%; 3 – $W_0$ = 28.6%. t : = (0 30 60 90 120)$^T$, Starch1 := (39.63 42.18 39.4 37.35 36.53)$^T$, Starch2 := (38.7 42.7 39.21 37.14 36.4)$^T$, Starch3 : = (37.38 42.6 38.92 36.72 35.86)$^T$.

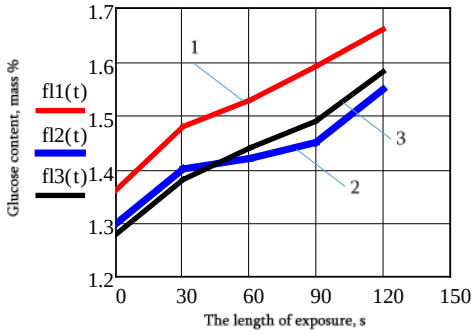


**Figure 6** The dependence of the change in glucose content on the length of exposure, „Tsanava“ bean variety. Note: The distance between the product and IR panel H = 75 mm; Moisture content: 1 – $W_0$ = 12.7%; 2 – $W_0$ = 18.3%; 3 – $W_0$ = 28.6%. t := (0 30 60 90 120)$^T$, Sugar1 := (1.36 1.48 1.53 1.59 1.66)$^T$, Sugar2 := (1.3 1.4 1.42 1.45 1.55)$^T$, Sugar3 := (1.28 1.38 1.44 1.49 1.58)$^T$.

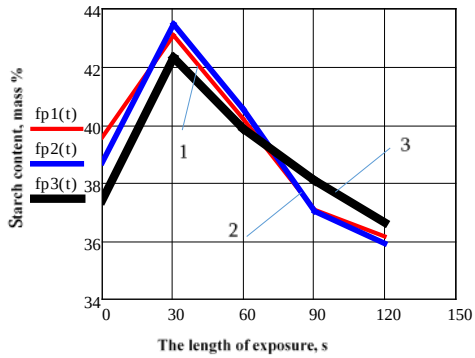


**Figure 7** The dependence of the change in starch content on the length of exposure, „Tsanava“ bean variety. Note: The distance between the product and IR panel H = 50 mm; Moisture content: 1 – $W_0$ = 12.7%; 2 – $W_0$ = 18.3%; 3 – $W_0$ = 28.6%. t := (0 30 60 90 120)$^T$, Starch1 := (39.6 43.1 40.2 37.1 36.14)$^T$, Starch2 := (38.71 43.45 40.54 37.04 39.94)$^T$, Starch3 := (37.34 42.34 39.87 38.11 36.64)$^T$.

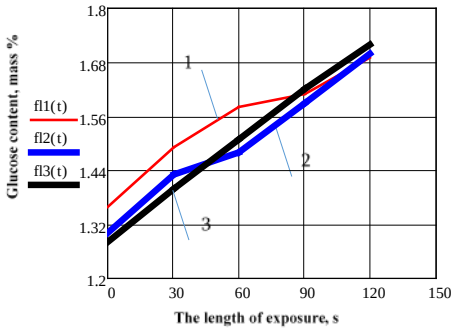


**Figure 8** The dependence of the change in glucose content on the length of exposure, „Tsanava“ bean variety. Note: The distance between the product and IR panel H = 50 mm; Moisture content: 1 – $W_0$ = 12.7%; 2 – $W_0$ = 18.3%; 3 – $W_0$ = 28.6%. t := (0 30 60 90 120)$^T$, Sugar1 := (1.36 1.49 1.58 1.61 1.69)$^T$, Sugar2 := (1.3 1.43 1.48 1.59 1.7)$^T$, Sugar3 := (1.28 1.4 1.51 1.62 1.76)$^T$.

To increase the nutritional value and quickly prepare the legumes, we have chosen heat treatment with IR rays.

The authors **(Iheke et al., 2017)** studied the effect of fermentation on the physicochemical properties and nutrients of carob fruits. The approximate composition, mineral composition, anti-nutritional factors, and physicochemical properties of both unfermented and fermented samples were determined. The results showed that fermented samples are better in nutritional value than unfermented seeds.

The authors **(Skurikhin and Tutelyan, 2002)** give the chemical composition of different varieties of beans without heat treatment, which is different from our data. After heat treatment, our data are approximated to their data.

The dependence of the change in starch content on the length of exposure during high-temperature micronization

**Table 2** Changes in starch content (mass %) for the ,,field red" bean variety with dependence on the duration of high-temperature micronization.

| No | Initial moisture content, $W_0$ % | Biological parameter | The distance between the product and IR panel, mm | The length of exposure, s | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 30 | 60 | 90 | 120 |
| 1 | 17.5 | Starch, mass % | 100 | 43.94 | 45.12 | 43.22 | 39.76 | 36.73 |
| 2 | | Sugar, mass % | | 1.05 | 1.28 | 1.41 | 1.63 | 1.95 |
| 3 | 29.8 | Starch, mass % | | 43.96 | 45.56 | 42.27 | 39.55 | 37.04 |
| 4 | | Sugar, mass % | | 1.01 | 1.22 | 1.68 | 1.97 | 2.12 |
| 5 | 34.8 | Starch, mass % | | 43.96 | 46.16 | 41.2 | 38.06 | 35.76 |
| 6 | | Sugar, mass % | | 0.95 | 1.55 | 2.0 | 2.36 | 2.54 |
| 1 | 17.5 | Starch, mass % | 75 | 43.94 | 45.42 | 43.63 | 39.16 | 35.93 |
| 2 | | Sugar, mass % | | 1.05 | 1.25 | 1.56 | 1.84 | 2.06 |
| 3 | 29.8 | Starch, mass % | | 43.96 | 45.87 | 43.44 | 38.78 | 36.23 |
| 4 | | Sugar, mass % | | 1.01 | 1.25 | 1.78 | 2.03 | 2.21 |
| 5 | 34.8 | Starch, mass % | | 43.96 | 46.02 | 41.82 | 37.86 | 35.14 |
| 6 | | Sugar, mass % | | 0.95 | 1.63 | 1.86 | 2.44 | 2.64 |
| 1 | 17.5 | Starch, mass % | 50 | 43.94 | 45.88 | 43.48 | 40.24 | 36.73 |
| 2 | | Sugar, mass % | | 1.05 | 1.28 | 1.64 | 1.95 | 2.07 |
| 3 | 29.8 | Starch, mass % | | 43.96 | 46.17 | 43.24 | 39.18 | 36.03 |
| 4 | | Sugar, mass % | | 1.01 | 1.31 | 1.85 | 2.1 | 2.3 |
| 5 | 34.8 | Starch, mass % | | 43.96 | 46.28 | 42.51 | 37.09 | 35.96 |
| 6 | | Sugar, mass % | | 0.95 | 1.72 | 1.93 | 2.52 | 2.73 |

**Table 3** Changes in starch content (mass %) for the ,,white lupine" variety with dependence on the duration of high-temperature micronization.

| No | Initial moisture content, $W_0$ % | Biological parameter | The distance between the product and IR panel, mm | The length of exposure, s | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 | 30 | 60 | 90 | 120 |
| 1 | 10.8 | Starch, mass % | 100 | 40.65 | 44.4 | 42.7 | 41.7 | 40.5 |
| 2 | | Sugar, mass % | | 1.16 | 1.32 | 1.48 | 1.78 | 2.01 |
| 3 | 20.5 | Starch, mass % | | 40.66 | 45.4 | 41.43 | 40.63 | 40.14 |
| 4 | | Sugar, mass % | | 1.1 | 1.30 | 1.5 | 1.83 | 2.0 |
| 5 | 26.8 | Starch, mass % | | 40.66 | 45.67 | 41.2 | 39.84 | 39.1 |
| 6 | | Sugar, mass % | | 1.05 | 1.35 | 1.53 | 1.85 | 2.03 |
| 1 | 10.8 | Starch, mass % | 75 | 40.65 | 44.62 | 42.45 | 41.1 | 39.46 |
| 2 | | Sugar, mass % | | 1.16 | 1.38 | 1.63 | 1.88 | 2.07 |
| 3 | 20.5 | Starch, mass % | | 40.66 | 45.48 | 41.02 | 39.2 | 38.96 |
| 4 | | Sugar, mass % | | 1.1 | 1.38 | 1.62 | 1.92 | 2.1 |
| 5 | 26.8 | Starch, mass % | | 40.66 | 45.65 | 42.56 | 40.56 | 38.72 |
| 6 | | Sugar, mass % | | 1.05 | 1.47 | 1.75 | 1.96 | 2.19 |
| 1 | 10.8 | Starch, mass % | 50 | 40.65 | 45.56 | 42.2 | 40.6 | 38.64 |
| 2 | | Sugar, mass % | | 1.16 | 1.44 | 1.7 | 1.94 | 2.12 |
| 3 | 20.5 | Starch, mass % | | 40.66 | 45.67 | 40.76 | 39.1 | 38.2 |
| 4 | | Sugar, mass % | | 1.1 | 1.54 | 1.69 | 2.0 | 2.15 |
| 5 | 26.8 | Starch, mass % | | 40.66 | 45.8 | 40.78 | 38.28 | 37.68 |
| 6 | | Sugar, mass % | | 1.05 | 1.6 | 1.91 | 2.05 | 2.2 |

at different distances between the product and IR panel for the "Tsanava" bean variety is shown in Figures 3, 5, and 7, while Figures 4, 6, and 8 illustrate the change in the glucose content.

Data on changes in starch content after high-temperature micronization for the "field red" bean variety are given in Table 2.

Data on changes in starch content after high-temperature micronization for the "white lupine" variety are given in Table 3.

As shown in Figure 2, the mass fraction of starch in the "Tsanava" beans increases at the initial stage of heat treatment, and then gradually decreases. The increase in the mass fraction of starch at the initial stage is more noticeable the higher the moisture content of the grain is. After 30 seconds of high-temperature micronization, at 12.7% moisture content of the grain, conditions of the starch content of the grain increases from 39.65% to 40.12%, while at 18.3% moisture content, it increases from 38.71% to 41.2%, and at 28.6% moisture content, it increases from 37.36% into 42.42%. This must be because, at the initial

stage of high-temperature micronization, moisture evaporation proceeds faster than the conversion of starch to dextrins and glucose.

This process is more pronounced when the infrared panel is less distant from the heat treatment surface, i.e. the higher the temperature in the heat treatment zone (Figures 5 and 7).

Similar processes are also observed for the beans "field red" (Table 2) and "white lupine" (Table 3), however, these processes are more intense when the geometrical dimensions of the grain are less (Tables 2 and 3), indicating that heat treatment takes place more intensively inside the grain.

As the mass fraction of starch decreases, the percentage of sugar increases slightly at the initial stage, in terms of the equivalent amount of glucose (Figure 3), and then the percentage of glucose increases relatively rapidly simultaneously with growth in the rate of starch deterioration. Similar processes take place in the case of "field red" bean (Table 2) and "white lupine" (Table 3).

In this context, it should be noted that the study of the process of inactivation of alkaloids in legumes by the method of high-temperature micronization should be the subject of further research.

## CONCLUSION

Our research and others have shown that the seeds of the studied legumes may be a promising alternative food source to overcome the malnutrition problem that people face. The legume seeds have a high protein content, an indispensable amino acid profile, and a low level of anti-nutritional compounds. When considering the composition of legumes, researchers focus on the content and importance of proteins in them, but also no less attention is paid to the content and importance of carbohydrates in legumes, which is less discussed in the scientific literature. It is noteworthy that in beans and white lupines, a sharp change in biological and chemical characteristics is possible as a result of high-temperature micronization. This method allows making full use of the nutritional potential of the studied legumes. It has been established that at this time, intensive partial hydrolysis of starch in legumes occurs, resulting in the formation of dextrins and glucose. We found that at the beginning of the high-temperature micronization of "Tsanava" beans at a grain moisture content of 12.7%, the starch content in the grain increases from 39.65% to 40.12%, then gradually decreases, and at a moisture content of 18.3% it increases from 38.71% to 41.2%, with a humidity of 28.6%, it increases from 37.36% to 42.42%. Similar processes are observed for the „field red" and "white lupine" beans. This should be due to the fact that at the initial stage of high-temperature micronization, moisture evaporation occurs faster than the transformation of starch into dextrins and glucose, and naturally the mass fraction of starch grows, then, with the decomposition of starch, its mass fraction gradually decreases.

As the mass fraction of starch decreases, the percentage of sugar (in terms of the equivalent amount of glucose) at a moisture content of 12.7% initially increases from 1.36% to 1.46%, and then the percentage of sugar increases relatively quickly to 1.64 %, at 18.3% humidity it increases from 1.3% to 1.38%, then increases to 1.51, at 28.6% humidity it increases from 1.28% to 1.35%, and then increases to 1.54. Similar processes are observed for the "field red" and "white lupine" beans. The method of high-temperature micronization makes it easier to carry out the decomposition of starch into dextrins and glucose and the products obtained thereby are much more easily absorbed by the human body. At the same time, the considered legumes become easier to prepare, and their nutritional value increases.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement

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# THE NUTRITIONAL PROFILE OF INDONESIAN SALMON VAN JAVA MAHSEER *T. SORO* SPECIES

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## ABSTRACT

*T. soro*, in Indonesia called salmon van Java is of high economic value, and due to high demand, its culture has been intensively studied and developed. This study aimed to assess the nutritional value of wild and cultured *T. soro*. The fish's proximate compositions, minerals, as well as amino and fatty acid profiles were analyzed. A t-test analysis was used to identify differences between treatments. Results showed that the fat content of wild *T. soro* was higher than that of cultured fish, but the protein, water, and ash contents between the two groups were not significantly different ($p$ >0.05). *T. soro* was considered a lean fish with higher concentrations of PUFAs (polyunsaturated fatty acids) than MUFAs (monounsaturated fatty acids). The amino acid profile was dominated by lysine, phenylalanine, and allo-isoleucine. Both groups of fish were a good source of macro- (Na, K, Ca) and microminerals (Zn, Fe), except for selenium (Se). The two groups were not significantly different ($p$ >0.05) in ω3, ω6, and PUFAs, indicating that culturing *T. soro* in proper ways could substitute for wild *T. soro*.

**Keywords:** amino acid and fatty acid profile; Indonesian *T. soro*; minerals; proximate composition

## INTRODUCTION

In some parts of the world, especially in developing countries, fish has been a major protein source in diet. The latest FAO data have shown that fish contributes 17% of animal protein consumption and 6.7% of total protein consumption globally. As food, fish offers a unique combination of high-quality protein and vital micronutrients such as vitamins and minerals. Fish can improve the quality of dietary proteins by providing certain essential amino acids that may be deficient in other foods. Vitamins and minerals in fish include B-complex vitamins, vitamin D, vitamin A (especially in oily fish), selenium, zinc, iodine, and iron. All vitamins and minerals as micronutrients are important for development and health maintenance. Small fish with bones is an important source of calcium in human diets. Fish is also a good source of certain fatty acids (omega 3 and omega 6) that are as vital to normal brain development in unborn babies and infants as they are vital to adult due to the roles of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in lowering the risk of coronary heart disease (CHD) mortality **(Abouel-Yazeed, 2013; Thilsted et al., 2014)**. These fatty acids cannot be produced by the human body and must be supplied by food. Fish is reported to be the only natural source of EPA and DHA **(Philibert et al., 2006)**.

Fish consumption in Indonesia is as high as 43.9 kg/capita/year **(Ministry of Marine Affairs and Fisheries, 2017)**, and it contributes up to 51% to animal protein consumption. In addition to saltwater fish, Indonesia is also blessed with numerous freshwater fish species that play important roles in the food security and livelihood of communities living close to freshwater areas. One of the freshwater fish is Tor fish. Some are endemic to Indonesian waters, and some are commonly found in the lakes and rivers in Aceh, West Java (Kuningan, Sumedang, Majalengka), East Java (Blitar, Senggaring), South, Central, and West Kalimantan, West and South Sumatera, and Jambi **(Arifin et al., 2019)**. The genus Tor, called true mahseer, is reported to represent 16 valid species so far **(Pinder et al., 2019)**. These species are related to high religious and cultural significance throughout South and Southeast Asia, including Indonesia. They live in clean, strong-current rivers as well as in lakes. The fish are usually found in lowland and warm habitats, but one species of Tor (*Tor putitora*) is also found in Himalayan rivers and lakes **(Sarma et al., 2015)**. There are 6 species of the mahseers found in Indonesia, 4 from the genus Tor and 2 from the genus Neolissochillus. The genus Neolissochillus is endemic to Toba Lake, North Sumatera, and currently included on the International Union for Conservation of Nature (IUCN) list as an endangered fish species **(Arifin et al., 2019)**.

The Tor species found in Indonesia are *T. tambroides*, *T. soro*, *T. douronensis*, and *T. tambra*, of which the morphology has been described by **Haryono and Tjakrawidjaja (2006)**. *Tor soro* is locally known as Kancra or Dewa in West Java, Pedih in Aceh, Jurung in North Sumatra, Sirang in Lampung, and Kelah in Kalimantan. It

is utilized as food and ornamental fish. In North Sumatera, *T. soro* is used as a substitution for Batak fish (*Neolissochilus thieenmanni*) for cultural ceremonies. As this fish grows slowly, a high level of utilization has led to overfishing. Tor fish has become less common due to the degradation of its habitats. Fortunately, there are places where Tor fish is considered sacred and kept in sanctuary ponds, for example, in Kuningan in West Java, Telaga Rambut Monte, Gandusari, Blitar in East Java, Aek Sirambe in North Sumatra, and Lubuk Larangan in West Sumatra. Studies and trials to culture Tor fish have been conducted **(Kristanto, Asih and Winarlin, 2007; Gustiano et al., 2013; Radona et al., 2015; Muchlisin et al., 2017)**. This effort is intended to save the fish from extinction, and nowadays, the culture of *T. soro* has been developed in Indonesia. Culture development not only helps Indonesia on the fish extinction but also to use this fish as alternative protein sources for the needy population. Character comparison such as the nutritional value of the cultured fish to the wild fish is very important to improve the culture technique of this fish.

The chemical composition of freshwater fish, including the Tor fish from cold-water environments, has been reported elsewhere **(Abouel-Yazeed, 2013; Sarma et al., 2011; Skibniewska et al., 2013; Basumatary et al., 2017)**. However, the information regarding this tropical freshwater fish has been limited. The objective of this study was to assess the chemical composition and to provide information on the nutritional value of Indonesian *T. soro*. As the cultivation of *T. soro* has been developed, in the current study, cultured *T. soro* was included in the analysis for comparison.

## Scientific hypothesis

Due to the similar characteristics and living environment, the chemical composition and nutritional value of wild and cultured Indonesian *T. soro* were not significantly different.

## MATERIALS AND METHODS

### Sample

This study used *T. soro* fish in consumable size. Wild *T. soro* was caught by fishermen from the Cipunagara River, Subang (West Java), Indonesia, in size of 36 ±3.6 cm in length and 535.5 ±24.2 g in weight (Figure 1). Identification was conducted by the Research Institute for Inland Water Fisheries. The 33.3 ±1.5 cm and 343.7 ±34.4 g cultured fish were from the Research Institute for Inland Water Fisheries' ponds in Cijeruk, West Java, Indonesia. The cultured fish was fed with commercial feed (SINTA pakan ikan), composing of the protein content of 32%, fat content of 5%, dietary fiber of 6%, ash content of 12%, and moisture content of 12%. The feed was applied at 2% per day per total biomass since the fish attained a weight of 200 grams at 10 months. The fish was cultured with a stocking density of 15 fish per cubic running water. The fish was harvested at 10 months old.

### Chemicals

The Chemical used in this study were Merck-based HCl, NaCl, $HNO_3$, $NA_2SO_4$, KOH, methanol, hexane, and isopropyl alcohol. Amino acid standard testing kit KG0-7167 and AG0-7184 (Phenomenex) were also used in this experiment.

### Instruments

The equipment used in this study were inductively coupled plasma optical emission spectrometry (ICP-OES) Agilent 720 (US), Gas Chromatography equipped with flame ionization detector (FID) (Perkin Elmer Technologies, US), centrifuge, microwave, vortex, oven, furnace, and freezer.

### Description of Experiments

#### *Sample Preparation*

*T. soro* fish used in this study was consumable size. Wild *T. soro* was caught by fishermen from the Cipunagara River, Subang (West Java), Indonesia, in size of 36 ±3.6 cm long and 535.5 ±24.2 g. The cultured fish in size of 33.3_±1.5 cm and 343.7 ±34.4 g were collected from Research Institute for Inland Water Fisheries, Cijeruk, West Java, Indonesia. The fishes were brought alive to the Research Center for Marine and Fisheries Product Processing and Biotechnlogy (BBRP2BKP) and were killed by immersing them in iced water and filleted as soon as the fish died.

Each group of fish (15 wild and 15 cultured *T. soro*) was divided into 3 sub-groups, filleted, minced, and homogenized using a domestic food processor (Panasonic, Indonesia). The homogenate (25 g) was then put into several small sterile plastics and stored at -20 °C before laboratory analysis. For fatty acid analysis, the homogenate was added with liquid nitrogen and stored at -80 °C before being analyzed in the following day. All analyses were conducted in triplicate.



**Figure 1** Wild (left) and Cultured (right) Indonesian Masheer *T. soro*

***Chemcal Composition (Proximate and Mineral Analysis)***

Proximates (protein, fat, ash, and moisture contents) were assessed according to the **AOAC (2000**). Protein was assayed by the Kjeldahl method, fat content by the Bligh and Dyer method, and ash and moisture contents by gravimetry.

Proximate analysis was conducted at BBRP2BKP Chemical lab. Minerals (Ca, Na, K, Zn, and Fe) were assayed at PT SIG Saraswanti's laboratory according to the method of **AOAC (2000)**. A total of 0.5 g of sample was added with 10 ml of $HNO_3$ and then destructed at 190 °C for 20 minutes. The sample was introduced into a flask and added with aquabidest until 50 mL in volume, and then filtered. Determination of mineral content was assayed using inductively coupled plasma optical emission spectrometry (ICP-OES) Agilent 720 at each wavelength of minerals.

***Amino Acid Analysis***

Amino acid analysis was conducted at BBRP2BKP's instrumentation laboratory. The fish sample (20 mg) was hydrolyzed according to **Csapo et al. (1997)**. That is, the sample was hydrolyzed with 1 ml of 6N HCl and heated using a microwave at 180 °C for 60 min. The hydrolysate (100 µL) was then analyzed for amino acid content using EZ, the fast amino acid testing kit Phenomenex (KG0-7167), against essential and non-essential amino acid standards (Phenomenex AG0-7184). An analysis of amino acids was conducted in duplicate using a gas chromatography flame ionization detector (GC-FID).

***Fatty Acid Analysis***

Fatty acid analysis was conducted at PT SIG Saraswanti's laboratory, Bogor, Indonesia. A Five-gram sample was weighed into a clean quencher tube, added with 4 mL of isopropyl alcohol, and shaken for 1 minute. Six mL of hexane was added. Then, vortexing was conducted for 1 minute, followed by centrifuging for 3 minutes (12.370 RCF) to completely dissolve the extract. The upper layer with hexane phase was removed into a screw tube. For a methylation process, about 1 mL of hexane extract was removed into a tube, added with 1.5 mL of 0.5 M KOH methanol, heated at 100 °C for 20 minutes, and cooled at room temperature. After that, it was added with 1.5 mL of 20% BF3 in methanol and heated again at 100 °C for 20 minutes. The solution was cooled and shaken until the temperature decreased to 30 °C. It was then added with 3 mL of NaCl and 2 mL of hexane before being subjected to vortexing for 2 minutes. After cooling and formation of two phases, one of which containing fatty acid methyl ester (FAME), the mix was transferred into a tube and dried with 0.1 – 0.2 g of anhydrous sodium sulfate ($NA_2SO_4$). The FAME solution was removed into a vial and injected into GC-FID. FAME was identified and measured by Perkin Elmer Technologies Gas Chromatography equipped with flame ionization detector (FID) with SP TM -2560 column of 100 m having a 0.35 mm ID and 0.20 µm film thickness, 0.5 µL split, and 100:1 injection. The initial oven temperature was set and held at 100 °C for 3 minutes before being increased to 240 °C at a rate of 2.5 °C/minute and held at that temperature for 23 minutes. The carrier gas was helium at a total flow rate of 18.0 cm/second with a column length of 100 m. The detector temperature was set at 240 °C, and the injector temperature at 224 °C.

**Statistical Analysis**

All analyses were conducted in triplicate. The results are reported as mean value ± standard deviation (*SD*). Differences between treatments for each parameter were tested for significance using t-test analysis.

**RESULTS AND DISCUSSION**

***Proximate composition and minerals***

The *T. soro* studied, both wild and cultured, were considered low-fat fish as the fat content of both was less than 2%. As shown in Table 1, except for fat, Ca, and Na, the proportions of all components were not different between wild and cultured *T. soro* ($p > 0.05$). Wild *T. soro* contained higher Ca, Na, and fat contents than the cultured ones ($p < 0.05$).

Studies on the proximate composition of wild and cultured fish of the same species have been conducted in many parts of the world. Inconsistent results in terms of the composition of the two groups were reported by different authors. Some reported that wild fish contained a higher content of certain chemical contents such as protein and fat, while others reported the opposite. Concerning protein content, wild fish were reported to be higher in the cases of yellow perch (Perca flavescens) **(Gonzales et al. 2006)**, Nile tilapia (*Oreochromis niloticus*) **(El-zaeem et al, 2012)**, and native fish *Dormitator latifrons* **(López-Huerta et al., 2018)**. Contrary results were reported on freshwater giant prawn (*Macrobrachium rosenbergii*), shrimp (*Penaeus monodon*) **(Islam et al., 2017)**, and sea bass (*Dicentrarchus labrax*) **(Alasalvar et al., 2002)**, in which case the cultured ones were reported to have higher protein and fat contents

**Table 1** Chemical compositions of wild and cultured *T. soro* compared with *T. Putitora.*

| Components | *Tor soro* | | *Tor putitora** | *Channa striata*** | |
|---|---|---|---|---|---|
| | **Wild** | **Cultured** | **(Golden Mahseer)** | **Wild** | **Cultured** |
| Moisture (%) | 77.7 ±6.3 | 79.4 ±0.6 | 76.24 – 79.24 | 78.88 ±0.29 | 76.90 ±0.99 |
| Ash (%) | 1.3 ±0.4 | 1.2 ±0.2 | 1.23 – 1.55 | 1.23 ±0.09 | 1.44 ±0.12 |
| Protein (%) | 17.8 ±0.9 | 18.6 ±0.6 | 15.59 – 17.29 | 19.85 ±0.59 | 19.71 ±0.28 |
| Fat (%) | 1.4 ±0.2[a] | 0.9 ±0.1[b] | 0.62 – 1.52 | 0.44 ±0.19 | 2.65 ±0.83 |
| Ca (mg,100 $g^{-1}$) | 182.5 ±6.4[a] | 253 ±2.8[b] | 1400 – 1600 | 12.15 ±2.33 | 73.23 ±36.86 |
| Na (mg,100 $g^{-1}$) | 90.9 ±7.0[a] | 26.7 ±3.6[b] | 200 | 18.35 ±3.04 | 34.82 ±2.65 |
| K (mg,100 $g^{-1}$) | 378 ±12.7 | 370 ±0.0 | 1400 – 1600 | 283.00 ±18.38 | 398.83 ±17.37 |
| Zn (mg,100$g^{-1}$) | 0.5 ±0.0 | 0.4 ±1.9 | 1 – 1.4 | 0.36 ±0.003 | 0.45 ±0.02 |
| Fe (mg,100 $g^{-1}$) | 0.6 ±0.0 | 0.6 ±0.0 | 0.6 – 1.3 | 0.17 ±0.01 | 0.45 ±0.02 |

Note: Values with a different word in a row means significant difference ($p < 0.05$). Source: ***Sarma et al. (2015)**; ** **Chasanah et al. (2015)**.

compared to the wild sources. Feeds, habitats, seasons, and sex were the factors considered as most responsible. In fish culture, feeding is usually controlled for feed composition and feeding time to achieve certain targets such as weight, protein content, fat content, or color of the fish.

In this study, cultured *T. soro* were not significantly different from wild *T. soro* in moisture, protein, and ash content ($p$ >0.05), but significantly different in fat content and minerals ($p$ <0.05). Wild *T. soro* contained higher fat content and minerals compared to the cultured *T. soro*. This result was supported by previous studies conducted by **Nettleton and Elmhurst (2000)** and **Job et al. (2015)**. **Nettleton and Elmhurst (2000)** found that among 3 fishes studied, the fat contents in cultured and wild rainbow trouts were not significantly different, while **Job et al. (2015)** found that the fat content of wild tilapia was higher than that of cultured tilapia. The cultured *T. soro* used in this study were harvested from ponds and were fed with 2% of total biomass per day of commercial feed **(Kristanto, Asih and Winarlin, 2007)**. The commercial feed had a protein content of 32.70 ±0.34%, moisture of 9.47 ±0.01%, fat content of 7.76 ±0.16%, and ash content of 14.61 ±1.16%. Meanwhile, the wild *T. soro* harvested from the Cipunagara River, Subang (West Java, Indonesia) consumed natural feed available in the river water. Tor fish is reported to be an active swimmer, fast-moving fish which eats a variety of foods available in the river. This means that the river from where the *T. soro* were harvested might be rich in natural feed containing high-fat content, which was not only used as an energy source but also conserved in the body. The main diet of wild *T. soro* is algae, detritus, insects, or smaller fish **(Haryono and Tjakrawidjaja, 2006)**. The study reported by **Sarma et al. (2015)** on the proximate composition of the Himalayan mahseer *T. putitora* showed comparable results with those of the warm water *T. soro* used in this study. Fat content is highly dependent on the species as well as fish ontogeny, feed, and environmental waters. In cold water environments, like Himalayas, the composition of fish was reported maximal during the monsoon season and lesser during the summer and winter seasons **(Sharma and Singh, 2020)**.

The microelement (Zn) and macroelements (Ca, Na, K) in *T. soro* flesh were in general lower compared to those in golden mahseer (*T. putitora*) flesh, but the Fe contents of the two species were comparable. The *T. soro* studied, both wild and cultivated in ponds, were poor in micromineral selenium (Se) (Table 2). Microelement Se (selenium) was reported to be used as an important, and good, antioxidant against aluminum toxicity **(Iordache et al., 2011)**. Himalayan mahseers, which live in the cold water of Himalaya, provide better sources of micro- and macroelement compared to *T. soro* which live in warm tropical water. However, compared to local fish Channa striata, *T. soro* was superior in terms of Ca, Na, Fe and comparable in terms of Zn and K contents. Therefore, *T. soro*, especially the wild ones, could be used as a good source of macrominerals. Potassium, calcium, and sodium are macrominerals essential to human health and involved in biological systems, while microminerals are involved in normal tissue metabolism and maintaining the health of the human body **(Sharma and Singh, 2020)**. The results of this study are different from those of the study conducted by

**Table 2** Amino acid profile of wild and cultured *T. soro* compared to *T. putitora*.

| Amino Acids (%) | *Tor soro* | | *Channa striata* | | *Tor putitora* (Golden Mahser)*** |
|---|---|---|---|---|---|
| | Wild | Cultured | River* | Swam water **(Merauke) | |
| **Non-Essential** | | | | | |
| Allo-isoleucine | 7.14 ±0.18[a] | 10.46 ±0.26[b] | | | |
| Alanine | 3.01 ±0.30 | 4.33 ±0.43 | 3.35 ±1.29 | 1.2 | |
| Aspartic acid | 2.88 ±0.77 | 3.72 ±1.0 | 2.93 ±0.30 | 2.3 | 7.61 |
| Proline | 2.63 ±0.14[a] | 3.39 ±0.18[b] | 2.27 ±1.07 | 0.5 | 6.68 |
| Glycine | 2.3 ±0.18[a] | 3.59 ±0.27[b] | 2.41 ±0.91 | 0.7 | 7.46 |
| Glutamine | 1.18 ±0.17 | 0.83 ±0.12 | 1.13 ±0.75 | 3.5 | 9.63 |
| Asparagine | 0.29 ±0.06 | 0.18 ±0.03 | 0.27 ±0.25 | | |
| β-Aminoisobutyric acid | 0.21 ±0.03 | 0.31 ±0.04 | 0.13 ±0.11 | | |
| Tyrosine | 0.17 ±0.08 | 0.28 ±0.06 | 0.62 ±0.50 | 0.7 | |
| α-Aminoadipic acid | 0.13 ±0.08 | 0.19 ±0.12 | 0.18 ±0.07 | | |
| Ornithine | 0.09 ±0.02 | 0.14 ±0.04 | 0.12 ±0.03 | | |
| Cysteine | 0.08 ±0.01[a] | 0.15 ±0.02[b] | 0.11 ±0.06 | 0.2 | |
| β-Amino butyric acid | 0.02 ±0.01 | 0.03 ±0.01 | 0.13 ±0.11 | | |
| **Essential** | | | | | |
| Lysine | 5.24 ±0.17[a] | 7.32 ±0.24[b] | 5.49 ±0.12 | 2.5 | 9.41 |
| Phenylalanine | 4.21 ±0.45[a] | 6.68 ±0.71[b] | 3.63 ±0.02 | | |
| Valine | 2.28 ±0.07[a] | 3.33 ±0.3[b] | 1.82 ±1.26 | 1.0 | |
| Leucine | | | 5.76 ±2.59 | 1.6 | 7.59 |
| Isoleucine | 2.27 ±0.19[a] | 3.62 ±0.1[b] | 0.22 ±0.15 | 0.9 | |
| Histidine | 0.91 ±0.41 | 1.58 ±0.71 | 0.83 ±0.10 | 0.4 | |
| Methionine | 0.1 ±0.02 | 0.16 ±0.03 | 0.12 ±0.05 | 0.9 | |
| Tryptophan | 0.1 ±0.03 | 0.1 ±0.03 | 0.15 ±0.00 | 0.2 | |
| **Total amino Acid** | 35.25 | 50.39 | | | |

Note: Values with a different word in a row means significant difference ($p$ <0.05). Source: *__Chasanah et al. (2015)__; **__Susilowati et al. (2016)__; *** __Sarma et al. (2015)__.

**Gonzales et al. (2006)**, who reported that both cultured and wild *Cichlasoma festae* living in tropical Ecuadorian rivers did not have significantly different values for K, Zn, and Fe, but did have significantly different values for P, Ca, and Mg, with the cultured fish having the higher values.

The value of Fe in *T. soro* flesh was following FAO's 2004 requirements for Fe content, that is, 0.23 – 2.1 mg.100g$^{-1}$ for human requirement **(FAO, 2004)**. The macro- and micromineral contents in the fish were highly dependant on the water environment from where the fish is harvested, and this study supported earlier reports that different geographic areas influence the proximate composition and minerals in fish. Exogenous factors such as environment/geographic area from where the fish is harvested as well as indigenous factors such as species, age, and sex will affect the chemical composition of the fish, including minerals.

***Amino acid profile***

Table 2 shows the amino acid profiles of the cultured and wild *T. soro*, which indicate that the cultured fish had a higher amount than the wild ones. However, the amino acid patterns of both fish were similar. The orders of amino acids in terms of amount were not much different. The essential amino acids in *T. soro* flesh were mostly lysine and phenylalanine, with some addition of isoleucine and valine, while the non-essential amino acids were composed of allo-isoleucine as the most abundant as well as alanine, glycine, proline, and aspartic acid.

In general, fish is well known as a prime nutrition source with a high quality of proteins. The essential amino acid composition in the *T. soro* studied, both wild and cultured, was dominated by lysine, followed by phenylalanine, valine, and isoleucine. Lysine was also reported as the dominant amino acid in freshwater fish from Indonesia, namely, *Channa striata* and *Osphronemus goramy* **(Chasanah et al., 2015**; **Susilowati, Sugiyono 2016)**. While **Mohanty et al. (2014)** reported lysine and aspartic acid as key amino acids in the freshwater fish harvested from cold water, the mahseer from Himalaya was reported to contain lysine and leucine and dominant amino acids. Even though the protein contents of *T. soro* and Himalayan mahseer (*Tor putitora*) were comparable, the concentrations of lysine and leucine were higher in Himalayan mahseer. As an essential amino acid, lysine plays an important role in human nutrition since this amino acid is required to obtain optimal growth. Hence, consumption of the fish can be used as one strategy to combat malnutrition and prevent stunting in children **(Marinda et al., 2018; Ngaisyah and Rohman, 2019)**. In this study, nonessential amino acid allo-isoleucine (7.14 ± 0.18%) in the wild group and 10.46 ±0.26% in the cultured group) was dominant in *T. soro*, while the Himalayan mahseer flesh was dominated by glutamic acid, aspartic acid, and glycine. Allo-isoleucine was detected as a dominant amino acid in *T. soro*, but nonexistent in *Tor putitora* and other freshwater fish such as *Channa striata* (Table 2). Since it is non-essential, this amino acid could be formed from a precursor in the feed. On the contrary, leucine which is an essential amino acid was absent in the *T. soro* studied. Allo-isoleucine is branched-chain amino acids (BCAAs) required for muscle formation and proper growth, as are leucine, isoleucine, and valine. This BCAA was found low in chronic renal failure (CRF) patients on hemodialysis **(Mohanty et al., 2014)**. Allo-isoleucine is associated with MSUD (Mapple syrup urinary disease) **(Merriam-Webster, 2020)**, and it has been used as a pathognomonic marker of branched-chain keto acid dehydrogenase complex (BCKDC) disorders **(Olson et al., 2014)**. *Tor soro*, both wild and cultured, are considered as sources of the functional amino acid (FAA) in human nutrition as it is rich in aspartic acid, glycine, proline, and glutamine. The non-essential amino acids glutamic acid, aspartic, and proline have participated in the healing process **(Sarma et al., 2015)**. Aspartic acid regulates the secretion of important hormones and is the precursor of methionine, threonine, isoleucine, and lysine **(Mohanty and Singh, 2018)**, while amino acid glycine is reported to be able to convert harmful materials in the body into harmless forms and to control gluconeogenesis and blood sugar. Meanwhile, glutamine plays an important role in regulating gene expression, intracellular protein turnover, nutrient metabolism, and oxidative defense **(Mohanty et al., 2014; Wu, 2010)**.

***Fatty acid profile***

Table 3 presents the fatty acid profile of *T. soro*. It shows that wild *T. soro*, in general, had higher fatty acids than those in the cultured ones, except arachidic acid (C20:0), eicosenoic acid (C20:1), EPA (C20:5), and linoleic acid (C18:2). The wild fish was richer in saturated fatty acid (SFA), while the cultured fish contained more monounsaturated fatty acid (MUFA) ($p$ <0.05). The wild and cultured fish were not significantly different in PUFA, total n-3, n-6 contents ($p$ >0.05), with similar patterns in the dominant fatty acids, i.e., oleic acid (C18:1 n9), palmitic acid (C16:0), linoleic acid (C18:2), DHA (C22:6), stearic acid (C18:0), and arachidonic acid (C20:4).

Polyunsaturated fatty acids (PUFAs), especially n-3, contribute positively to human health. Consumption of n-3 fatty acids has been reported to reduce the incidence of coronary heart diseases, depression, stroke, blood pressure disorders, glycemic index, triglycerides, and cancer **(Taşbozan and Gökçe, 2017; Williams et al., 2017)**. Among n-3 fatty acids, docosahexaenoic acid (DHA) 22:6 n-3 and eicosapentaenoic acid (EPA) C20:5 n-3 correlate to good health benefits **(Swanson et al., 2012; Mohanty and Singh, 2018)**. Our results show that wild and cultured *T. soro* had insignificant differences in total n-3, total n-6, DHA, and total PUFA, but cultured *T. soro* had higher EPA (eicosapentaenoic acid C20:5 n-3) compared to the wild one. The ratio of n-6 to n-3 in the fish studied was around 2, i.e., 1.99 ±0.06 and 2.17 ±0.13 for wild and cultured *T. soro*, respectively. The n-6/n-3 ratio of 1:5 is considered a good diet, but 1:2 is not harmful to human health **(Ana et al., 2019)**.

The EPA and DHA contents of the fish were derived from the feed and their endogenous metabolism system. Being an omnivore, the wild *T. soro* derived the EPA and DHA from freshwater plankton and algae as natural feeds available in the river. On the other hand, the cultured *T. soro* consumed commercial feed. Consequently, the fatty acid profile of the fish was very dependent on the fatty acid in the feed. Commercial feeds are usually composed of a variety of alternative plant-based ingredients such as legume seeds, oilseed cakes, leaf meals, vegetable oils, and other materials. In the case of the cultured *T. soro* in this study,

**Table 3** Fatty acid (FA) profile of wild and cultured *T. soro* compared with another freash water fish, *C. Striata*.

| Fatty acids (FA) | *Tor soro* | | *Channa striata** |
|---|---|---|---|
| | Wild (%) | Cultured (%) | |
| **SFA** | | | |
| C 4:0 (butiric acid) | 0.17 ±0.03[a] | 0.06 ±0.01[b] | 1.5 ±0.3 |
| C 12:0 (lauric acid) | 0.52 ±0.00[a] | 0.14 ±0.06[b] | |
| C 14:0 (miristic acid) | 2.53 ±0.03[a] | 1.71 ±0.09[b] | |
| C 15:0 (pentadecanoic acid) | 0.34 ±0.01[a] | 0.27 ±0.01[b] | |
| C 16:0 (palmitic acid) | 24.52 ±0.58[a] | 21.07 ±0.66[b] | 23.3 ±0.3 |
| C 17:0 (heptadekanoic acid) | 0.36 ±0.04 | 0.29 ±0.01 | |
| C 18:0 (stearic acid) | 8.08 ±0.08 | 8.06 ±0.01 | 9.8 ±0.6 |
| C 20:0 (arachidic acid) | 0.18 ±0.01[a] | 0.26 ±0.01[b] | |
| C 22:0 (behenic acid) | | 0.25 ±0.02 | |
| C 24:0 (lignocerate acid) | | 0.12 ±0.04 | |
| **TOTAL SFA** | 36.69 ±0.57[a] | 32.20 ±0.81[b] | 38.4 ±0.8 |
| C 14:1 (miristoleic acid) | | 0.08 ±0.01 | |
| 16:1 (palmitoleic acid) | 2.25 ±0.10 | 2.62 ±0.13 | 3.1 ±0.3 |
| C 20:1 (eicosenoic acid) | 0.78 ±0.03[a] | 1.41 ±0.08[b] | |
| C 18:1 n-9 (oleic/n-9 acid) | 25.77 ±0.13[a] | 29.29 ±0.11[b] | 23.2 ±1.0 |
| C 22:1 n-9 (erucic acid) | | 0.11 ±0.00 | |
| C 24:1 (nervonic acid) | | 0.16 ±0.02 | |
| **TOTAL MUFA** | 28.80 ±0.00[a] | 33.65 ±0.07[b] | 34.2 ±1.7 |
| C 20:2 (eicosadienoic acid) | 0.57 ±0.04[a] | 0.45 ±0.04[b] | |
| C 22:2 (docosadienoic acid) | 0.27 ±0.16[a] | 0.18 ±0.03[b] | |
| **Omega 3** | | | |
| C 18:3 (linolenic acid) | 0.98 ±0.02 | 1.14 ±0.01 | 2.7 ±0.7 |
| C 20:5 (EPA) | 0.86 ±0.12[a] | 1.27 ±0.03[b] | 0.9 ±0.2 |
| C 22:6 (DHA) | 9.44 ±0.34 | 8.16 ±0.64 | 4.0 ±0.9 |
| Total omega 3 | 11.27 ±0.44 | 10.57 ±0.66 | 11.3 ±0.2 |
| **Omega 6** | | | |
| C 18:3 (linolenic acid) | 0.32 ±0.00[a] | 0.21 ±0.00[b] | 0.5 ±0.1 |
| C 18:2 (linoleic acid) | 16.40 ±0.07[a] | 18.13 ±0.06[b] | 8.7 ±1.1 |
| C 20:3 (eikosatrienoic acid) | 1.57 ±0.05 | 1.20 ±0.01 | 0.4 ±0.1 |
| C 20:4 (arachidonic acid ) | 4.12 ±0.13[a] | 3.43 ±0.09[b] | 6.0 ±1.3 |
| Total omega 6 | 22.41 ±0.25 | 22.96 ±0.03 | 15.5 ±2.4 |
| TOTAL PUFA | 34.51 ±0.57 | 34.15 ±0.75 | 26.8 ±2.4 |
| Ratio PUFA/SFA | 0.94 ±0.03 | 1.06 ±0.05 | |
| Ratio n-6 /n-3 | 1.99 ±0.06 | 2.17 ±0.13 | |
| Ratio n-3 /n-6 | 0.50 ±0.01 | 0.46 ±0.03 | 0.8 ±0.1 |

Note: Values with a different word (superscript) in a row means significant difference ($p$ <0. 05). Source: ***Jaya-ram et al. (2018)**.

the feed consumed contained more EPA than those consumed by the wild ones. Although freshwater fish has been known not to be as good as marine fish at providing PUFA essential fatty acids, including n-3 fatty acids, this study showed that *T. soro* in both groups could be a better source of DHA than other freshwater fish such as *Channa striata*. DHA is a primary structural component of the human brain, cerebral cortex, skin, and retina **(Swanson et al., 2012)**. **Abouel-Yazeed (2013)** reported that the fatty acid compositions of marine and freshwater fish species from Egypt were comparable, indicating that fresh water could be used as the source of polyunsaturated fatty acids (PUFAs). Meanwhile, **Coutinho et al. (2019)** reported that *A. gigas*, a freshwater fish from Brazil's farm, was a great source of DHA (22:6 n-3) but had a low content of EPA (20:5 n-3). This agrees with **Jaya-ram et al. (2018)**, who reported that the freshwater fish from Bukit Merah Reservoir contained considerable amounts of beneficial omega-3 PUFA, thus usable as a source of EPA and DHA.

The *T. soro* studied were rich in linoleic acid (C18:2 n-6), and this amino acid, along with α-linolenic acid (ALA C18:3 n-3), can be converted into the long-chain fatty acids EPA and DHA by freshwater fish body systems **(Thilsted et al., 2014)**. The rates of conversion by endogenous metabolism are reported to vary between 1 and 10% by species **(Robert et al., 2014; Williams et al., 2017)**. Therefore, it can be inferred that the essential fatty acids, EPA and DHA found in both groups studied were from either feed or endogenous conversion. Phytoplankton is the primary producer of these essential fatty acids. Therefore,

besides diet, the fatty acid composition and another nutritional status of the fish will be ruled by many factors like species and environmental factors like salinity, temperature, seasons, geographical location, and weather, and this applies to both the farmed or wild fish **(Robert et al., 2014; Williams et al., 2017; Sarma et al., 2015)**.

## CONCLUSION

The *T. soro* studied, both wild and cultured, contained high protein and low-fat values. The proximate contents were comparable with *T. putitora* from the cold water of Himalaya. However, allo-isoleucine was detected only in the *T. soro* studied here, with the cultured fish containing higher amounts than the wild fish. *T. soro* could be used as a good source of macrominerals and microminerals, except for Se (selenium). Wild *T. soro* contained more lipid than the cultured one. However, the cultured *T. soro* was as good as the wild one in terms of PUFA, n-3, n-6, and DHA. The ratio of PUFA to SFA of the *T. soro* met the optimal ratio of PUFA to SFA in food diets, i.e., 1.0 – 1.5. Based on this study, the *cultured T. soro* could provide nutrition as well as the wild one, but increasing leucine while reducing allo-isoleucine is important.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE ANTIMICROBIAL EFFECT OF THYME AND ROSEMARY ESSENTIAL OILS AGAINST *LISTERIA MONOCYTOGENES* IN SOUS VIDE TURKEY MEAT DURING STORAGE

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## ABSTRACT

The research was aimed to study the impact of sous vide thermal treatment on the microbiological quality of fresh turkey breast meat after treatment with thyme and rosemary EOs and the survival of *Listeria monocytogenes* on the turkey meat samples. The samples were vacuum-packed and cooked at 55 °C, 60 °C, and 65 °C for 5, 15, 30 and, 60 min. There was an amount of 5 g (5 ±0.2 g) of the sample placed in PA/PE film bags and inoculated with 100 µL of *L. monocytogenes* inoculum. The sample was incubated at 37 °C for 18 h after bag sealing. The samples were tested on the 1st and 3rd days of experiments. The microbiological quality of fresh turkey breast meat was assessed by the detection of total microbial counts and meat microbiota was identified by mass spectrometry using MALDI-TOF MS Biotyper (Bruker Daltonics, Germany). Microbial counts differed significantly depending on temperature and time and the microbial counts ranged from 2.21 log cfu.g$^{-1}$ to 8.26 log cfu.g$^{-1}$ on the 1st and 3rd day of the experiment. The study shows that the sous vide method with essential oils combination is an effective method and it can be used to protect the microbiota of turkey meat and *L. monocytogens* survival, however, the quality of raw material is crucial.

**Keywords:** bacteria; MALDI-TOF MS Biotyper; turkey breast; essential oils; sous vide

## INTRODUCTION

Sous vide is a professional widely used food cooking and preservation technology and it is applied in catering, food industry, and home-made food production. The sous vide technology may also be referred to as lapping, vacuum cooking, vacuum-packed cooking, or baking-cooling in vacuum **(Nyati, 2000; Todd, 2014; Yikmi et al., 2018)**. Sous vide helps to improve food characteristics that meet customers' demands for "fresh-like" processed foods of good quality **(García-Linares et al., 2004; Stringer et al., 2012)**. *Listeria* monocytogenes is a foodborne pathogen that causes listeriosis. Listeriosis may be characterized by serious disorders as sepsis, meningitis, meningoencephalitis in immunocompromised patients, which may result in lifelong harm and/or death. Listeriosis cases were connected with the consumption of raw milk and dairy products, meat and poultry, fish, and Ready-to-eat (RTE) products **(Liu et al., 2012)**. Cooked chicken meat can be contaminated with *L. monocytogenes* during processing or post-processing activities **(Goh et al, 2014)**.

The poultry meat as was shown can be contaminated with pathogens so control of pathogens is a great challenge for poultry-processing companies to avoid economic losses and minimize public health risks **(Ferreira Moura et al., 2016)**.

Chemical food preservatives were recognized as an effective method to control spoilage and pathogenic bacteria. Nowadays, consumers demand healthy foodstuff without the addition of chemical preservatives and replace them with natural compounds. Essential oils (EOs) attracted the interest of the food industry to satisfy consumer needs. Essential oils (EOs) are aromatic oily liquids produced from different parts of plants such as leaves, seeds, flowers, or roots **(Burt, 2004)**. Natural extracts were reported to inhibit the growth and survival of *L. monocytogenes* in meat **(Mytle et al., 2006; Djenane et al., 2011)**.

*Thymus vulgaris* L. (thyme) is an aromatic plant of the *Lamiaceae* family **(Solomakos et al., 2008)** and its EO showed antibacterial activity **(Solomakos et al., 2008)**. Thymol is the main antibacterial compound of *T. vulgaris* EO and comprises over 50% of its chemical composition **(Rota et al., 2008; Govaris et al., 2011; Pesavento et al., 2015)**.

The eucalyptol is the main compound of *Rosmarinus officinalis* L. (Rosemary) is recognized for its antioxidative and antimicrobial activities **(Ojeda-Sana et al., 2013)** with activity against bacterial membrane **(Van Vuuren and Van Vijoed, 2007)**.

The study aimed to examine the effect of sous vide thermal treatment on the microbiological quality of fresh turkey

breast meat with thyme and rosemary EO and the survival of *Listeria monocytogenes*.

### Scientific hypothesis

The use of the sous vide method, temperature with time combination and essential oils addition allows to reduce microbiological contamination, reduce the number of bacteria, and survival of *L. monocytogenes* in food to a safe level.

## MATERIAL AND METHODOLOGY

### Sample

Fresh turkey breast meat purchased in a commercial chain was used for the study.

### Chemicals

Buffered peptone water (BPW, pH 7.0, Oxoid code CM0509, Basingstoke, UK), Tryptone Soya Agar (TSA, Oxoid, UK), Oxford Agar with the supplement of oxford supplement (OA, Oxoid, UK).

### Animal and Biological material

*L. monocytogenes* CCM 4699 was got from the Czech Collection of microorganisms (Brno, Czech Republic).

### Instruments

MALDI-TOF MS Biotyper (Bruker, Daltonics, Bremen, Germany).

### Laboratory method

### Microbiological analyses

There was 5 g of the turkey breast transferred into a sterile stomacher bag containing 45 mL of 0.1% buffered peptone water (BPW, pH 7.0, Oxoid code CM0509, Basingstoke, UK) and it was homogenized for 60 s at room temperature.

Appropriate serial decimal dilutions were prepared in 0.1% BPW solution for each sample. The amount of 0.1 mL of serial dilutions was spread on the surface of Tryptone Soya Agar (TSA, Oxoid, UK) for detection of total viable counts (TVC). They were counted on after incubation for 2 days at 30 °C. There was an Oxford Agar with the supplement of oxford supplement inoculated with 0.1 mL of sample. The incubation was carried out at 37 °C for 24 h.

### Identification of the bacteria

The colonies were resuspended in 300 µL of sterile distilled water after incubation, and there was 900 µL of absolute ethanol added. The mixture was centrifuged at 10,000 x g for 2 min. The pellet was centrifuged again after discarding the supernatant. The precipitate was allowed to dry at room temperature.

Then 30 µL formic acid (70%) and 30 µL of acetonitrile were added and mixed thoroughly with the pellet. The solution was centrifuged at maximum speed for 2 min and 1.5 µL of the supernatant was spotted on a polished MALDI target plate (Bruker Daltonics, Bremen, Germany). There was 1.5 µL of the matrix solution added to each spot and allowed to dry immediately after drying,

The samples were processed on a MALDI-TOF MS spectrometer with Flex Control software (Bruker Daltonics). Each spectrum was obtained by averaging 40 laser shots obtained in automatic mode with the minimum laser power necessary to ionize the samples. The spectra were analyzed and compared to the database according to real-time software, v3.1 classification.

### Description of the Experiments

### Sample preparation

The samples of fresh turkey breast meat were prepared as follow (Table 1, 2):

MC: turkey was vacuum packed in polyethylene bags and stored anaerobically at 4 °C, treated at 55 – 60 °C for 5 – 30 min;

MT: turkey with 0.1% thyme EO was vacuum packed in polyethylene bags and stored anaerobically at 4 °C, treated at 55 – 60 °C for 5 – 30 min;

MR: turkey with 0.1 % rosemary EO was vacuum packed in polyethylene bags and stored anaerobically at 4 °C, treated at 55 – 60 °C for 5 – 30 min;

MB: turkey with *L. monocytogenes* was vacuum packaged in polyethylene bags and stored anaerobically at 4 °C, treated with 55 – 60 °C for 5 – 30 min;

MBT: turkey with *L. monocytogenes* and 0.1% thyme EO was vacuum packed in polyethylene bags and stored anaerobically at 4 °C, treated at 55 – 60 °C for 5 – 30 min;

MBR: turkey with *L. monocytogenes* and 0.1 % rosemary EO was vacuum packed in polyethylene bags and stored anaerobically at 4 °C, treated with 55 – 60 °C for 5 – 30 min.

The samples were prepared under sterile conditions with 800 g of turkey which was divided into 78 samples. Meat (10 ±0.2 g) was placed in knurled vacuum bags, *Listeria monocytogenes*-infected samples were packed after inoculation in a vacuum sealer (Proficook PC-VK 1015). The control sample was prepared from raw meat on 0 days. The next day, EOs were added to samples, and maceration for 24 h was performed. The samples were prepared in CASO SV1000 sous vide device. *L. monocytogenes* CCM 4699 was prepared in concentration $10^8$ cfu and 100 µL was added to samples.

**Sample preparation:** 78
**Number of samples analyzed:** 78
**Number of repeated analyses:** 3
**Number of experiment replication:** 3

### Statistical analysis

All analyses were performed in triplicate. Statistical variability of data was processed using Microsoft-Excel® software. Analysis of variance (ANOVA) was used to evaluate the results. Comparison of the treatment means was based on Tukey's Honest Significant Difference (HSD) test.

## RESULTS AND DISCUSSION

The present study aimed to examine the control of microbiological hazards of food with the application of heat treatment and EOs. The raw meat without any antibacterial treatment is prone to microbiological spoilage and the growth of all groups of bacteria was demonstrated. The microbiological analysis confirmed that the thyme oil had the best inhibitory effect on *Listeria* and TVC.

TVC in meat samples without the addition of EOs ranged from 2.21 ±0.02 to 8.26 ±0.02 log cfu.g$^{-1}$ (Figure 1).

Food spoilage is a food deterioration during storage due to the proliferation of microorganisms resulting from external contamination or the proliferation of natural microbiota of meat. Common preservation methods help to prolong the shelf-life of products and delay microbial growth. There is a need to pay more attention to additional ingredients in food together with the development of food technology, that may improve the overall quality of food. Plant compounds such as EOs which have often been used in traditional and

**Table 1** Heat treatment conditions with the sous vide method of control samples.

| Control meat (MC) | | | Control meat with thyme EO (MT) | | | Control meat with rosemary EO (MR) | | |
|---|---|---|---|---|---|---|---|---|
| **No.** | **Temperature (°C)** | **Time (min)** | **No.** | **Temperature (°C)** | **Time (min)** | **No.** | **Temperature (°C)** | **Time (min)** |
| 1. | 4 | - | 14. | 4 | - | 27. | 4 | - |
| 2. | 55 | 5 | 15. | 55 | 5 | 28. | 55 | 5 |
| 3. | 55 | 15 | 16. | 55 | 15 | 29. | 55 | 15 |
| 4. | 55 | 30 | 17. | 55 | 30 | 30. | 55 | 30 |
| 5. | 55 | 60 | 18. | 55 | 60 | 31. | 55 | 60 |
| 6. | 60 | 5 | 19. | 60 | 5 | 32. | 60 | 5 |
| 7. | 60 | 15 | 20. | 60 | 15 | 33. | 60 | 15 |
| 8. | 60 | 30 | 21. | 60 | 30 | 34. | 60 | 30 |
| 9. | 60 | 60 | 22. | 60 | 60 | 35. | 60 | 60 |
| 10. | 65 | 5 | 23. | 65 | 5 | 36. | 65 | 5 |
| 11. | 65 | 15 | 24. | 65 | 15 | 37. | 65 | 15 |
| 12. | 65 | 30 | 25. | 65 | 30 | 38. | 65 | 30 |
| 13. | 65 | 60 | 26. | 65 | 60 | 39. | 65 | 60 |

**Table 2** Heat treatment conditions with the sous vide method of inoculated samples.

| Meat with L. monocytogenes (MB) | | | Meat with L. monocytogenes with thyme EO (MBT) | | | Meat with L. monocytogenes with rosemary EO (MBR) | | |
|---|---|---|---|---|---|---|---|---|
| **No.** | **Temperature (°C)** | **Time (min)** | **No.** | **Temperature (°C)** | **Time (min)** | **No.** | **Temperature (°C)** | **Time (min)** |
| 40. | 4 | - | 53. | 4 | - | 66. | 4 | - |
| 41. | 55 | 5 | 54. | 55 | 5 | 67. | 55 | 5 |
| 42. | 55 | 15 | 55. | 55 | 15 | 68. | 55 | 15 |
| 43. | 55 | 30 | 56. | 55 | 30 | 69. | 55 | 30 |
| 44. | 55 | 60 | 57. | 55 | 60 | 70. | 55 | 60 |
| 45. | 60 | 5 | 58. | 60 | 5 | 71. | 60 | 5 |
| 46. | 60 | 15 | 59. | 60 | 15 | 72. | 60 | 15 |
| 47. | 60 | 30 | 60. | 60 | 30 | 73. | 60 | 30 |
| 48. | 60 | 60 | 61. | 60 | 60 | 74. | 60 | 60 |
| 49. | 65 | 5 | 62. | 65 | 5 | 75. | 65 | 5 |
| 50. | 65 | 15 | 63. | 65 | 15 | 76. | 65 | 15 |
| 51. | 65 | 30 | 64. | 65 | 30 | 77. | 65 | 30 |
| 52. | 65 | 60 | 65. | 65 | 60 | 78. | 65 | 60 |

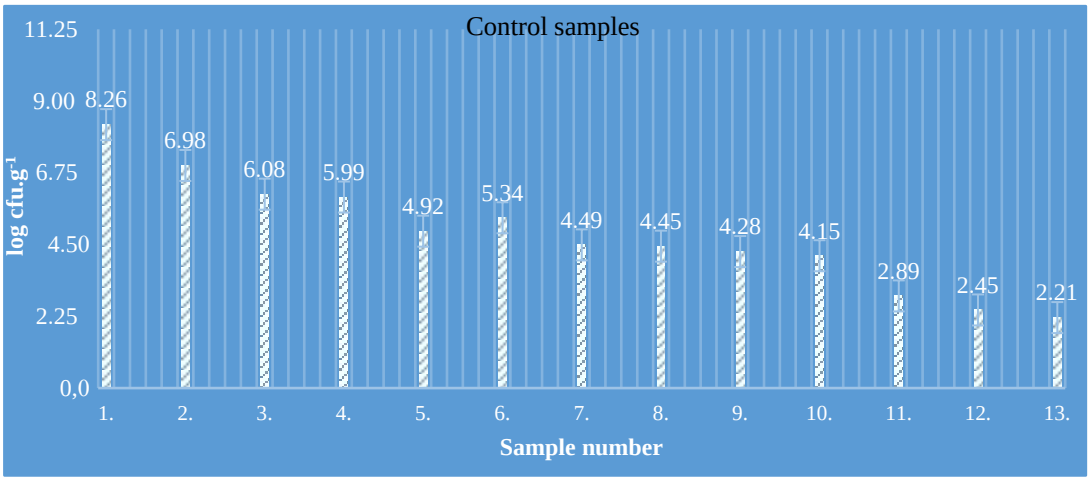


**Figure 1** The total number of microorganisms in meat samples without addition of Eos.

natural medicine for centuries are widely used natural substitutes for food preservation.

EOs are becoming more popular because of the increased palatability of products and inhibitory properties on spoilage microbiota **(Król et al., 2013)**. It seems that for microbial growth in Sous vide products, products stored at 3 and 10 °C have longer shelf life than 40 days, while the microbial growth started on day 9 in products stored at 20 °C **(Yıkmı et al., 2018)**.

TVC in meat samples with the addition of thyme EO ranged from 1.96 ±0.02 to 6.38 ±0.02 log cfu.g$^{-1}$ (Figure 2) and rosemary EO ranged from 1.89 ±0.02 to 7.7 ±0.02 log cfu.g$^{-1}$ (Figure 3).

This study showed that thyme EO was more effective than rosemary EO in the reduction of TVC. This fact can be related to the chemical composition of this EO which contains phenolic compounds in high concentrations.

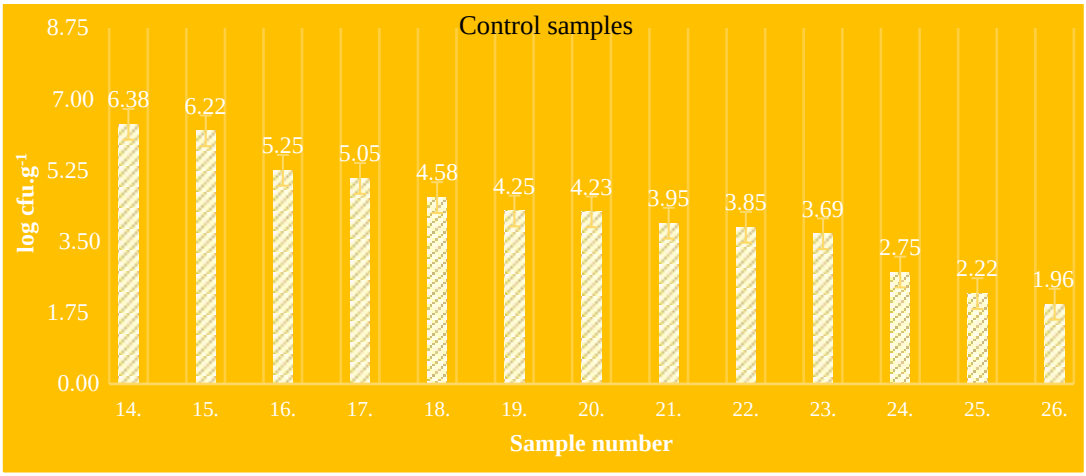


**Figure 2** The total number of microorganisms in meat samples with addition of thyme EO.

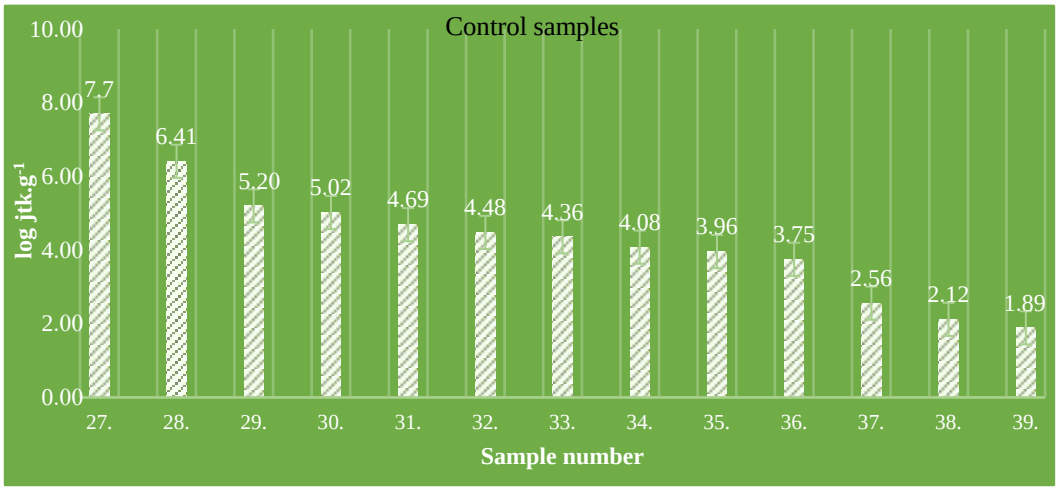


**Figure 3** The total number of microorganisms in meat samples with addition of rosemary EO.

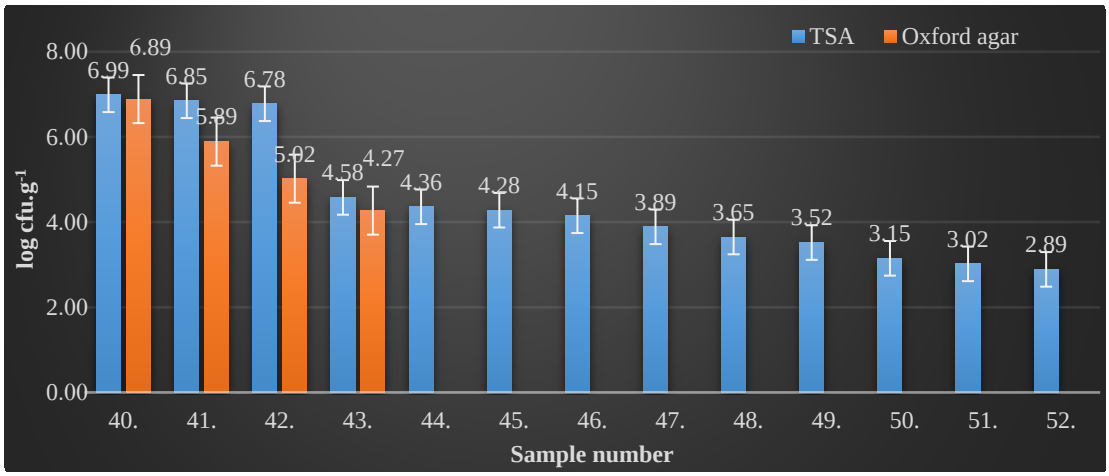


**Figure 4** The total number of microorganisms and *L. monocytogenes* in meat samples without addition EO.

Thyme EO exhibited an antimicrobial effect on the growth of *L. monocytogenes* and these results were in agreement with **Pesavento et al. (2015)** who reported a higher antimicrobial effect against *L. monocytogenes* with the addition of thyme EO at different concentrations in minced meat stored at 4 °C.

The use of essential oils in food inhibits the growth of pathogenic microorganisms **(Nazzaro et al., 2013)**. There were different effects of EO on the growth of Gram-positive and Gram-negative bacteria observed with a more pronounced effect of EOs on Gram-positive bacteria than Gram-negative bacteria were identified due to differences in the cell wall structure **(Król et al., 2013)**.

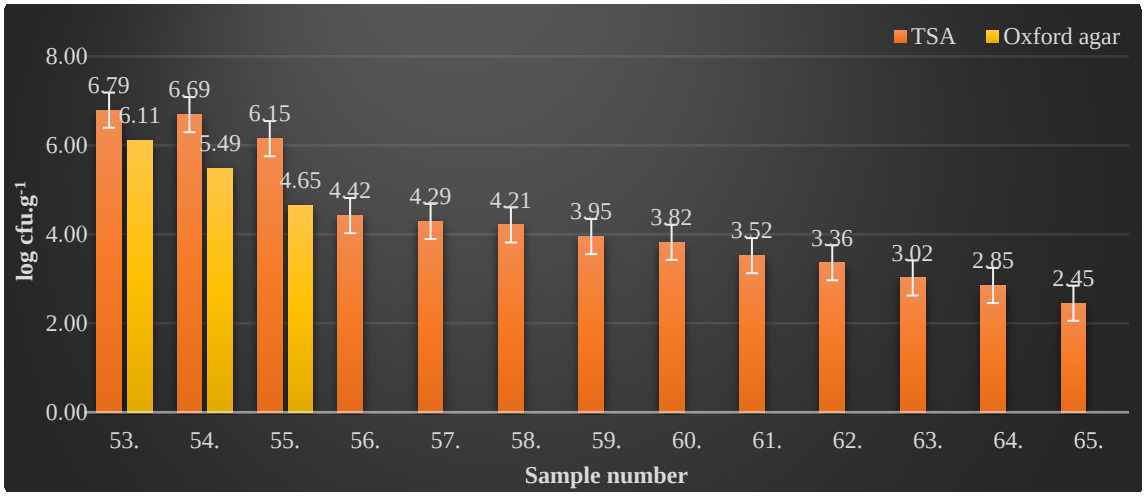


**Figure 5** The total number of microorganisms and *L. monocytogenes* in meat samples with addition of thyme EO.

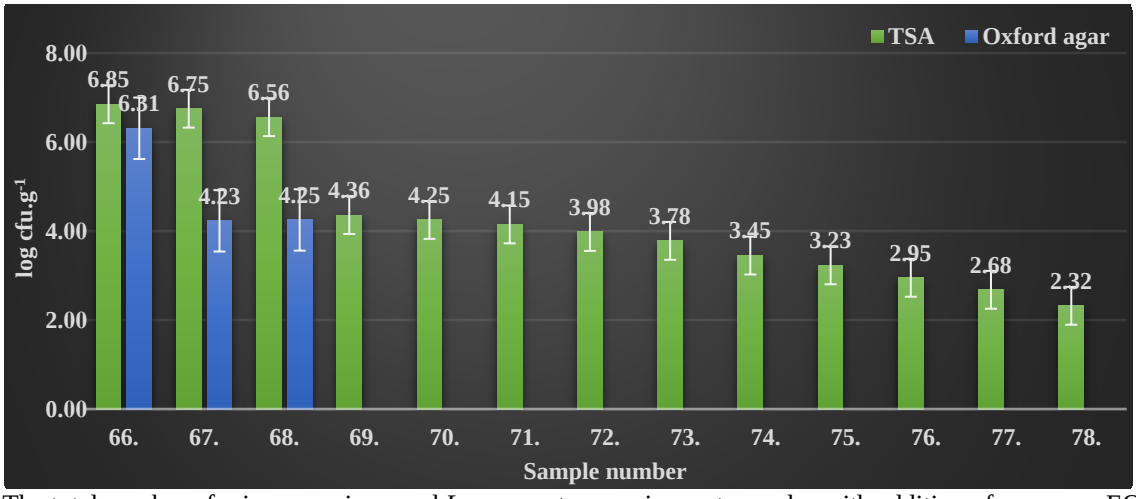


**Figure 6** The total number of microorganisms and *L. monocytogenes* in meat samples with addition of rosemary EO.

**Moura-Alves et al. (2020)** used saga EO and sous vide treatment of beef meat with addition of *L. monocytogenes* and they investigated, that sage as a natural preservative, must be combined with other agents to control microbial growth more effectively.

TVC in meat samples with the addition of *L. monocytogenes* ranged from 2.89 ±0.02 to 6.88 ±0.02 log cfu.g$^{-1}$ (Figure 4). TVC in meat samples inoculated with *L. monocytogenes* and thyme EO ranged from 2.45± 0.02 to 6.79 ±0.02 log cfu.g$^{-1}$ (Figure 5) and *L. monocytogenes* and rosemary EO ranged from 2.32 ±0.02 to 6.85 ±0.02 log cfu.g$^{-1}$ (Figure 6).

Significant differences ($p$ <0.05) were between samples stored at 4 °C, but differences were not found between control samples with Listeria and samples with *Listeria* with thyme EO, between control samples with *Listeria* and samples with *Listeria* with rosemary EO and between samples with *Listeria* with thyme EO and samples with Listeria with rosemary EO. Significant differences ($p$ <0.05) were noted between samples treated at 55 °C during 15'. No significant differences were between control samples and samples with *Listeria* with thyme EO, control samples with thyme EO and control samples with rosemary, and between control samples with *Listeria* and samples with *Listeria* with rosemary EO. There were significant differences ($p$ <0.05) between samples treated at 55 °C during 30', no significant differences were only between the control group with thyme EO and the control group with rosemary EO. Significant differences ($p$ <0.05) were noted between samples treated at 55 °C during 60', no significant differences were only between samples with *Listeria* and thyme EO and samples with *Listeria* and rosemary EO. Significant differences ($p$ <0.05) were noted between samples treated at 60 °C during 5', no significant differences were between the control group with *Listeria* and control group with thyme EO and between the control group with thyme EO and samples with *Listeria* with thyme EO. Significant differences ($p$ <0.05) were found between samples that were treated at 60 °C during 15' and 30'. There weren´t significant differences only between samples with *Listeria* and thyme EO and samples with *Listeria* and rosemary EO. There were significant differences ($p$ <0.05) between all analyzed samples which were treated at 55 °C during 5', 60 °Cduring 60' and 65 °C during 5', 15', 30' and 60'.

The study of **Abel et al. (2020)** investigated the heat inactivation efficiency of *L. monocytogenes* in nutritive solution (BHI) and under sous vide heating conditions of game meat. The results showed that the heat inactivation was strongly affected.

**Table 3** Isolated bacterial strains from turkey without addition of EOs.

| MC | | MR | | MT | |
|---|---|---|---|---|---|
| 1. | *Escherichia coli, Proteus mirabilis* | 14. | *Escherichia coli, Proteus mirabilis* | 27. | *Escherichia coli, Proteus mirabilis, Stenotrophomonas maltophilia* |
| 2. | *Proteus mirabilis* | 15. | *Proteus mirabilis* | 28. | *Escherichia coli* |
| 3. | *Escherichia coli* | 16. | *Escherichia coli* | 29. | *Rhizobium radiobacter, Stenotrophomonas maltophilia* |
| 4. | *Escherichia coli, Proteus mirabilis* | 17. | *Escherichia coli* | 30. | *Proteus mirabilis, Serratia liquefaciens* |
| 5. | | 18. | *Escherichia coli* | 31. | *Serratia liquefaciens* |
| 6. | *Escherichia fergusonii* | 19. | *Escherichia coli* | 32. | *Escherichia coli* |
| 7. | *Escherichia coli* | 20. | *Escherichia coli* | 33. | *Serratia liquefaciens* |
| 8. | *Escherichia coli* | 21. | *Serratia liquefaciens, Stenotrophomonas maltophilia* | 34. | *Proteus mirabilis, Stenotrophomonas maltophilia* |
| 9. | *Escherichia coli* | 22. | *Proteus mirabilis, Staphylococcus saprophyticus* | 35. | *Escherichia coli* |
| 10. | *Stenotrophomonas maltophilia* | 23. | *Escherichia coli, Proteus mirabilis* | 36. | *Escherichia coli* |
| 11. | *Serratia liquefaciens* | 24. | *Enterococcus faecalis, Escherichia coli* | 37. | *Serratia liquefaciens* |
| 12. | *Serratia liquefaciens* | 25. | *Escherichia coli* | 38. | *Stenotrophomonas maltophilia* |
| 13. | *Escherichia coli* | 26. | *Stenotrophomonas maltophilia* | 39. | *Escherichia coli* |

Farag proved that thyme and caraway EOs have the strongest antibacterial activity compared to other EOs **(Farag et al., 1989)**. A study of inhibitory properties of 60 different EOs on *P. putida* strain isolated from meat showed that oregano oil had high antibacterial activity **(Oussalah et al., 2006)**. The anise extracts also characterized by strong antibacterial activity against *P. aeruginosa* and *Candidia albicans* **(Chanwitheesuk et al., 2005)**. **Karyotis et al. (2017)** described log-linear inactivation kinetics for *L. monocytogenes* and *Salmonella* in marinated chicken breast heated at different temperatures between 55 °C and 60 °C in another study.

**Betts and Gaze (1995)** studied the growth and heat resistance of bacteria in sous vide products and found the relationship between the temperature and time of processing and the growth of bacteria during storage. An increase of temperature up to 90 °C may significantly reduce the number of microorganisms **(Betts and Gaze, 1995)**.

**Lee et al. (2017)** compared in their study the growth curves of *L. monocytogenes* inoculated in beef with storage temperatures between 5 and 25 °C. There was the development of *L. monocytogenes* at 5 °C observed.

The addition of rosemary EO at 1.25% (v/w) was found to be effective against *L. monocytogenes*. However, no statistical differences were observed after the addition of rosemary EO at 0.2% (v/w) in poultry fillets stored at 4 °C after 7 days of storage **(Kahraman et al., 2015)**. The antimicrobial effect of rosemary EO could be associated with eucalyptol which is the main chemical compound. The oxygen groups of eucalyptol can also disrupt the cell membrane structure even in subinhibitory concentrations **(Sousa et al., 2015)**.

**Mizi et al. (2019)** reported that the combined usage of sage (powder) and high-pressure processing in beef burgers using two concentrations of sage (0.3% and 0.6%) does not result in any antimicrobial activity against *L. monocytogenes*.

The differences between the present and previous reports were explained with *L. monocytogenes* strain characteristics and the main compounds of EOs **(Abdollahzadeh et al., 2014)**. The antimicrobial effect of EOs is related to intrinsic factors such as food composition, as well as extrinsic factors such as including temperature and presence of oxygen **(Hayouni et al., 2008)**.

**Table 4** Isolated bacterial strains from turkey without addition of EOs and *L. monocytogenes*.

| MB | | MBR | | MBT | |
|---|---|---|---|---|---|
| 40. | *Listeria monocytogenes* | 53. | *Listeria monocytogenes* | 66. | *Listeria monocytogenes, Acinetobacter dijkshoomiae, Proteus mirabilis* |
| 41. | *Acinetobacter pittii, Listeria monocytogenes* | 54. | *Listeria monocytogenes* | 67. | *Escherichia coli, Listeria monocytogenes* |
| 42. | *Acinetobacter pittii, Escherichia coli, Listeria monocytogenes* | 55. | *Listeria monocytogenes, Escherichia coli* | 68. | *Listeria monocytogenes, Proteus mirabilis* |
| 43. | *Listeria monocytogenes, Escherichia coli* | 56. | *Escherichia coli* | 69. | *Escherichia coli* |
| 44. | *Enterococcus faecalis, Escherichia fergusonii* | 57. | *Escherichia coli* | 70. | *Serratia liquefaciens* |
| 45. | *Escherichia fergusonii* | 58. | *Serratia liquefaciens* | 71. | *Escherichia fergusonii* |
| 46. | *Enterococcus faecalis,* | 59. | | 72. | *Serratia liquefaciens* |
| 47. | *Escherichia coli* | 60. | *Serratia liquefaciens, Stenotrophomonas maltophilia* | 73. | *Stenotrophomonas maltophilia* |
| 48. | *Stenotrophomonas maltophilia* | 61. | *Proteus mirabilis, Staphylococcus saprophyticus* | 74. | *Escherichia coli* |
| 49. | *Serratia liquefaciens* | 62. | *Escherichia coli, Proteus mirabilis* | 75. | *Stenotrophomonas maltophilia* |
| 50. | *Serratia liquefaciens* | 63. | *Enterococcus faecalis, Escherichia coli* | 76. | *Serratia liquefaciens* |
| 51. | *Stenotrophomonas maltophilia* | 64. | *Stenotrophomonas maltophilia* | 77. | *Stenotrophomonas maltophilia* |
| 52. | *Serratia liquefaciens* | 65. | *Bacillus* spp. | 78. | *Serratia liquefaciens* |

**Moura-Alves et al. (2016)** found out that the counts of *L. monocytogenes* in beef samples with rosemary EO stored at 2 and 8 °C decreased about 2 $\log_{10}$ cfu.

There were some results in the study **Giarratana (2016)** which revealed that the mixture of rosemary and thyme EOs had a bacteriostatic activity against *L. monocytogenes* and both 0.025 and 0.05% of tested EOs significantly inhibited *L. monocytogenes* growth compared with the control sample.

Additionally, in a study carried out by **Raeisi et al. (2016)**, the effects of sodium alginate coating with nisin, cinnamon, and rosemary EOs individually and in combinations on the fate of *L. monocytogenes* in chicken meat during 15 days of refrigeration were studied. The control sample and the sample coated with alginate solution had the highest growth rate of *L. monocytogenes*, while other treated samples, especially those with the combined use of tested antimicrobial agents, resulted in the inhibition of *L. monocytogenes*, whereas the combination of cinnamon and rosemary EOs, rosemary EOs and nisin, and cinnamon EOs and nisin had the lowest final population, respectively, indicating the synergistic effect of these EOs and nisin in controlling *L. monocytogenes.*

Similarly, **Pavli et al. (2019)** found out that the incorporation of oregano EO into sodium alginate edible films in ham slices led to a 1.5 log cfu.$g^{-1}$ decrease in population of *L. monocytogenes* at the end of the storage (40 days) at 8 and 12 °C and an approximately 2.5 log cfu.$g^{-1}$ reduction at 4 °C. They finally indicated that a significant reduction or absence of *L. monocytogenes* was achieved in ham slices by application of high hydrostatic pressure and an edible film containing oregano EO, together.

Increased heat treatment temperature harmed the sensory properties of food and may reduce digestibility. Lower storage temperature of heat treatment reduces the storage time **(Vaudagna et al. 2002)**.

The results of **Kluz et al. (2016)** study suggest the possibility of using caraway and anise EO as natural food preservatives and a potential source of antimicrobial ingredients for chicken breast meat.

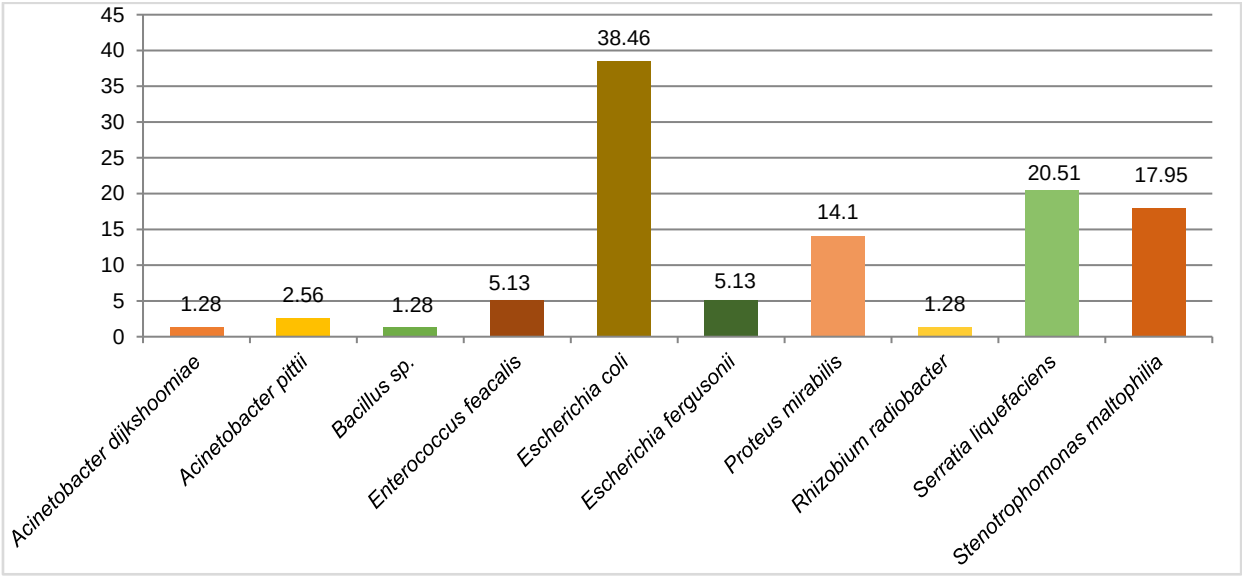


**Figure 7** Isolated bacterial strains (%).

*Pimpinella anisum*, *Mentha spicata* var. *crispa*, *Thymus vulgaris* L., *Origanum vulgare* L. EOs exhibited promising results in the study of **Kačániová et al. (2016)** for natural food preservatives and potential sources of antimicrobial ingredients for the food industry for chicken meat.

There were *Acinetobacter dijkshoomiae* (1.28%), *Acinetobacter pittii* (2.56%), *Bacillus* spp. (1.28%), *Enterococcus feacalis* (5.13%), *Escherichia coli* (38.46%), *Escherichia fergusonii* (5.13%), *Proteus mirabilis* (14.1%), *Rhizobium radiobacter* (1.28%), *Serratia liquefaciens* (20.51), and *Stenotrophomonas maltophilia* (17.95%) isolated from turkey in the present study (Tables 3 – 4, Figure 7).

**Kunová et al. (2017)** isolated *Staphylococcus warneri* from the control group stored in the air conditions, *Kocuria rhizophila* from control vacuum-packed control samples, *Staphylococcus warneri*, *Aeromonas salmonicida* and *Aeromonas popoffii* from control group treated with EDTA, *Staphylococcus hominis*, and *Staphylococcus epidermidis* from meat treated with caraway EO. There were in total, 15 genera identified from meat after EO and vacuum packaging: *Aeromonas*, *Aromatoleum*, *Buttiauxella*, *Clostridium*, *Enterobacter*, *Hafnia*, *Lactobacillus*, *Lysinobacillus*, *Pantotea*, *Pseudomonas*, *Rahnella*, *Raoultella*, *Serratia*, *Staphylococcus*, and *Yersinia* **Kačániová et al. (2019)**.

## CONCLUSION

Turkey meat supports microbial growth including pathogenic microbiota. Storage and processing of meat may influence the microbiological contamination of turkey meat. Our study shows that the sous vide method is an effective method for the treatment of fresh turkey breast meat to protect the meat from spoilage. There were evaluated in our study that sous vide method with combination with essential oils is effective against the total count of microorganisms and *L. monocytogenes*. The best results were found by treatment samples with rosemary EOs. Temperature and application of EOs have a positive effect against the total count of bacteria and especially against *L. monocytogenes*.

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# THE STATE AND THE SHARE OF RURAL WOMEN ON THE AGRICULTURAL ENTREPRENEURSHIP ACTIVITIES IN THE SLOVAK REPUBLIC

***Zuzana Kapsdorferová, Silvia Jacková, Petronela Švikruhová***

## ABSTRACT

The economic empowerment of rural women in the recent development of agriculture through entrepreneurship is considered an important challenge. Rural women often manage complex households and pursue multiple livelihood strategies. Their activities typically include cultivation of crops, farming animals, processing and preparing food, working for wages in agricultural or other rural enterprises, collecting fuel and water, engaging in trade and marketing, caring for family members, and maintaining their homes. The research aimed to collect up-to-date data on Slovak rural women dealing with business in the field of agriculture, women's role and status in society in rural areas, on the farm, women's participation in education, training, and agricultural services. The main focus is dedicated to their share on the management of agricultural entities, land cultivation, employment of rural women and men, and their deal on the selected entrepreneurial activities of Slovak agriculture. Women doing business in agriculture dispose of with considerable knowledge and experience in the agricultural and food sectors. The realized research showed that the scale of farms managed by women is 325 hectares. Rural women in the surveyed group are taking care of about 2.5% of the total area of agricultural land in Slovakia and they are employing 2.40% of the working forces in the agricultural sector in Slovakia. According to our survey, rural women are under-represented in managerial and ownership positions of agricultural entities.

**Keywords:** agribusiness; agricultural entities; rural women; gender gap; agricultural entrepreneurial activities

## INTRODUCTION

The mission and tasks of rural women are differentiating according to individual states, regions, continents. Despite this, women everywhere in the world have to face in their professional and private lives various specific limitations. According to **FAO (2011)**, women represent approximately 43% of the world's working forces in agriculture, but they do own less than 20% of agricultural land in the world. If women would have a better approach to financial and production sources, across the world, this could significantly downsize the number of people affected by hunger. Moreover, the nutrition status of the world population would immensely improve owing to the higher yields of about 20 to 30%. This would virtually mean fewer hungry and undernourished people about 12 – 17% in the world **(FAO, 2011)**. Historically rural women always played in the society important key role, both in the care of the members of their families as well as in crop production together with animal rearing. Throughout the millennials was, and this is persisting, the great share of rural women at the arrangement of nutrition in families through their works in kitchens, on fields, gardens, orchards, forests, and in stables. If the rural women would be more involved in agriculture and rural development as the owners of the farms or managers, this would lead to the improvement of their social status and upgrade the living standards of their families. Very essential is also the fact that this would generate new working opportunities for females and men. Therefore, it is desirable for women living and acting in the countryside to create appropriate space for their self-realization and education opportunities through which, the involvement of rural women in entrepreneurial activities can be reasonably upgraded. The main problems of rural women can be divided into four main categories economic, social, family level, and individual. Most of the problems are connected to the social and political change and transition taking place in the countries. For many rural women and families, the transition from one system to another has meant increased economic problems and a loss of paid labor and unemployment. In private agriculture, the main problem is low-income levels. For many rural women, the economic problem is dependence on their husband's income. Social problems are connected with economic problems; unemployment and a lack of income are usually connected with malnutrition, illness, and alcoholism. In many CEE countries, the social infrastructure in the villages was connected with the agricultural production system; giving up the state and cooperative farms led to the loss of

social services such as kindergartens as well as health and maternity care provided by the state and cooperative farms. In addition, many cultural activities were connected with the state and cooperative farms and have been lost during the transition **(Siiskonen, 1996)**. The objective of the submitted scientific article is to evaluate the status of the agricultural business environment in the Slovak Republic with a focus on those enterprises in which the owners are women or eventually in which the women are undertaking managerial positions. Furthermore, the objective is to find out the share of rural women in agricultural entrepreneurial activities.

### Scientific hypothesis

This research paper aims to collect up-to-date data on Slovak rural women dealing with business in the field of agriculture, women's role and status in society, in rural areas, on the farm, and women's participation in education, training, and agricultural services.

Several hypotheses were formulated:

Hypothesis 1: Rural women have equal business rights when asking for various kinds of state or EU supports.

Hypothesis 2: We assume that most women operate their businesses in the form of an individual farmer.

Hypothesis 3: There exists dependency between the size of farms in hectares and the legal business forms.

Hypothesis 4: There exists a dependence between several years of practical experience of rural women in agriculture and the legal form of business.

## MATERIAL AND METHODOLOGY

In this part of a scientific article, we do identify the share of rural women in various economic activities performed in the frame of agricultural and food sector, as well as the share of women on the acreage of agricultural land, number of agricultural entities with different legal statuses, or their share on the number of employees. Moreover, we deal with the evaluation of rural women's share in the rearing of farm animals. For rendering primary data was as the main research technique used questionnaire survey, furthermore with rural women had been conducted face-to-face interviews. The gathering of data was carried out in two rounds; the first round was focused on a pilot survey which was carried out during summer 2017. In this way, it was tested the functionality and correct construction of the questionnaire survey. Furthermore, following above mentioned steps, those agricultural companies that were led by women have been approached with consistent analytical and synthetical research in 2018. The total number of agricultural enterprises involved in the questionnaire survey was 130. The following formula was used to estimate the minimum number of samples (*n*) **(Szreder, 2004)**:

$$n = \frac{\frac{1}{4} \times N}{N \times \frac{d^2}{z^2_{\alpha/2}} + \frac{1}{4}}$$

Where:

*N* – number of entities; $z^2_{\alpha/2}$ – the value of random variable Z of normal standard distribution; *d* – statistical error.

In the studies, it was assumed that the maximum statistical error of the results may amount to +/- 5%. The necessary minimum sample size was set at 116 business entities. After the collection of required data, this had been classified, processed, and evaluated. To statistically elaborate the obtained data, was utilized program XLStat and as the key statistical methods had been applied nonparametric statistical tests such as Kruskal-Wallis test Fisher´s test, Friedman´s test, and Dunnett method. Computation of the Kruskal-Wallis test involves arranging the score from all the groups in order and assigning ranked scores. The sum of ranks is computed for each group and used in the equation below to determine whether there are group differences i the ranks (equation adapted from **Siegel and Castellan, 1988**).

$$KW = \left[\frac{12}{N(N+1)} * \sum_{i=1}^{k} n_i \bar{R}_i^2\right] - 3(N+1)$$

Where:

$k$ – represents the number of groups; $n_i$ – represents the number of cases in the *i group*; $\bar{R}_i$ – mean rank from the *i group*; *N* – represents the total sample size.

The test determines whether the medians of two or more groups are different. Like most statistical tests, you calculate a test statistic and compare it to a distribution cut-off point.

Rural women from the surveyed group have in general university education, mainly in the field of agriculture. The average age of female farmers is 49 years. Rural women are doing their business as the individual farmers managing the micro-companies, or small companies, although they are prepared to manage even larger companies, so they are daring enough to take over a larger responsibility in the position of managerial staff. This later is in recent years considered as a very natural trend as the women more and more are managing larger companies and occupy seats on the top managerial positions. Concerning the average size of farms managed by rural women, this achieves 325 hectares of agricultural land. In most cases, the rural women in their business companies own only a small part of the land and the rest is rented for a long-term period from the local community, individual owners, or from State Land Fund. Rural women are doing their business mainly in the field of crop production rather than in animal production. Also, they do concentrate their effort on the provision of services, such as agro-tourisms, or the sale of primary or processed agricultural products. Doing agribusiness activities means from rural women's value point of view, first of all, continuation in family traditions. They like this work since they grew up with it. If rural women would be once more given the opportunity to select the field of their entrepreneurial activities, they will again commence with business linked to agriculture and food processing. It should be emphasized that businesswomen in agriculture do have a wide experience and knowledge in the given fields. The average years of practical experience are recording 23 years. Rural women consider themselves as emancipated personalities and they do not feel any discrimination concerning their business activities.

**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) Kruskal Wallis test
2) Friedman´s test
3) Fisher´s test and
4) Dunnett method

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

Women possess good personality traits for business, such as excellent organizational and communication skills, they are well motivated, dedicated, consistent, and able in an empathic way to navigate their colleagues into positive thinking and to lead them to high performances **(Kadlečíková and Veselá, 2011)**. Women are farmers, workers, and entrepreneurs, but almost everywhere they face more severe constraints than men in accessing productive resources, markets, and services. This "gender gap" hinders their productivity and reduces their contributions to the agriculture sector and the achievement of broader economic and social development goals. Closing the gender gap in agriculture would produce significant gains for society by increasing agricultural productivity, reducing poverty and hunger, and promoting economic growth **(Patil and Venkatachalapathi, 2018)**. Rural women's role and mission in agriculture are not irreplaceable at the land cultivation and achievement of the nutrition for inhabitants **(Bujňáková, 2010)**. According to **FAO (2011)** the most significant recent challenges in agriculture, besides the others, are that women have no sufficient resources and opportunities for their own business. In various states is very complicated to assign, that what is the share of rural women in agricultural activities **(Doss, 2011)**. A very important precondition for rural women 's business the education. In this connection, they state that tertiary agricultural education achieved in 2012 about 8.4% more women than men, by this they proved to have a considerable place in the economy, also in science, and politics **(Jacková et al., 2016)**. In line with authors **Schneiderová (2015)** states that from universities are graduated significantly more women than men, even with better study results. The same author concluded that women-entrepreneurs in the same age category are achieving the top positions in business as their male colleagues. This means the age around 50 years with already completed long career rout around of 20 – 25 years in the agribusiness sector.

In the Slovak countryside is living 42.1% of women of productive age, **(ILO 2018, Fares et al., 2007)**. In the years 1999 – 2008 was the share of the European women in the age of 15 – 49 years at the level of 49.5%. In the rural areas, this indicator was a little bit lower (47.7%), while in cities this achieved even 50.4%. In Slovak circumstances have been achieved analogical results about first indicator 49.2%, then in countryside 48.2 % and finally in the city agglomerations, it was 50.1%. **Grznár and Szabo, (2015)** and **Buchta (2013)** highlighted that a serious challenge is employees' sharp decline in the agricultural sector. These authors do propose the development of supporting investment programs that will encourage the employment of young and female farmers.

The share of unemployed rural women in Slovakia is rather high. In 2012 this represented even 18.4%. During the period 2007 – 2014 the average share of unemployed rural women dropped by 16.55%. In comparison with other states, rural women's employment was more favorable e.g. in the Czech Republic with 8.19% and Austria with 3.53% **(ILO, 2018)**. In the environment of the Slovak Republic comparing with average data, fewer women are involved in entrepreneurial activities in agriculture and food processing. According to the Statistical Office of the Slovak Republic in agribusiness is employed only 26.5% of women as the physical working staff, managers, or owners of the farms **(Statistical Office of Slovak Republic, 2018)**. Women in the past were working behind the men and they were invisible on the farm for decades as farmers' wives with their unpaid work. Historic inequality in ownership and heritage of land and control are cause and consequence of the opinion that farming is not considered a suitable career for women **(Robertson, 2017)**.

Stemming from the above-cited literature sources, it would be important to achieve a more balanced equal status of men and women in agriculture and to shift the attitudes in favor of an increasing number of rural women who will run their business entities.

### The Share of Rural on the Development of Agricultural Activities

In this part of a scientific article, we do identify the share of rural women in various economic activities performed in the frame of agricultural and food sector, as well as the share of women on the acreage of agricultural land, number of agricultural entities with different legal statuses, or their share on the number of employees. Moreover, we deal with the evaluation of rural women's share in the rearing of farm animals.

#### *The Share of Rural Women on the Cultivated Agricultural Land*

In 2016 the acreage of the utilized agricultural land represented 1 918 878 hectares. In comparison with the year 2015, there was a noted decline of about 2 685 hectares (0.14%) and in comparison with the year 2012, the decline was even about 8 572 hectares. The acreage of tillage in 2016 was 1 347 293 hectares, comparing with 2012 it was noted downsizing about 12 686 hectares, so every day in the indicated period was lost 9.2 hectares of tillage. This result is documenting irresponsible behavior to the environmentally friendly approach about the care of natural resources, which to the end can very seriously harm the state food security very soon.

In 2017, women involved in undertaken research, in total cultivated 39 418,25 hectares of land (2.05% from the overall area of agricultural land). This percentage can be

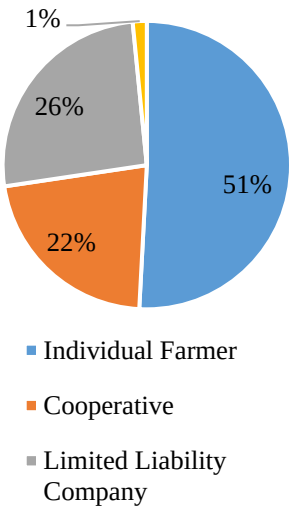


**Figure 1** Classification of Companies According of Legal Forms in %. Note: Own processing based on questionnaire survey.

considered as a negligible extend and it is proof that the involvement of rural women in agricultural entrepreneurial activities is so far very low. The same is valid about the number of farms managed by rural women. Taking into consideration that in Slovakia the number of total agricultural entities is 27 833 **(Green Report, 2017)** and that our research had been involved 130 farms managed by rural women, then their share on the management and ownership of agricultural entities is only 0.46%. The size of one agricultural entity managed by the rural woman in our case is 325 hectares. This is significantly higher than the Slovak average with 80.7 hectares. In this respect, it should be positively regarded the courage of rural women to establish larger entrepreneurial entities with determination to further expand their business. The management of larger enterprises is more demanding from a managerial point of view, but it has to be reiterated, that women from our research group exceed with excellent organizational traits, great determination, and intuition.

***The Share of Rural Women on Agricultural Employment***

The share of agriculture in total employment in the national economy was just 2.89% in 2017 **(Green Report, 2017)**. This result is connected with the permanent outflow of working forces from the sector to the other branches of the national economy which commenced with the transition process of the national economy and continued with country accession to the EU.

In 2017 was employed in Slovak agriculture with 52,5 thousands of employees, from which women represented 13,1 thousand (24.95%) and men 39,4 thousand (75.05%) **(Green Report, 2017)**. The number of women employed in agriculture in the analyzed period had relatively stable development, the least women worked in the sector in 2015 (12,8 thousand).

The agricultural entities led by women in our research group are employing in total 1 471 persons, this presents 2.40% from the overall number of people employed in agriculture (2017). Stemming from the total number of women employed in agriculture (13,1 thousand), rural women in our group employ merely 3.48% of them. However, it should be reiterated that on average, rural women who manage the agricultural entities in our group employ 30.99% (456) of women, which is a slightly higher share as it is prevailing in the sector (24.95%). The results of our survey claim that women in their business incline to prefer female working forces. In this case, we cannot perceive such an approach as a negative trend from the gender point of view. This is more about the cause interlinked to the finalization of agricultural commodities, such as processing and selling of ready-made products, as well as providing services connected to the rural businesses, where is more preferable female working force. This is also another reason why it is important to support the entrepreneurial activities of women in the agricultural and food processing sector because the materialization of this approach will be created more employment opportunities both for women and men in the countryside.

***The Number and Types of Agricultural Business Entities Managed by Women***

As it was previously mentioned in Slovak agriculture operated 27 833 entities in 2017 which applied various legal business forms. This is representing a share of 1.16% of the overall number of agricultural entities in the state.

In the surveyed sample the strongest group is represented by female individual farmers with 63 farms, this is almost half of the entities in our research group. (48.5%). Few women are operating their businesses in the form of Limited liability companies (Ltd.), in total 32 companies (24.6%). In the legal form – cooperative is doing their agribusiness 27 companies (20.8%). With trade certificates are operating 6 respondents (4.6%) and only 2 entities from the research group (1.5%) have indicated that they provide their business in the shareholding companies (Figure 1).

The size of cultivated land in an agricultural business entity was analyzed concerning the legal business form of the company. The objective was to recognize, whether there are some connections or differences concerning the size of companies and applied legal business forms. For this purpose, it was utilized Kruskal-Wallis test (Table 1).

Kruskal-Wallis test is confirming that there do exist differences between hectares of cultivated land and the applied legal forms of business. For the sake of more detailed exploration was this test completed by the Dunnett method.

**Table 1** Kruskal- Wallis Test for Recognition of Dependence between Scale of Entities and Legal Forms.

| | |
|---|---|
| $H_0$ | There are no statistically significant differences between the size of farm in hectars and the legal business forms. |
| $H_1$ | There are statistically significant differences between the size of farm in hectars and the legal business forms. |
| *p – value* | <0.0001 |
| **Test Conclusion** | **H1 is accepted** |

Note: Own results, XL Stat.

Dunnett's method separated the agricultural entities into two groups, A and B. Those in group A have statistically significantly less land than the entities involved in group B. The least land has companies that are doing their business upon the trade certificates, and the biggest sizes in hectares have shareholding companies and cooperatives.

There is a statistically significant difference according to the company size in hectares of agricultural land among shareholding companies and cooperatives on the one side and on the other side between Ltd., individual farmers, and those who do the business upon the trade certificate.

Furthermore, our goal was to ascertain that what kind of effect has the number of years of manager's experience in agriculture on the selected legal forms of business by rural women. For this purpose, was applied Kruskal-Wallis test (Table 2).

The dependence between several years of practical experience of rural women in agriculture and the legal form of business confirmed by the Kruskal-Wallis test was also proved and more profoundly specified by the Dunnett method. According to Dunnett methods have been companies divided into two groups. Women who are managing the companies in the first A group have significantly fewer years of practical experience in agriculture than the women who are managing the companies in group B. The last years of practical experience in agriculture have women in the Ltd., the most years the women who are managing shareholding companies and the cooperatives. The highest statistically significant difference was in the number of years in agriculture between the women in the Ltd. and the women who are leading the cooperatives.

From this analysis are stemming two conclusions:

1. 1.Rural women are willing to take over higher responsibility which is connected to the management of the agricultural entities on a larger scale;
2. Rural women do prefer to manage companies whose legal business forms are requiring a wide scope of technical, financial, managerial, and leadership capacities.
3.

***Production focus of rural women***

In business activities, rural women concentrate on the balanced approach both to crop and animal productions. They have no intention to simplify their production programs by cultivating monocultures typical for industrial agriculture.

The ratio between crop and animal productions is rather equal, whereas the rural women dedicate their business efforts to the cultivation of crop breeds in 77.70% of cases, while with animal rearing deal 64.60% of women. For women of our surveyed group, except for traditional agricultural production is the characteristic sale of the agricultural products (30.50% of respondents). With this is connected the processing of agricultural primary products. This activity is ranked as number 4 with a share of 20.80%. An almost identical result was achieved at the sale of agricultural products. This is once more activity typical for the female working force, as the women have better assumptions for providing a different kind of services in a more complex way. Less significantly is represented agrotourism with 10.50%. Interestingly, women doing their business in the countryside alongside production activities are oriented also on the extension services in such an area as the correct accomplishment of the requests for direct payments and other subsidy's entitlements, or the work with information technologies.

The rent of the agricultural machinery is represented less significantly, just with 2.30% (Table 3).

The number of farm animals reared by rural women and their share of the total number of farm animals in 2017 is mentioned in Table 4. The share of women in livestock

**Table 2** Kruskal- Wallis Test for Exploration of Dependence between Number Years of Experience in Agricultural and the Legal Form of Business Entities.

| | |
|---|---|
| $H_0$ | There are no statistically significant differences between number of years of experiences in agriculture and the selected legal business forms. |
| $H_1$ | There are statistically significant differences between number of years of experiences in agriculture and the selected legal business forms |
| *p – value* | 0.0172 |
| **Test Conclusion** | **H1 is accepted** |

Note: Own results, XL Stat.

**Table 3** Production focus of companies managed by rural women in 2017.

| Production focus of company | Number | In **[%]** |
|---|---|---|
| Crop production | 101 | 77.70 |
| Animal production | 84 | 64.60 |
| Processing of agricultural products | 27 | 20.80 |
| Sale of the agricultural products | 41 | 30.50 |
| Agrotourisms | 15 | 11.50 |
| Providing of services | 26 | 20.00 |
| Extension services | 7 | 5.40 |
| Rent of agricultural equipment | 3 | 2.30 |
| Others | 3 | 2.30 |

Note: Own results, XL Stat.

**Table 4** Number of farm animals reared by rural women and its share on the total number of farm animals in Slovak Republic in 2017.

| **Farm animal category** | **Number** | **In [%]** |
|---|---|---|
| Livestock | 9.789 | 2.19 |
| Pigs | 274 | 0.05 |
| Sheep | 14.915 | 4.04 |
| Goats | 406 | 1.12 |
| Poultry | 240.815 | 1.99 |
| Horses | 141 | 2.20 |

Note: Own results, XL Stat.

production is only 2.19%. The lowest share is noted by rural women in the production of swine with 0.05%. With rearing of swine deal only 6 companies. Poultry represents 1.99% of the total number of this breed in Slovakia, while the number of goats is represented by the share of 1.12%.

We presume that these results are influenced by the fact that the majority of rural women are doing their business by farming system, moreover in the less favorable natural areas. Therefore, women strive to the rear first of all sheep, goats, as well as cows, to process milk on the finalized food commodities, which would be sold in their farm shops, or on the farm, markets organized nearby of their farms. Except for these, there is the opportunity created in last years to directly place local food products in the supermarkets. With regard, the horse rearing recent trend is very much interlinked with the diversification of agricultural production to create riding areas with other leisure time activities, which are very popular in the last years. Our results confirm that businesswomen prefer the finalization of products with higher added value. In comparison with agribusiness men, rural women prefer traditional agricultural production which combines crop and animal production intending to take advantage of the interconnection of these two basic farming branches.

Agriculture and food production do represent one of the most important sectors of the national economy and fulfill a key role in the achievement of the food security of citizens. **Bujňáková (2010)** underlines the insatiable role of agriculture in the cultivation of agricultural land and citizens' high-quality nutrition. These meaningful tasks of agriculture are fully taken into consideration by rural women involved in undertaken research. At the management of companies, they do orient their attention first of all on the fact, that Slovakia is considered as the country with strong agriculture background and also that the state can ensure its food self-sufficiency. **FAO (2011)** deals with certain recent challenges of agriculture and among those most serious is alongside others listed the fact that women lack enough resources and business opportunities. In contrary to this FAO conclusion, Slovak rural women do not feel this kind of disadvantage. They consider themselves emancipated. They pose with a high level of self-awareness and their access both to the production resources, as well as to financial resources is considered as good. According to our research, any discrimination in this respect is refused. In individual states of the world, is rather complicated to assign that what is the share of rural women to the development of the respective agricultural sector **(Doss, 2011)**. The results of our research are documenting that share of rural women on the realization of agricultural activities is so far symbolic, especially if we speak about the top managerial and ownership positions, their shares on cultivated land, rural employment, or on rearing farm animals.

**Jacková et al., (2016)** wrote that tertiary agricultural education achieved about 8.4% more women than men in 2014. According to our findings, the results are analogical when we take into consideration the achieved education, as in the group of rural women are graduated from agricultural universities 29%. This finding is also in line with author **Schneiderova (2015)**, who states, that nowadays are completing university education far more women than men, even frequently with better results. With this author, we also coincide in the statement that every businesswoman is in a similar age as a man when she/he is acting in the supreme managerial position. This virtually means age about fifty years and so woman and man have behind of their relatively long professional carrier.

## CONCLUSION

Women doing business in agriculture dispose of with considerable knowledge and experience in the agricultural and food sectors. The average of years of practical experience in our group of rural women reached 23 years. If we deal with the analysis of the gender differences, the rural women do consider their overall business rights are equal when asking for various kinds of state or EU supports, for approval of projects, etc.

Stemming from the achieved results of a carried out survey it had been formulated following conclusions: 1. It is important to strengthen processes that promote the economic development of rural women and their managerial and social inclusion to overcome the over-helming representation of men in the agribusiness sector. 2. Rural women dealing with business in the field of agriculture and food processing industry should intensively cooperate with young people, especially with females at the universities and vocational schools in the form of delivering lectures, organizing workshops, and practical activities in their farms. With this could be enhanced presence of young people including of women in the sector. In addition, the agricultural environment could be for future young farmers more attractive. 3. It is necessary to strengthen institutional support agencies to proactively address women's needs. In this respect, it is important to involve rural women in the work of associations and clusters through which rural women could enforce their requirements and needs having in mind the improvement of their respective businesses. The changing nature of farming as a profession has an impact on agricultural education changes too. It allows women to establish their professional careers in the field of their interest. The accessibility of training is supporting them to be able to embrace new technologies and managerial techniques. With this, their low representation in the managerial and ownership position in the sector can be significantly changed in comparison with recent unfavorable results revealed by this research.

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# EVALUATION OF THE BASIC SACCHARIDES CONTENT IN TOMATOES

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## ABSTRACT

Saccharides are the basic dry matter components of all fruits and vegetables. The dominant tomatoes saccharides are fructose and glucose, minor ones are sucrose but also arabinose, xylose, and galactose. The objective of this paper is to analyze carbohydrates such as glucose, fructose and sucrose in the selected tomatoes varieties intended for the direct consumption and industrial processing. We used 14 varieties and 3 newly selected tomatoes varieties. The glucose content in the studied varieties was in the range of 4.87 – 15.9 $g.kg^{-1}$, the fructose content was 11.1 – 22.27 $g.kg^{-1}$ and sucrose content was 0.07 – 1.73 $g.kg^{-1}$. The highest fructose and sucrose content was detected in the Tomanova variety, the highest sucrose content was found out in the Bovita variety. When comparing the glucose content it was found out that the higher content on average was achieved in the varieties intended for the industrial processing (10.10 $g.kg^{-1}$) than in the varieties for the direct consumption (7.96 $g.kg^{-1}$). The varieties intended for industrial processing were generally characterized by higher glucose and fructose content than the varieties intended for the direct consumption. When comparing the fructose content, we found a higher average content in the varieties intended for industrial processing (15.70 $g.kg^{-1}$) than in the varieties for the direct consumption (14.40 $g.kg^{-1}$). In most of the studied varieties sucrose was present in low content (<1.0 $g.kg^{-1}$), only in the Tomanova variety sucrose content represented more than 1 $g.kg^{-1}$.

**Keywords:** tomato; saccharides; glucose; fructose; sucrose

## INTRODUCTION

Carbohydrates and organic acids are the dry matter key ingredients with influence on tomatoes quality and the preference of the variety for consumers in terms of taste intensity **(Baldwin et al., 2011; Bastias et al., 2011; Ponce-Valadez et al., 2016; Wang et al., 2016)**. Sugars and organic acids represent more than 60% of tomatoes' dry matter content.

Most studies show that the dominant carbohydrates present in tomatoes are fructose, glucose, and sucrose, and organic acids such as malic, citric, or eventually ascorbic acid are dominant **(Osvald et al., 2001; Fulton et al., 2002; Ruggieri et al., 2014)**. Fructose and glucose levels are low during the growth and development of tomatoes, but their concentration increases rapidly during the tomato maturation time **(Carrari et al., 2006)**.

**Thakur et al. (1996)** reported that a small percentage of sucrose, raffinose, arabinose, xylose, and galactose are also present in the tomatoes. **Schauer et al. (2005)** detected alcoholic sugars such as galactitol, maltitol and sorbitol in tomatoes. **Kelebek et al. (2017)** determined that sucrose is in the smallest amount present among tomatoes carbohydrates.

The indicators, representing the basic composition of tomatoes such as soluble dry matter content, acid content, carbohydrates content, are often the most important indicators that determine the variety success for both the consumer and processor.

**Tijskens and Schouten (2009)** reported that consumers firstly perceive the content of sugars and acids in the tomatoes, which they convert to the sweetness and acidity of the tomatoes and finally to the tomatoes flavor attribute, which is crucial for tomatoes success. Also, **Kader (2008)** and **Baldwin et al. (2008)** state that the tastiness of tomatoes is determined by the presence of sugars, organic acids, mineral substances, and volatile substances that contribute to the tomatoes' flavor. The fruit taste and aroma are of decisive importance for the perception of the overall tomato acceptability.

The second most important factor, after genotype determining final tomatoes quality, is the tomatoes harvest at the right maturity level **(Kader, 2008)**. At the time of ripening, the proportion of sugars in tomatoes increases, organic acids are degraded, and volatile substances are intensively synthesized.

The picking of tomatoes at the right degree of maturity also determines the use of the harvested tomatoes, either for the direct consumption without the necessity of storage or for storage or canning processing. The objective of this paper is to analyze the basic carbohydrates, such as glucose, fructose, and sucrose in the selected tomato varieties.

## Scientific Hypothesis

Carbohydrates are the basic component of tomato dry matter. The dominant carbohydrates are fructose and glucose. Sucrose is a minor in tomato fruits. The variety has a statistically significant effect on the carbohydrate content.

## MATERIAL AND METHODOLOGY

### Samples

We used 14 varieties and 3 newly selected tomato varieties. Out of the varieties intended for the industrial processing, we analyzed varieties Uno Rosso F1, Pavlína, Mobil, Zömök, Denár, Bovita, Danuša, Salus. Out of the varieties dedicated for the direct consumption, we analyzed varieties Niki Zel F1, Ady Zel F1, Ambros F1, Žofka, F1, Jerguš F1, Tomanova, and the newly-selected varieties 987/17, 1037/17, and 1018/17. The newly-selected varieties 1037/17 and 1018/17 belonged to the small cherry tomato types.

### Chemicals

To prepare samples, an extraction solution in the composition of demineralized water and ethanol was used (Ethanol ≥99.5%, suitable for HPLC, CAS 64-17-5, Sigma-Aldrich. St Louis, MO, USA) in a ratio of 20:80.

They were used to clarify the samples:

Carrez solution I for determination of sugars and starch (1 L contains: 219 g zinc acetate – dihydrate, 30 g acetic acid min. 99.8 %, water pure, CAS R.9944.1, P-LAB, ČR, Praha

Carrez solution II for determination of sugars and starch (1 L contains 106 g potassium ferrocyanide – trihydrate, water pure, CAS R.9950.1, P-LAB, ČR, Praha)

Calibration curves were prepared from solution:

Glucose (D-(+)-Glucose ≥99.5% (GC)**,** CAS 50-99-7, Sigma-Aldrich. St Louis, MO, USA)

Fructose (D-(−)-Fructose ≥99%**,** CAS 57-48-7 Sigma-Aldrich. St Louis, MO, USA)

Sucrose (Saccharose ≥99.5% (GC), CAS 57-50-1, Sigma-Aldrich. St Louis, MO, USA).

### Biological Material

Samples of cultivars Uno Rosso F1, Pavlína, Mobil, Zömök and Denár were supplied by the Botanical Garden of the Slovak University of Agriculture in Nitra. The location is in a mild climate. The climate is continental with an average annual temperature of 9.5 °C and an average annual rainfall of around 595 mm.

Samples of cultivars Niki Zel F1, Ady Zel F1, Ambros F1, Žofka, F1, Jerguš F1, Tomanova, Bovita, Salus, 987/17, 1037/17 and 1018/17 were supplied by the Zelseed Breeding Station in Horná Potôň. The location is in a mild climate. The climate is continental with an average annual temperature of 9.9 °C and average annual precipitation of about 513 mm.

### Instrument

The following instrument was used for the measurement:

Liquid Chromatography Equipment (HPLC) LC-20 AD, Shimadzu Corporation.

### Description of the Experiment

#### *Collection of samples for analysis*

The tomatoes assigned for the experiment were picked at the stage of technological maturity, which concerned the varieties intended for industrial processing. The varieties targeted for the direct consumption were harvested at the stage of consumer maturity. The ripeness of the tomatoes was characterized by intense deep color, characteristic full tomato flavor and aroma, and a minimum value of soluble dry matter of 4.5 °Brix. Maturity in the field conditions was monitored subjectively by the color intensity of the tomatoes, and also objectively by the soluble dry matter content using a Krüss HR18-01 digital hand refractometer.

#### *Laboratory Methods*

Determination of glucose, fructose, and sucrose content in the samples was performed by HPLC-RID (Refractive Index Detector) method **(Sanz and Martínez-Castro, 2007)**. The liquid chromatography was used for the analysis with the Agilent Zorbax column and aminopropyl stationary phase. The mixture of acetonitrile and water at the ratio of 80:20 with the flow rate of 1.6 $mL.min^{-1}$ was used as the mobile phase. The sample preparation consisted of extracting 2 g of homogenized tomato mass in 20 mL of the soluble mixture consisting of ultrapure ethanol and water at a ratio of 4:1 for 1 hour. The clarification of the sample was performed with Carrez reagents in amounts of 2.5 mL Carrez I and 2.5 mL of Carrez II. After the clarification for 15 minutes, the samples were centrifuged and filtered by syringe filters before the analysis. Calibration curves were prepared from a stock solution of glucose, fructose, and sucrose at the concentration of 0.5 – 20 V.

## Statistical Analysis

For statistical evaluation of the influence of the variety on the content of the monitored parameters, a one-factor resp. two-way analysis of variance ANOVA at $p < 0.01$. In post-hoc testing, the Tukey HSD multiple comparison test was used to find statistically significant differences at $p < 0.05$. All statistical analyzes were performed using the Statsoft Statistica 12.5 statistical package (Statsoft Inc., Tulsa, USA).

## RESULTS AND DISCUSSION

### Soluble dry matter

The determination of the soluble dry matter content expressed as °Brix is in practice used as a basic measurable indicator to determine tomato ripeness. A minimum of 4.6 °Brix is required for tomatoes to be processed and, as reported by **Patané and Cosentino (2010)**, the low soluble dry matter content and the high-water content of tomatoes cause a reduction of economic profits during processing. De **Castro Vilas Boas et al. (2017)** also state that at the point of purchase the quality of tomatoes intended for processing is currently determined subjectively by color and measured by the soluble dry matter in °Brix, while other quality characteristics are often overlooked. In addition to the soluble dry matter content, the ratio of carbohydrates to organic acids **(Zhao et al., 2016)** is significant in assessing the quality of ready-to-eat tomatoes.

The soluble dry matter content in the monitored tomato varieties ranged in the values of 4.5 to 7.5 °Brix. According to the average dry matter content, the varieties were divided into 5 homogeneous groups. The newly selected varieties 987/17 and 1018/17 reached statistical significance ($p < 0.05$) with the lowest values (4.5 °Brix), while the highest content of soluble dry matter was detected in the variety Tomanova (7.5 °Brix) intended for the direct consumption. The varieties Danuša, Bovita, and 1037/17 also showed high dry matter content (6.5 °Brix). According

to their way of utilization Danuša and Bovita varieties are classified among the varieties intended for industrial processing with the possible way of use for the direct consumption. The varieties with the soluble dry matter content of 5.0 – 5.2 °Brix represented the largest group of varieties with a balanced dry matter content. This group included the varieties intended for the industrial processing such as Uno Rosso F1, Mobil, Zömök, Salus, as well as the varieties intended for the direct consumption, such as Niki Zel F1, Ady Zel F1, Ambros F1, Žofka F1 or Jerguš F1 (Table 1).

**Sun et al. (2012)** states that the fruit lycopene content and the total soluble solids content are important factors that determine the quality of tomato fruit.

**Anthon et al. (2011)** in their publication also monitored the soluble dry matter content. According to their results, the dry matter of tomatoes is mainly composed of simple sugars such as glucose and fructose. The authors monitored the dry matter content of tomatoes intended for industrial processing and found out that the dry matter content ranged from 4.6 to 5.4 °Brix. During the time of storage just before processing they detected an increase of dry matter content up to 5.6 °Brix in most varieties, which was partly due to transpiration and breathing processes that are accompanied by loss of water of tomato pulp, but also by tomato post-harvest ripening and synthesis of the various dry matter components. However, along with the prolonged tomato storage time and during storage at temperatures above 20 °C, there is a risk of tomatoes self-heating and the development of undesirable microorganisms. Therefore, it is not recommended to store tomatoes before processing with the purpose of post-harvest ripening, and thus to increase the dry matter content. If the storage before processing is necessary, the technology should include at least a minimum of cooling.

**Patané et al. (2017)** analyzed the soluble dry matter content by using the Digital Brix refractometer HI 96801 in the Italian tomato varieties Pizzottello di Montallegro and Locale di Filicudi grown in Italy, Catania. They reported that the content of dry matter was 8.2 °Brix and 8.15 °Brix, which is higher than we found out in our study. Undoubtedly, the climatic conditions of the growing area, in particular, higher average temperatures, higher sums of temperatures during vegetation, and also the intensity of sunlight had the positive effect on the higher dry matter content, as it was documented in the publication of **Patané et al. (2017)**.

The dry matter content of tomatoes grown in the northern Tunisia was monitored by **Ilahy et al. (2016)**. In their paper, they evaluated newly selected tomato varieties with a high content of lycopene HLT-F71 and HLT-F72 and they used Tunisian traditionally grown variety Rio Grande as a reference variety. The Rio Grande variety reached an average dry matter content of 5.8 °Brix, while newly selected varieties achieved the dry matter content up to 6.7 °Brix. These values correspond to our varieties, in which we found a higher content of soluble dry matter, namely variety Danuša, Bovita, and newly selected variety 1037/17.

**Radzevičius et al. (2016)** evaluated the soluble dry matter content of tomatoes by using the varieties Rutuliai during ripening time. They utilized the classical methods and by using NIR spectrophotometer. The authors recorded that the dry matter content measured by the NIR spectrophotometer was slightly lower than the dry matter content determined by the classical method. The values that were detected by the authors at the stage of tomato ripening were 3.98 – 5.20 °Brix. These values are comparable to our findings.

**Domínguez et al. (2016)** monitored the content of soluble dry matter in the varieties Delitia and Pitenza. Tomatoes were grown in the greenhouse conditions in Spain and they are picked at the stage of full ripeness. The authors found out a soluble dry matter content of 5.6 °Brix in the variety Delitia right after harvest.

**Duma et al. (2017)** monitored the soluble dry matter content in varieties Sunstream F1, Sakura F1, Black Cherry F1, Golden Nudget F1, and Rhianna F1 cultivated in Lithuania. The authors detected the highest dry matter content in the variety Golden Nudget F1, represented 7.9 °Brix and the lowest in the variety Sunstream 5.2 °Brix. These values are also comparable with our results.

**Majidi et al. (2011)** analyzed the dry matter content of the tomatoes grown in the Iranian region and found out the dry matter content within the range of 5.2 – 6.0 °Brix. The authors decided to evaluate the dynamics of changes in the dry matter content during the cold storage as well as the storage with a regulated atmosphere. It was stated that the dry matter content slightly increased during 90 days of storage in conditions of the cold storage during the first 20 days and later it decreased sharply, while in the storage using regulated atmosphere the dry matter content decreased after 40 days of storage

## Glucose, fructose, sucrose

Sugars and organic acids are the key components impacting tomato quality and customer preferences. They account for over 60% of the dry matter, and contribute to soluble solid content and also are essential to the flavor intensity **(Goff and Klee, 2006; Bastias et al., 2011; Sauvage et al., 2014; Zhang et al., 2010; Zhang et al., 2016)**.

In fruit, sugars provide sweetness, which is the most important determinant of fruit quality. The relationship between sugar content, measured as the soluble solids content, and fruit yield has been evaluated in a number of horticultural studies **(Ruggieri et al., 2014; Anthon et al., 2011; Kanayama et al., 2005)**.

Generally known, carbohydrates are the main dry matter components of fruits and vegetables. As we learned from our results, the main carbohydrate in the analyzed tomato varieties was fructose, which was present at the amount of 11.10 – 22.27 $g.kg^{-1}$ in fresh tomatoes. Glucose was present in lower quantity and it reached 4.50 – 15.93 $g.kg^{-1}$ in the individual varieties. The lowest content was represented by sucrose, which was analyzed in amounts of 0.07 – 1.73 $g.kg^{-1}$ in fresh tomatoes (Table 1).

According to the average glucose content by comparing the analyzed varieties, they were divided into 6 homogeneous groups. The statistically significant highest ($p$ <0.05) glucose content was found out in the variety Tomanova (15.93 $g.kg^{-1}$). Higher values were also detected in the varieties Bovita (15.10 $g.kg^{-1}$) and Pavlína (14.37 $g.kg^{-1}$). On the other hand, the statistically significant lowest values were recorded in the varieties intended for the direct consumption, such as Niki Zel F1 (4.5 $g.kg^{-1}$), Ady Zel F1 (5.0 $g.kg^{-1}$), 987/17 (5.07 $g.kg^{-1}$), 1018/17 (5.17 $g.kg^{-1}$) and also in the variety Salus (4.87 $g.kg^{-1}$)

intended for the industrial processing. In the case of fructose, these varieties were also of low content. Both the variety Salus and newly selected variety 1018/17 had statistically significant ($p$ <0.05) the lowest fructose content (homogeneous group a). In the case of varieties intended for the direct consumption, the low content of simple sugars may cause the less attractive taste to the consumer, and in case of the variety Salus, intended for the industrial processing, a lower sugar content may cause a lower product yield during the tomato purée production.

According to the average fructose content, the varieties were divided into 9 homogeneous groups. The statistically significantly highest ($p$ <0.05) fructose content was analyzed in the variety Bovita (22.27 g.kg$^{-1}$) and high content was also analyzed in the variety Tomanova (20.83 g.kg$^{-1}$). Both varieties showed not only significantly higher fructose content but also high glucose content.

The biggest homogeneous group regarding glucose content was the group of 15.9 – 17.0 g.kg$^{-1}$, which included 6 varieties, such as 4 varieties intended for industrial processing particularly variety Denár (15.93 g.kg$^{-1}$), Mobile (16.10 g.kg$^{-1}$), Pavlína (16.83 g.kg$^{-1}$) and Danuša (17.0 g.kg$^{-1}$) and 2 varieties intended for the direct consumption, such as newly selected variety 1037/17 and Žofka F1 variety, both had a the comparable average fructose content of 16.53 g.kg$^{-1}$. Based on the statistical results, it cannot be directly stated that the varieties intended for the direct consumption in comparison with the varieties intended for industrial processing were characterized by a higher or lower content of simple carbohydrates.

Based on the results of the Tukey HSD test of multiple comparisons of the average sucrose content, the varieties were only divided into 2 homogeneous groups. As already mentioned, sucrose is not a dominant carbohydrate present in tomatoes. It is represented in the smallest amount and the majority of the monitored varieties showed in the given parameter lower values below 1 g.kg$^{-1}$ and these varieties formed a separate homogeneous group containing up to 15 varieties. The variety Pavlína with an average sucrose content of 0.83 g.kg$^{-1}$ represented the variety that was not statistically significantly different from the group of varieties with very low sucrose content, but nor from the variety Tomanova (1.73 g.kg$^{-1}$), which had the statistically significant highest sucrose content.

**Bastias et al. (2011)** reported the fructose content in mature tomatoes at the level of 1.19 g.100g$^{-1}$ FM and glucose content of 0.91 g.100g$^{-1}$ FM. **Beullens et al. (2006)** monitored the sugar content in tomatoes by using high-performance gas chromatography and found out the fructose content in the range of 1.21 – 1.98 g.100g$^{-1}$ FM, glucose content of 1.17 – 2.16 g.100g$^{-1}$ FM and the lowest one was sucrose content in the range of 0.41 – 0.84 g.100g$^{-1}$ FM.

**Kelebek et al. (2017)** monitored the glucose, fructose and sucrose content of the variety Shasta in tomatoes grown in Turkey. Tomatoes were intended for the industrial processing, and similarly to our research, the authors found out the lowest content of sucrose of 1.75 – 1.81 mg.100g$^{-1}$. They also detected that the highest content of carbohydrates showed fructose, representing 14.44 – 14.73 mg.100g$^{-1}$, which was similar to our results. The authors also monitored the changes in carbohydrates that occur during the tomato processing and found out that the content of all monitored carbohydrates increased gradually during the individual processing stages, while the mutual ratios between the individual carbohydrates remained the same.

**Ayvaz et al. (2016)** monitored glucose and fructose content in 66 tomato varieties that were not specified in detail according to the method of tomato identification. The authors determined the soluble dry matter content in the range of 3.9 to 6.3 °Brix. Glucose content of 2.7 – 18.1 g.dm$^{-3}$ was recorded, as well as a fructose content of 3.8 – 14.5 g.dm$^{-3}$. Especially in the case of fructose, these values differ from our findings because in our results we found out the fructose content more than 11 g.dm$^{-3}$.

**Wilkerson et al. (2013)** analyzed the glucose and fructose content of the tomato varieties intended for processing, but

**Table 1** Average values of soluble dry matter content, content of glucose, fructose and sucrose and homogenous groups based on multiple Tukey HSD test results.

| Variety | dry matter (°Brix) | glucose (g.kg$^{-1}$) | fructose (g.kg$^{-1}$) | sucrose (g.kg$^{-1}$) |
|---|---|---|---|---|
| Uno Rosso F1 | 5.2 b | 10.13 ±0.06 cd | 13.43 ±0.23 de | 0.07 ±0.02 a |
| Pavlína | 5.5 c | 14.37 ±0.35 ef | 16.83 ±0.56 g | 0.83 ±0.05 ab |
| Mobil | 5.0 b | 9.90 ±0.20 cd | 16.10 ±0.36 g | 0.77±0.15 a |
| Zömök | 5.0 b | 6.33 ±0.41 b | 12.60 ±0.17 cd | 0.17 ±0.01 a |
| Denár | 5.0 b | 9.93 ±0.21 cd | 15.93 ±0.23 g | 0.33 ±0.02 a |
| Bovita | 6.5 d | 15.10 ±0.10 ef | 22.27 ±0.38 i | 0.53 ±0.12 a |
| Salus | 5.0 b | 4.87 ±0.38 a | 11.43 ±0.06 a | 0.60 ±0.20 a |
| Danuša | 6.5 d | 10.27 ±0.12 d | 17.00 ±0.17 g | 0.67 ±0.15 a |
| Niki Zel F1 | 5.0 b | 4.50 ±0.13 a | 11.93 ±0.25 abc | 0.27 ±0.06 a |
| Tomanova | 7.5 e | 15.93 ±0.12 f | 20.83 ±0.15 h | 1.73 ±0.32 b |
| Ady Zel F1 | 5.0 b | 5.00 ±0.11 a | 12.57 ±0.12 bcd | 0.10 ±0.00 a |
| Ambros F1 | 5.0 b | 9.10 ±0.22 c | 14.57 ±0.12 f | 0.63 ±0.06 a |
| Žofka F1 | 5.0 b | 9.10 ±0.36 c | 16.53 ±0.06 g | 0.33 ±0.03 a |
| Jerguš F1 | 5.0 b | 6.80 ±0.10 b | 14.13 ±0.21 ef | 0.17 ±0.01 a |
| 987/17 | 4.5 a | 5.07 ±0.25 a | 11.47 ±0.12 ab | 0.37 ±0.11 a |
| 1037/17 | 6.5 d | 10.93 ±0.2 e | 16.53 ±0.21 g | 0.17 ±0.06 a |
| 1018/17 | 4.5 a | 5.17 ±0.40 a | 11.10 ±0.10 a | 0.20 ±0.10 a |

Note: average value in fresh matter; ±standard deviation, different letters at averages represent statistically significant differences among varieties ($p$ <0.05).

they do not specify the name of varieties. Tomatoes were grown in field conditions in the different areas of California. The authors found out the results related to glucose content 10.0 – 21.4 $g.dm^{-3}$ and fructose content 11.0 – 20.6 $g.dm^{-3}$, which are results that are comparable with our measurements.

**Pinela et al. (2012)** evaluated the content of individual carbohydrates in tomato commercial varieties grown in Portugal. In the study they used the varieties Amarelo, Batateiro, Comprido and Coração and found out that sucrose had the smallest content, almost zero, regarding to the studied carbohydrates and among individual varieties they indicate the content of 0.0 – 0.02 $g.100g^{-1}$. The glucose content ranged from 2.15 to 3.42 $g.100g^{-1}$ in the evaluated varieties. The highest values of the monitored carbohydrates were achieved by fructose, which was present in the samples in the quantity of 2.15 – 3.42 $g.100g^{-1}$. When comparing the varieties, the highest content of carbohydrates was present in the Amarelo variety and the lowest content in the Comprido variety.

**Hernández-Suárez et al. (2008)** monitored the glucose and fructose content of the varieties Dorothy, Boludo, Thomas, Dominique, and Dunkan grown in Tenerife and claimed that tomatoes had lower glucose content than fructose, although the differences between the varieties were not as significant as in our case. The highest content of glucose and fructose was found out in the variety Dominique, such as 1.16% of glucose and 1.24% of fructose.

## CONCLUSION

By evaluating the carbohydrate content we detected that glucose and fructose were the dominant carbohydrates present in the analyzed tomatoes. Their content of glucose ranged of 4.87 – 15.9 $g.kg^{-1}$ and in the case of fructose of 11.10 – 22.27 $g.kg^{-1}$. According to the obtained results sucrose can be considered as a minor tomato carbohydrate. The sucrose content in tomatoes was in the range of 0.07 – 1.73 $g.kg^{-1}$. When comparing the glucose content it was found out that the higher content on average was achieved in the varieties intended for the industrial processing (10.10 $g.kg^{-1}$) than in the varieties for the direct consumption (7.96 $g.kg^{-1}$). In both groups of varieties, there were exceptions for which the statement for the glucose content cannot be applied. Low glucose values were detected in the group of varieties intended for the industrial processing, such as the varieties Salus and Zömök, and on the other hand, significantly higher glucose content was found out in the group of the varieties intended for the direct consumption, especially in the variety Tomanova. When comparing the fructose content, we found a higher average content in the varieties intended for industrial processing (15.70 $g.kg^{-1}$) than in the varieties for the direct consumption (14.40 $g.kg^{-1}$). Significantly higher fructose content was recorded in the varieties Bovita (22.27 $g.kg^{-1}$) and Tomanova (20.83 $g.kg^{-1}$). Only the minimal differences between the varieties regarding the content of minor saccharide sucrose were registered by using the statistical data testing and multiple comparisons of average values of sucrose content by Tukey test. In most varieties, we found out statistically insignificant ($p$ >0.05) differences in sucrose content. The exception was the variety Tomanova (1.73 $g.kg^{-1}$) with the highest sucrose content.

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# THE EFFECT OF ANTIOXIDANTS ON XANTHINE OXIDASE ACTIVITY IN FRESH OVINE MILK

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## ABSTRACT

In the present, the consequences of nitrate pollution of the environment are very pronounced. In humans and animals, microorganisms can reduce nitrates to nitrites, which cause cancer. Purified and homogeneous xanthine oxidase (XO) of cow's milk can restore these compounds, which makes the article extremely relevant. The purpose of the article is to determine the effect of antioxidants on the activity of xanthine oxidase in fresh ovine milk. Various natural and artificial antioxidants were examined for the detection of xanthine oxidase (XO) activity in ovine milk. Among the natural antioxidants, L-cysteine was more effective in the stabilization of XO in heated milk. XO of sheep milk activated by heat treatment in the presence of cysteine and molybdenum became able to convert nitrate and nitrite to nitric oxide (NO). Therefore, L-cysteine was used for double purposes: as the protector of enzyme active center against the oxidation during heat treatment of milk and as a reagent for S-nitrosothiol formation. Hypoxanthine, as a natural substrate of XO, is an effective electron donor for nitrate reductase (NR) and nitrite reductase (NiR) activities. Heat treatment of the milk in the presence of exogenous lecithin increased the activity of NR and NiR of XO and CysNO formation. Thus, during the heat treatment: a) excess of exogenous phospholipids disintegrates the structure of milk fat globule membrane (MFGM) and b) enzyme molecules denatured partially and their active center became available for exogenous cysteine, molybdenum, hypoxanthine, and nitrate or nitrite.

**Keywords:** antioxidant; molybdenum; tungsten; phospholipids; nitrate; nitrite

## INTRODUCTION

At present time the pollution of the environment with nitrates is a big problem in the world. In the environment and in the human and animal body microorganisms may reduce nitrates to nitrites.

It is well known that nitrates ($NO_3^-$) and nitrites ($NO_2^-$) cause various diseases, including cancer. It is well known that nitrites irreversible bind to hemoglobin forming methemoglobin which losses the ability to transport oxygen **(Reynolds et al., 2007)**. Deficiency of oxygen causes asphyxiation and it is particularly hazardous to health for babies. Furthermore, nitrites easily bind to primary amines, such as cadaverine, putrescine, spermidine, and form potential carcinogens – nitrosamines **(Bryan, 2006; Gladwin et al., 2005)**.

For the first time in 1980 were observed that purified and homogeneous xanthine oxidase (XO) of cow's milk can reduce $NO_3^-$ and $NO_2^-$ **(Alikulov et al., 1980)**. Later, other groups of scientists have found that animal XO converts $NO_3^-$ and $NO_2^-$ to a physiologically important substance – nitric oxide (NO) **(Millar et al., 1998; Zhang et al., 1998; Hord et al., 2011)**. It is generally recognized that NO is one of the major biological messenger molecules, regulating blood pressure and blood flow, neurotransmission and brain function, immune system function, wound healing inhibition of platelet aggregation. NO is also involved in defense mechanisms against pathogens and some kinds of cancer cells **(Milkowski et al., 2010)**. In 1992 the nitric oxide was recognized as a molecule of the year. Scientists, studying its properties, have been awarded the Nobel Prize later in 1998 **(Bryan et al., 2009)**.

Because of the ability to form nitric oxide, the XO of milk harbors an antimicrobial activity. It is known that NO as the oxidant is a strong antibacterial agent. Antibacterial functions of XO are associated with peroxynitrite (ONOO-) which is the product of the reaction between NO and $O_2$ (superoxide anion). XO is also involved in protective and antiviral response by catalyzing the conversion of retinaldehyde to retinoic acid. Retinoic acid derivatives can inhibit viral replication and, thus, preventing the spread of viral disease **(Taibi et al., 2001; Taibi and Nicotra, 2007)**.

Xanthine oxidoreductase or dehydrogenase/oxidase (XO; EC 1.1.3.22) – molybdenum and iron-containing flavoprotein. Each of the two subunits of XO contains one molybdenum center, two iron-sulfur center, and one FAD. XO of the breast milk of women shows a molecular mass of

160 kDa subunit, XO of the cow's milk – 150 kDa. It is believed that the main biological function of XO is catalysis the final step of purine oxidation in eukaryotes; it catalyzes the sequence of hydroxylation that converts hypoxanthine to xanthine, then to uric acid **(Harrison, 2004)**. However, the enzyme has broad substrate specificity and is capable of reducing oxygen to generate the reactive oxygen species (ROS), superoxide and hydrogen peroxide, as well as oxidative transformation of pteridines and some aliphatic and aromatic aldehydes. Xanthine oxidase (XO) is not strongly specific to the oxidation of hypoxanthine or xanthine; it may catalyze the oxidation of about thirty nitrogen-containing heterocycles and aldehydes **(Harrison, 2004)**. Therefore, because of its multifunctional enzymatic reactions, XO is considered a potential enzyme detoxifying different xenobiotics **(Beedham, 2001)**. It is known that numerous heterocyclic xenobiotics (including pesticides) are carcinogens. Thus, in the case of contamination of milk with harmful xenobiotics, the active XO makes possible their biotransformation into harmless forms. XO reduces nitrite (and nitrate), yielding reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite **(Harrison, 2006)**.

In cow's milk XO is localized in the inner membrane layer of the fat globules (XO content is about 8% of total proteins of cove milk fat globules) **(Mondy and Keenan, 1993)**. However, XO of milk fat globule membranes (MFGM) contains tenth times less molybdenum or does not contain it which results in very low enzyme activity **(Godber et al., 1997; Bray et al., 1999; Atmani et al., 2004)**. A study of the cofactor composition of XO isolated from cow's and women's milk showed that more than 85-90% of enzyme molecules don't contain molybdenum in their active center and are inactive molecules **(Bray et al., 1999)**. The occurrence of xanthine oxidase in animal milk was a matter of slight controversy for a long time because in the milk xanthine oxidation has never been observed, although the levels of XO in the milk are relatively high. Presumably, this is because, before the inclusion of Mo atoms in the active center of the enzyme, Mo-free molecules of XO already is associated with the inner membrane of milk fat globules. As a result, XO molecules become molecules without molybdenum. It is proposed that XO is involved in the secretion of milk fat globules in a process dependent on the enzyme protein rather than on its enzymatic activity **(Harrison, 2006)**.

The ability of XOR to generate a diverse range of Reactive Oxygen and Nitrogen Species (RONS) has been the driving force for the intense study of the XOR enzyme. The XOR generation of ROS and the reactive nitrogen species (RNS) have been demonstrated **(Godber et al., 2000)**. The hypothesized scheme for $O_2$•- and $H_2O_2$ generation during the re-oxidation of reduced XO with $O_2$ was confirmed in 1981, it was concluded that a fully reduced XO monomer could generate two molecules of $H_2O_2$ and $O_2$•-, respectively **(Porras et al., 1981)**. The discovery of XOR nitric oxide synthase and peroxynitrite synthase activity has added further interest to the XOR. The enzyme XOR is capable of reducing organic and inorganic nitrate to nitrite, and nitrite to NO•, under hypoxic conditions. According to Millar et al. **(1998)**, all redox centers need to be inserted and be active. However, in the absence of active cofactors, for example, Mo, it is feasible to suggest that the FAD redox center alone could generate RONS under XOR–NADH oxidase activity **(Abadeh et al., 1992)**.

In the presence of the molybdenum atom in the active center, XO is active but in the absence of the metal, it becomes inactive. Like other molybdoenzymes, in the active center, XO contains the so-called molybdocofactor which has a pterin nature (therefore, it is called also molybdopterin) **(Kramer et al., 1987)**. The molybdenum atom is bound to the pterin via its vicinal thiol groups (Figure 1). This pterin is synthesized in vivo independently in the presence of molybdenum, i.e. in the absence of molybdenum cellular XO contains a normal amount of the cofactor. The cofactor is buried deep within the interior of the enzyme molecule and a tunnel-like structure makes it accessible to the appropriate substrates **(Kisker et al., 1997)**.

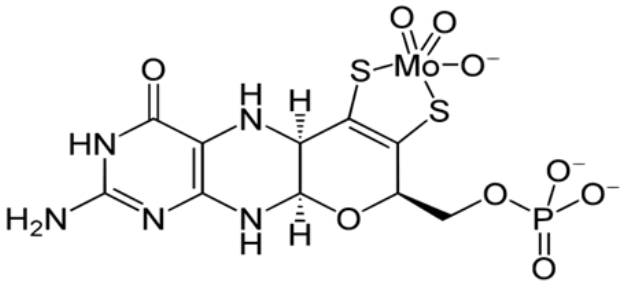


**Figure 1** Structure of molybdocofactor and its bond with molybdenum atom via vicinal SH-groups in the active center of XO **(Kramer et al., 1987)**.

## Scientific Hypothesis

As mentioned above, milk XO exists in molybdenum-free form and is localized in the inner layer of MFGM. Therefore, the activation of milk XO requires the incorporation of exogenous molybdenum into its active center. It is generally known that XO belongs to heat-stable enzymes – it remains active at a 75 – 80 °C temperature in several minutes **(Alikulov et al., 1980)**. However, at this temperature, the enzyme molecules undergo partially reversible denaturation. Therefore, one of the possible ways for the availability of XO molecules for exogenous molybdenum is the disintegration of milk fat globule membranes and partial denaturation of enzyme molecules. Thus, during the heat treatment:

a) excess of exogenous phospholipids disintegrate the structure of MFGM and

b) enzyme molecules denatured partially and their active center became available for exogenous cysteine, molybdenum, hypoxanthine, and nitrate or nitrite. Cysteine not only protects SH-groups of Mo-co against oxidation but also promotes the binding of molybdenum to the cofactor.

## MATERIAL AND METHODOLOGY

### Samples

Fresh milk samples were obtained from healthy female animals at a local farm near Astana city.

### Chemicals

Chemicals were purchased from Sigma-Aldrich Chemical Co. All common chemicals and solvents used were of analytical grade.

**Instruments**

Ethylenediaminetetraacetic acid (EDTA) to bind heavy metals was used for binding heavy metals from milk. Milk was boiled in narrow conical tubes in a volume of 2 ml. Special reagents were used dor detecting different activities of XO. Egg yolk lecithin from AppliChem (Panreac, ITW Companies, Darmstadt, Germany) was used in the experiments.

**Laboratory Methods**

Method for determination of the intrinsic activity of xanthine oxidase (XO). To determine the intrinsic activity of XO 200 μL aliquot of milk was mixed with 700 μL 0.1 M sodium phosphate buffer containing 10 μM EDTA, 5 μL phenylmethylsulfonylfluor (for inhibition of protease activity). To this mixture was added 100 μM 10 mM hypoxanthine. The mixture was incubated at 30 °C for 10 min under aerobic conditions. The proteins in the mixture are precipitated with trichloroacetic acid. After centrifugation, the amount of uric acid (under the influence of XO converted hypoxanthine to uric acid) in the supernatant was determined by measuring the absorbance of the reaction mixture in a spectrophotometer at 295 nm **(Suzuki et al., 2015)**. The amount of uric acid also is determined by its chemical oxidation with potassium permanganate ($K_2MnO_4$) or hydrogen peroxide ($H_2O_2$), thereby forming allantoin **(Poje and Sokolić-Maravić, 1986)**. Allantoin in biological liquids is easy to very accurately determined by a known method **(Vogels and Van der Drift, 1970)**.

Method for the determination of nitrate- and nitrite-reducing activity of XO. The nitrate-reducing activity of XO is determined by the disappearance of the added nitrate ($NO_3^-$) or by the appearance of nitrite ($NO_2^-$) in the reaction medium **(Alikulov et al., 1980)**. The nitrite-reducing activity of XO is determined by the disappearance of the nitrite to the reaction medium or by the appearance of nitric oxide (NO) **(Kuo et al., 2003)**.

**Sample preparation:**

Preparation of the milk for the detection of the various enzymatic activities. Before treatment in the milk of domestic animals added 10 μM ethylenediaminetetraacetic acid (EDTA) to bind heavy metals. For boiling fresh milk is poured into the narrow conical tubes in a volume of 2 mL. Then, for further determination of the enzymatic activity the tubes are placed in the water bath at 35 °C temperature and kept for 10 min, then using the special reagents different activities of XO are determined.

## Statistical Analysis

All determinations were conducted in triplicate or more and all results were calculated as mean ± standard deviation (SD). In this study Statistical analysis was performed using Student's t-test for significance and analysis of variance (ANOVA). The *p*-values less than 0.05 were considered statistically significant. The statistical analysis data were produced by Microsoft Excel and Statistica 15.

## RESULTS AND DISCUSSION

### Effects of different antioxidants in the stabilizing of heat-denatured milk XO

Our preliminary experiments showed that the optimal heating temperature and its duration for detection of all activities in fresh milk XO were 80 °C and 7 min (data not shown). Besides, the presence of 0.1 mM concentrations of cysteine and exogenous molybdenum was also necessary for the detection. Thus, to detect the activity of MFGM-bound enzyme is required heat treatment and the addition of cysteine as an antioxidant and molybdenum to fresh sheep milk. In the next experiments, we examined various natural and artificial antioxidants for the detection of milk XO activity. Natural antioxidants – cysteine, glutathione, lipoic acid, and ascorbic acid, and artificial antioxidants – dithiothreitol, unithiol and mercaptoethanol (2,3-dimercapto-1-propane sulfonic acid) were used in different concentrations (Figures 2, 3, 4).

Results present in the Figures 2, 3, 4 show that among the natural antioxidants L-cysteine was more effective in the stabilization of XO in heat-treated milk. In the same concentrations the effectiveness of cysteine-containing antioxidant – glutathione was considerably lower. This is because the tripeptide molecule of glutathione contains only one cysteine residue. Although both the oxidized (disulfide) and reduced (dihydric) forms of lipoic acid (dithiol) show antioxidant properties **(Milkowski et al., 2010; Rochette et al., 2013)**, its stabilizing effect for milk XO was lower than glutathione. Among the artificial antioxidants, monothiol mercaptoethanol was more effective in the stabilization of heated milk XO. Although dithiothreitol and unithiol contain two sulfhydryl groups they don't stabilize heat-treated milk XO. Recently we found that dithiothreitol in the presence of sulfanilamide forms a stable blue complex with molybdate ion (based on this finding we developed a new fast method for molybdenum determination in the biological materials).

Results in the Figures 5, 6, 7 show that the highest levels of the associated activities of milk XO observed with artificial electron donor – methyl viologen reduced by dithionite ($Na_2S_2O_4$). Among the physiological electron donors, the highest associated activities were obtained using reduced NADH. The natural substrate of XO – hypoxanthine was also effective in the donating of electrons for NR and NiR activities of the enzyme in fresh sheep milk. Thus, the electron transfer may also occur from the hypoxanthine (the reducing substrate) to nitrite and nitrate (the oxidizing substrates) **(Abadeh et al., 1992; Godber et al., 1997)**. Because in the presence of tungstate in the reaction mixture the associated activities were not detected, during nitrate reduction by hypoxanthine, the XO molybdenum center may participate directly in both the oxidative (hypoxanthine oxidation) and the reductive (nitrate and nitrite reduction) half-reactions.

### Detection of the products of nitrate- and nitrite reduction by XO in heat-treated sheep fresh milk

It is found that animal XO converts $NO_3^-$ and $NO_2^-$ to physiologically important gas – nitric oxide (NO) **(Millar et al., 1998; Zhang et al., 1998; Hord et al., 2011)**. NO effectively reacts with L-cysteine or reduced glutathione (GSH) at pH 7.0 and 7.4, to form orange-pink products of S-nitrosocysteine (CySNO) or S-nitrosoglutathione (GSNO). These products exhibited a peak absorbance at around 340 and 540 nm **(Kuo et al., 2003)**.

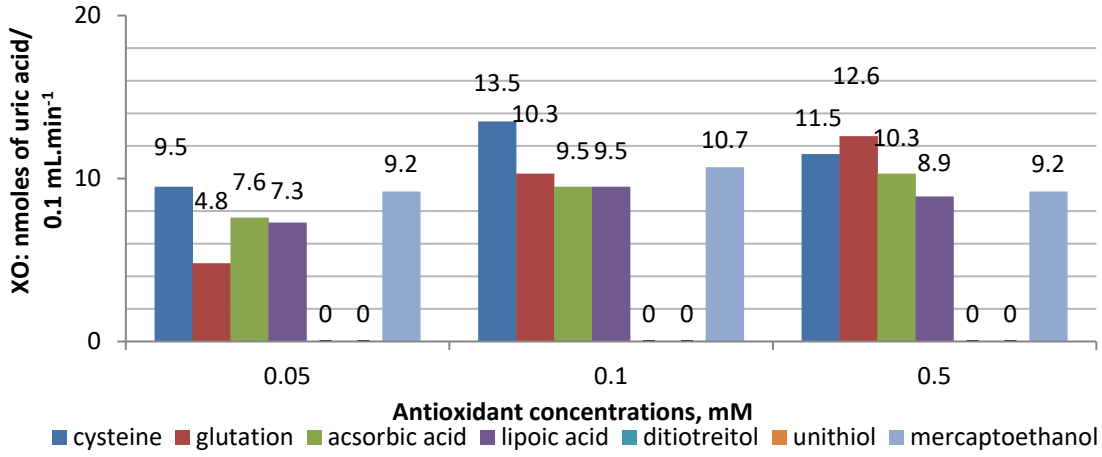


**Figure 2** Effects of different antioxidants on the stability of XO during the heat treatment of fresh sheep milk in the presence of $Na_2MoO_4$.

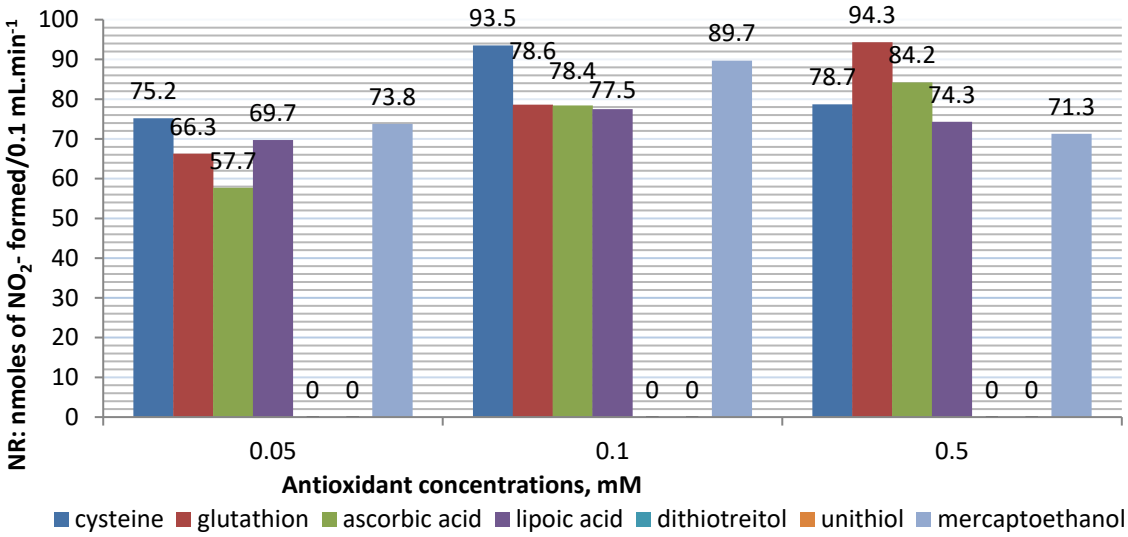


**Figure 3** Effects of different antioxidants on the stability of NR activity during the heat treatment of fresh sheep milk in the presence of $Na_2MoO_4$.

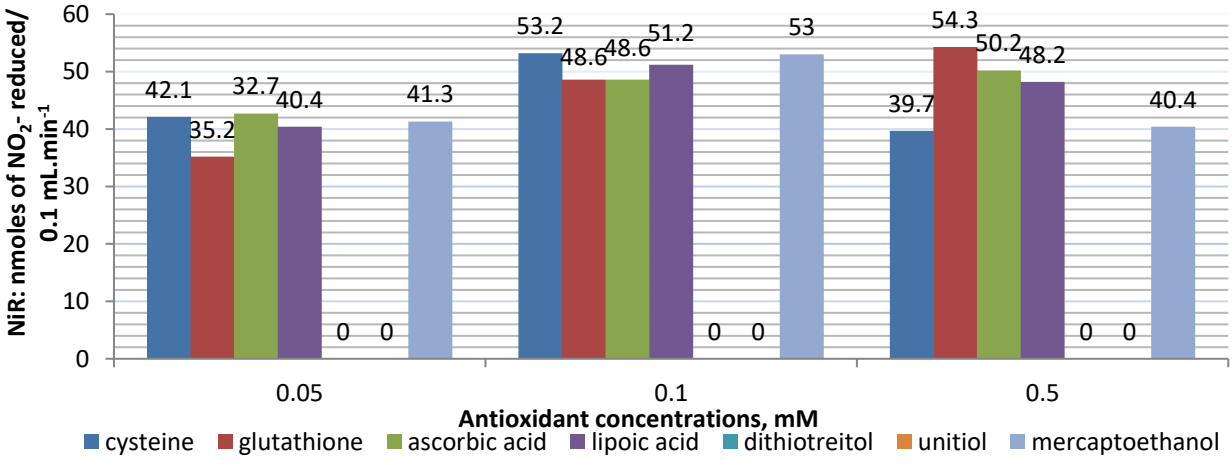


**Figure 4** Effects of different antioxidants on the stability of NiR activity during the heat treatment of fresh sheep milk in the presence of $Na_2MoO_4$.

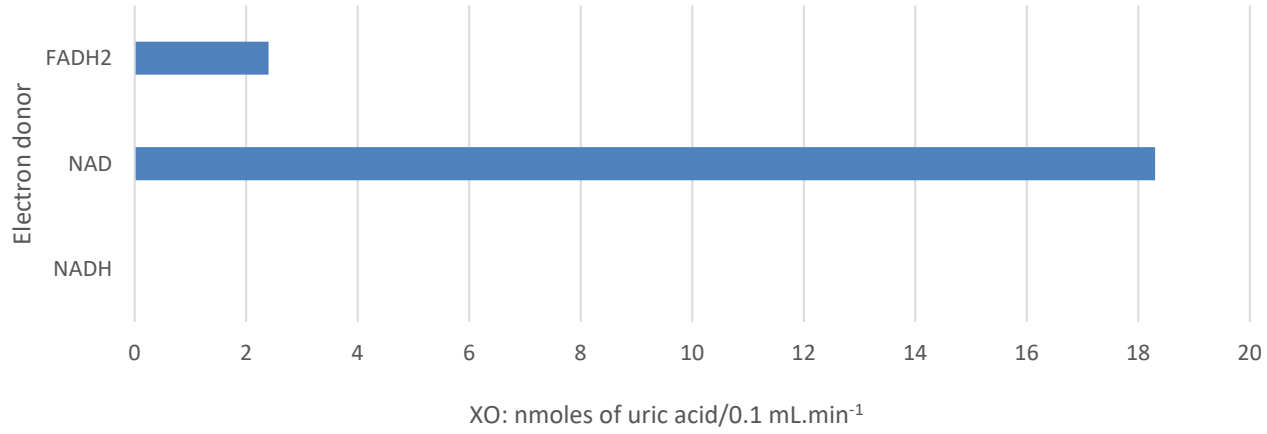


**Figure 5** Effects of different electron donors on XO activity of sheep milk in the presence of cysteine.

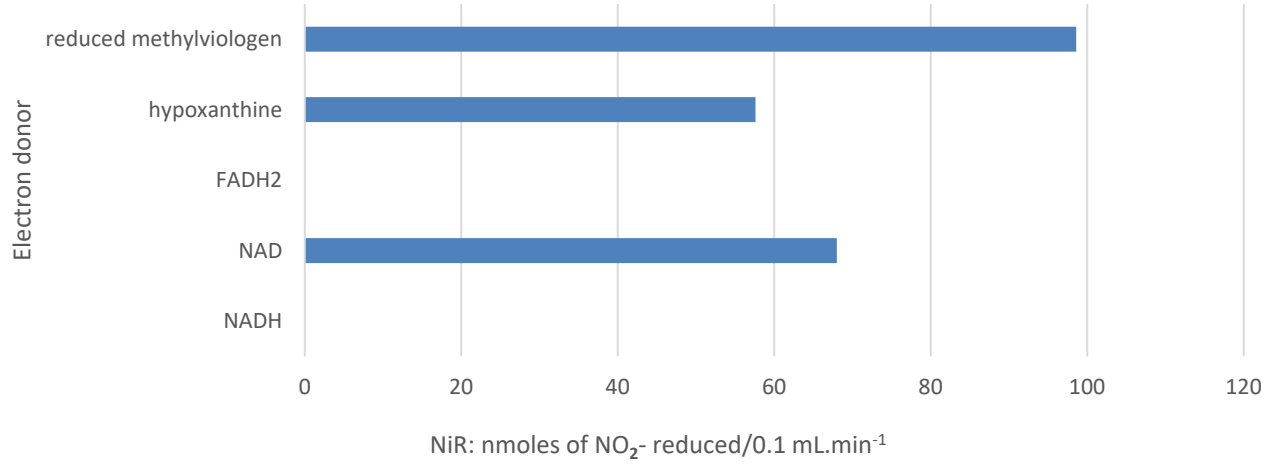


**Figure 6** Effects of different electron donors on nitrite reductase activity of sheep milk in the presence of cysteine.

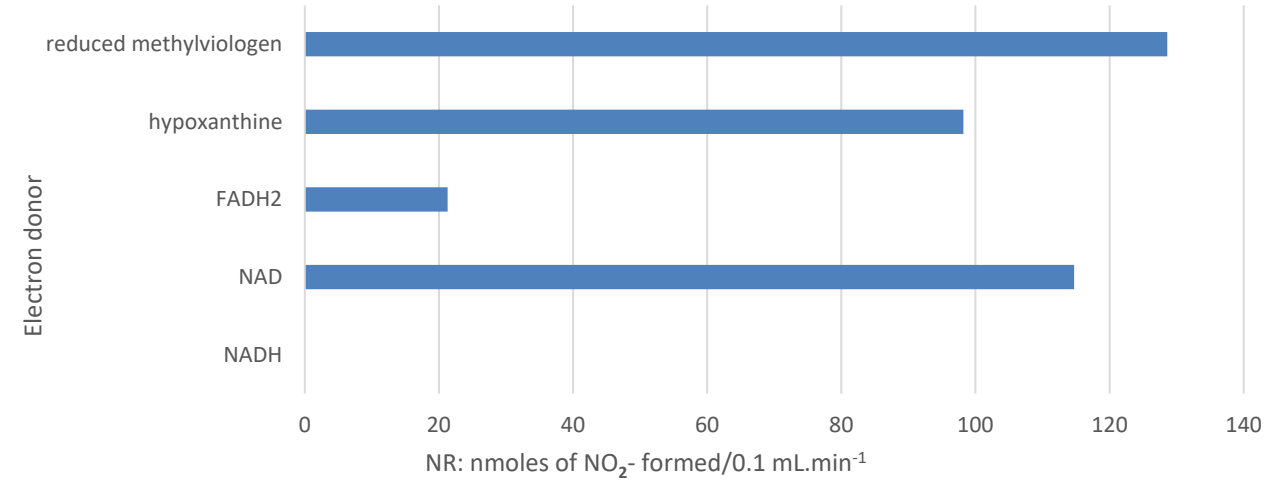


**Figure 7** Effects of different electron donors on nitrate reductase activity of sheep milk in the presence of cysteine.

**Table 1** Formation of CySNO in fresh sheep milk after heat treatment in the presence of cysteine and molybdenum or tungsten (n = 3, ±*SD*).

| Treatments of the milk | A substrate in reaction mixture | Absorbance, nм | Amount of CySNO, mM/0.1 mL.min$^{-1}$ |
|---|---|---|---|
| Control – PBS only without milk | | 340 | 0.0 |
| | $NO_3$- | 540 | 0.0 |
| | | 340 | 0.0 |
| | $NO_2$- | 540 | 0.0 |
| Control – milk without heat treatment | | 340 | 2.7 ±0.3 |
| | $NO_3$- | 540 | 0.0 |
| | | 340 | 5.8 ±0.2 |
| | $NO_2$- | 540 | 0.0 |
| Heating + $Na_2MoO_4$ at 80 °C, 7 minutes | | 340 | 12.6 ±0.8 |
| | $NO_3$- | 540 nм | 3.2 ±0.2 |
| | | 340 nм | 35.5 ±3.5 |
| | $NO_2$- | 540 nм | 7.3 ±1.3 |
| Heating + $Na_2WO_4$ at 80 °C, 7 minutes | | 340 nм | 0.0 |
| | $NO_3$- | 540 nм | 0.0 |
| | | 340 nм | 0.0 |
| | $NO_2$- | 540 nм | 0.0 |
| Heating of milk without $MoO_4^=$ or $WO_4^=$ | | 340 nм | 0.0 |
| | $NO_3$- | 540 nм | 0.0 |
| | | 340 nм | 0.0 |
| | $NO_2$- | 540 nм | 0.0 |

**Table 2** Effect of increasing concentrations of the lecithin on nitrate- and nitrite-reducing activities and CysNO formation in sheep milk (n = 3, ±*SD*).

| Lecithin content | Associated activities | | Amount of CySNO* | |
|---|---|---|---|---|
| | NR | NiR | From NR | From NiR |
| Control | 92.8 ±8.5 | 57.6 ±7.6 | 13.2 ±1.2 | 33.7 ±5.2 |
| 0.01 mg.mL$^{-1}$ | 91.2 ±10.2 | 58.4 ±6.8 | 14.3 ±2.6 | 33.5 ±7.3 |
| 0.05 mg.mL$^{-1}$ | 92.7 ±9.5 | 57.8 ±9.3 | 14.4 ±1.8 | 34.7 ±7.1 |
| 0.1 mg.mL$^{-1}$ | 95.8 ±11.6 | 62.9 ±9.7 | 18.4 ±3.2 | 42.9 ±8.2 |
| 0.5 mg.mL$^{-1}$ | 122.5 ±14.7 | 117.6 ±12.8 | 33.6 ±4.5 | 78.9 ±12.2 |
| 1.0 mg.mL$^{-1}$ | 128.6 ±12.8 | 135.2 ±18.6 | 36.7 ±7.3 | 93.8 ±11.7 |

It is known that NADH is one of the potential physiological electron donors for XO and it also has an absorbance at 340 nm (reduced NADH exhibits strong UV absorption at 340 nm whilst the oxidized form has virtually no absorption at this wavelength).

Therefore, to avoid mutual interference between the optical density of NADH and CysNO at 340 nm, instead of NADH we used hypoxanthine as an electron donor for NR and NiR activities of milk XO. It was demonstrated that heat treatment (80°C, 10 min) of homogeny XO resulted in the release of molybdenum cofactor (Mo-co) from the active center of the denatured enzyme molecule. During the heat treatment of XO ascorbic acid was the potential protector against the oxidation of released Mo-co. However, in the absence of ascorbic acid, it is quickly inactivated by oxygen (even in anaerobic conditions) **(Alikulov et al., 1980; Vogels and Van der Drift, 1970)**. Later we showed that glutathione and cysteine were the more powerful protectors for isolated Mo-co **(Alikulov and Mendel, 1984; Poje and Sokolić-Maravić, 1986)**.

However ascorbic acid decomposes S-nitrosocysteine **(Stsiapura et al., 2018)** and, therefore, we used L-cysteine as the protector against the oxidation of the cofactor in the active center of XO localized in MFGM. Thus, in our experiments L-cysteine was used for double purposes: as the protector of enzyme Mo-co against the oxidation during heat treatment of milk and as a reagent for S-nitrosothiol formation. For the construction of the calibration curve, we used nitroprusside as a donor of NO. Increasing concentrations of nitroprusside from 10 nM to 1.0 μM mixed with constant 0.1 mM concentration of cysteine in milk serum. Micromolar concentrations of nitroprusside release Nanomolar concentrations of NO **(Porras et al., 1981; Kramer et al., 1987)**. The serum was obtained by heating fresh milk, precipitating its proteins with acetic acid, and centrifuging. To obtain a transparent milk serum, its filtration was carried out through a membrane (33 mm Millex Filter).

For NO determination sheep milk mixed in the ratio of 1:1 with 0.2 M chlorinated phosphate buffer (PBS), pH 6.5, containing 10mM NEM, 2.5 mM EDTA **(Kisker et al., 1997; Marley et al., 2001)**, 0.2 mM $Na_2MoO_4$ or $Na_2WO_4$ and 0.1 mM cysteine. After incubation in 15 minutes at a temperature of 36 °C, the milk proteins were precipitated by diluted acetic acid added to the reaction mixture until pH 4.0. After centrifugation in the supernatant absorbance at 340 nm and 540 nm were measured. For many years tungsten was considered to be a biological antagonist of molybdenum and was used for the study of the properties and functions of molybdenum in Mo-enzymes. This was because tungsten can replace molybdenum in Mo-enzymes, forming catalytically inactive analogs **(Kletzin and Adams, 1996; Bray et al., 1999; Beedham, 2001; Taibi et al., 2001)**. Therefore, to make sure that it is the molybdenum enzyme that catalyzes the formation of NO, instead of molybdenum we incubated the milk in the presence of tungsten, and the results obtained (Tables 1, 2) convincingly show that the heat treatment of fresh sheep

milk in the presence of exogenous molybdenum activates XO and the enzyme becomes capable of converting nitrate and nitrite to nitric oxide. However, when nitrates were used as a substrate NO formation was very low. At the same time, using nitrites as substrate resulted in a 10 times higher amount of formed NO (i.e. CySNO) in comparison with nitrate substrates. The levels of CysNO determined by absorbance at 340 and 540 nm were completely different. This was likely due to the difference in the sensitivity of the absorption at the ultraviolet and visible wavelengths of the spectrophotometer.

### Effect of exogenous phospholipid on the associated activities of sheep milk XO

Phospholipids (PLs) are formed by glycerol, phosphoric acid, fatty acids, and a hydroxyl compound (e.g., choline, ethanolamine, serine, inositol). It is well known that milk fat globule membranes (MFGM) is composed of four main species of PLs: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS). The most abundant PLs in milk fat expressed as a percentage of the total, are PE (26.4% – 72.3% of total PLs), PC (8.0% – 45.5%), PI (1.4% – 14.1%), and PS (2.0% – 16.1%). Of the lipids, phosphatidylcholine is largely located on the outside while the neutral lipids, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol are concentrated on the inner surface **(Garcia et al., 2012; Suzuki et al., 2015)**. Amphiphilic properties of PLs derive from the presence of both a hydrophobic tail and a hydrophilic head make them natural detergents. Thus, under high-temperature phospholipids may effectively disrupt hydrophobic bonds between fatty acids, e.g., the integrity of all membrane layers of MFGM **(Godber et al., 2000; Gladwin et al., 2005)**. Therefore, the destruction of MFGM makes the enzyme molecules available for exogenous molybdenum and its substrates (hypoxanthine, nitrate, and nitrite).

In the next experiments, we examined the effect of lecithin on NR and NiR activities of heat-treated fresh sheep milk. Egg yolk lecithin was used in the experiments. The main phospholipids of this lecithin are phosphatidylcholine and phosphatidylethanolamine, i.e. they are components of the outer and inner layers of the membrane. The lecithin was dissolved in isopropyl alcohol and then added to the milk before heat treatment. The other components required for the heat treatment of milk have already been written above. Hypoxanthine was used as an electron donor for NR and NiR activities.

## CONCLUSION

During the research, it was found that dithiothreitol associated with molybdenum does not show a stabilizing or antioxidant effect. The absence of such a stabilizing effect of unithiol remains unclear. During nitrate reduction by hypoxanthine, the XO molybdenum center may participate directly in both the oxidative and the reductive half-reactions. Using nitrates as a substrate did not result in high NO formation while using nitrites as substrate increased the amount of formed NO. It can be explained by the difference in the sensitivity of the absorption at the ultraviolet and visible wavelengths of the spectrophotometer.

The results show that exogenous lecithin in relatively high concentrations (0.5 – 1.0 $mg.mL^{-1}$) increases the activity of NR, NiR, and the formation of CysNO. Apparently, during heat treatment, exogenous lecithin does partially decomposes the membrane of MFGM of milk, and enzyme molecules become more accessible. Thus, the results obtained may be important in the cleaning of animal milk contaminated with nitrates or nitrites. Thus, our results suggest the possible use of XO activation by heat treatment to remove nitrates from milk. Usually fresh sheep's milk is consumed after heat treatment.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# INCIDENCE OF HORMONAL GROWTH STIMULANT AND ANTIBIOTICS RESIDUES IN CHICKEN MEAT

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## ABSTRACT

The purpose of this study is to determine the level of antibiotic residues in poultry meat sold in the city of Almaty, which is a megacity of Kazakhstan, and compare it with the Minimum Residue Level set by World Health Organization and Food and Agriculture Organization. Determination of hormonal growth stimulators (progesterone, testosterone, estradiol-17β) and antibiotics (tetracycline, chloramphenicol, and streptomycin) in poultry was carried out using immunoassay according to the manufacturer's instruction. The results of monitoring showed that in the Almaty region annually wholesale deliveries of meat products from the USA, Belarus, Ukraine, and Russia are carried out regularly. On average in the region from 2016 to 2019, the specific weight meat imports averaged 47.9% of total consumption. A total of 105 samples were examined by us between 2019 and 2020, including 63-import meat and 42 samples of the meat of local production. Due to the particular importance of this problem, the main focus of the article`s goal is to conduct monitoring studies on imported and domestic meat for the residual content of hormones and antibiotics in poultry meat. As a result of the enzyme-linked immunosorbent assay of poultry meat, an excess level of maximum residue was detected in eight brands of imported meat.

**Keywords:** growth promoters; ELISA; level of maximum residue; animal products; import

## INTRODUCTION

Meat is an integral part of the traditional dishes of the Kazakh people. Every citizen of Kazakhstan consumes an average of 78 kg of meat and meat products per year. According to the Ministry of Agriculture, in 2018, Kazakhstan 12 thousand tons of beef imported from Ukraine, Paraguay, and Belarus, 17 thousand tons of horsemeat brought from Argentina and Uruguay, 13 thousand tons of pork and 132 thousand tons of chicken from Russia and the USA. 7.8 thousand tons of lamb came to Kazakhstan from Russia and Mongolia, and almost 30 thousand tons of sausage and meat products were put on store shelves by Russia and Belarus. According to the chairperson of the Meat Union Maksut Baktibaev, the need for poultry meat in Kazakhstan is very difficult to provide. Imported meat is cheaper than domestic if, in Paraguay, a kilogram of meat costs two dollars, and in Kazakhstan, it costs five dollars. But most often this meat does not go to the shelves, but to the sausage production, which is bought by socially vulnerable segments of the population – students and retirees **(Kuchma, 2016; Aitzhanova, 2019)**.

Antibiotics are widely used for various purposes, including therapeutic and preventive, as well as growth stimulants. There is a risk that the infection will spread very quickly among birds; so many farmers use the antibiotic in large quantities, without any prescription, without a system. In poultry farming, antibiotics are given with drinking water or food, which makes their use even more affordable. Dangerous residues of the drug accumulate in the edible parts of food animals, for example, in the pectoral muscles of chickens **(Kadim et al., 2020)**. High levels of maximum residues (MRL) pose a serious threat to public health and the development of antibiotic resistance. When consuming meat with antibiotic residues, the work of the human intestinal microflora is disrupted, pathogenic bacteria increase, this leads to hypersensitivity of the body and a violation of the normal intestinal microflora. Growth promoters can cause the accumulation of residues in animals (meat, milk, and eggs), and the environment (water and soil contamination) and wastewater treatment systems are other important routes through which antibiotics and hormones can enter the environment with negative consequences **(Al-Doski et al., 2015; Gómez-Pérez et al., 2015; Parr et al., 2015; Akhmet et al., 2019)**. A key goal of protecting the planet through a more sustainable diet is to reduce the number of antibiotics and synthetic growth promoters, in particular, to reduce antibiotic resistance and allergies. Analytical methods play a crucial role in food analysis to determine the presence of antibiotics and other additives. Modern methods based on liquid chromatography with ultraviolet, fluorescent, or mass spectrophotometry detection, which can identify more than 300 compounds in

samples, but requires a lot of time and cost. In General, there is a need for a monitoring program aimed at informing the public about the dangers associated with residues of antibiotics and synthetic stimulants in animal products and an accelerated method for determining them **(Sokolova et al., 2015; Robert et al., 2015)**.

The main danger is the risk of health effects on human residual amounts of these substances in livestock products. Numerous studies have proven high toxicity and the danger of steroid hormones when they enter the human body. The action of exogenous hormones can cause endocrine disorders balance in the body, which is expressed by unpredictable remote effects of steroid exposure at prepubescent age on reproductive function through a mechanism called "hormonal imprinting" **(Gómez-Pérez et al., 2015; Summary report, 2015; Van Boeckel et al., 2015)**. The scientific Veterinary Committee of the European Commission notes that there is a relationship between the use of steroid hormones and some cancers that meat consumption may increase the risk of breast and prostate cancer, and cites as an example North America, where the volume of consumption of meat produced from using hormones, the largest, and where the incidence of breast cancer and the prostate is also the highest **(Codex texts, 2015)**. Oncological diseases have a great impact on society in Kazakhstan and around the world. In 2018, 32.228 new cases of cancer were diagnosed in Kazakhstan, and 14.369 people died from this disease **(Kaidarova et al., 2020; Indicators of the Oncological Service, 2021)**. The most common cancers in both sexes (listed in descending order according to estimates of new cases in 2018) are breast cancer, lung, and bronchial cancer, colorectal cancer, stomach, cervical, esophageal, prostate, kidney, ovarian, pancreatic, endometrial, and liver cancers **(Wild et al., 2020)**. Unsafe food is associated with the death of approximately 2 million people each year, mostly children **(Afshin et al., 2019; James, 2019)**. Pathogenic bacteria, viruses, parasites, or chemicals found in food cause more than 200 diseases, from diarrhea to cancer. New threats to food safety are constantly emerging. Changes in food production technology, distribution, and consumption; environmental changes; new and emerging pathogens; bacterial resistance all these factors create problems for national food safety systems **(Khan et al., 2015)**.

At the first stage of the study, the analysis of the receipt of various imported types of meat in the Republic of Kazakhstan was carried out. The top ten countries importing meat and offal include Brazil, Argentina, the United States, Canada, Poland, Paraguay, Uruguay, Australia, Mongolia, India, and others, according to the customs control Committee, the United States is the largest supplier of meat in Kazakhstan. Poultry producers of the United States of America in the period from 2016 to 2019 are poultry meat for more than half of all imported meat to Kazakhstan. The traditional product supplied to Kazakhstan from the United States is chicken meat or chicken meat, so-called "Bush legs". For example, the share of poultry imports exceeds 51%. Last year, more than 191 thousand tons were imported to the country and three years ago, there were more than 165 thousand tons **(Mamyshev, 2019)**.

### Scientific hypothesis

The purpose of this study is to determine the level of antibiotic residues in poultry meat sold in the city of Almaty, which is a megacity of Kazakhstan, and compare it with the Minimum Residue Level set by World Health Organization (WHO) and Food and Agriculture Organization (FAO).

## MATERIAL AND METHODOLOGY

### Samples

Chicken samples (n = 105) (three replicate each) represented fresh, frozen, broiler chicken meat of local and international brands was collected from major supermarkets in Almaty, Kazakhstan. Samples of local production belong to brands such as Ala-Tau Kus, Alel Agro, and Sarybulak, and imported chicken samples were from Ukraine, Russia, and the United States of America (USA).

### Instruments

The method of enzyme-linked immunosorbent assay (ELISA) was used to determine the number of hormonal stimulants and antibiotics. To determine the content of hormones, various instrumental research methods are used (radioimmune, chromatographic, spectrometric, etc.), which are labor-intensive, require expensive equipment, are difficult to set up, and can only be used in laboratory conditions. From a practical point of view, the most acceptable method is ELISA, which has a high sensitivity, low time, and cost.

### Laboratory Methods

According to different methods of sample preparation, analyses were performed for three various hormones and antibiotics. The purpose of preparing the sample for research was to transform the sample put the meat in a suitable form for subsequent analysis – a solution, as well as for concentration and dilution of the analyte and getting rid of interfering analysis components. Preparation of meat samples for research consisted of a set of actions: grinding, homogenization, extraction, hydrolysis, and sample deposition. Test principle: the basis of the test is the antigen-antibody reaction. The microtiter wells (lunula) are coated with capture antibodies directed against the antigen (chloramphenicol, tetracycline, streptomycin, progesterone, testosterone, estradiol-17β). The microtiter plate (pallet) is already functionalized with antigen antibodies, which are bound by the immobilized capture antibodies. Any unbound conjugate is then removed in a washing step (at the stage of flushing). The Conjugate converts the chromogen into a blue product. The addition of the stop solution (inhibitor removal buffer) leads to a color change. The measurement is made photometrically at 450 nm.

Determination of hormonal growth stimulators (progesterone, testosterone, estradiol-17β) and antibiotics (tetracycline, chloramphenicol, and streptomycin) in poultry was carried out using immunoassay according to the manufacturer's instruction. RIDASCREEN® ELISA kits purchased from R-Biopharm AG (Darmstadt, Germany) were used for the immunoassay determination for the quantitative determination of growth stimulators and antibiotics in animal products. Research on the definition of antibiotics and hormonal stimulants in poultry meat was conducted in the laboratory "Antigen", located in Almaty, Kazakhstan.

**Sample preparation**

Samples were collected from major supermarkets in Almaty, Kazakhstan. Permissible levels for antibiotics tetracycline, chloramphenicol in meat is not allowed (<0.01 $mg.kg^{-1}$), streptomycin (<0.5 $mg.kg^{-1}$). For meat, the level of progesterone and testosterone is not more than 0.015 $mg.kg^{-1}$, for estradiol-17β no more than 0.0005 $mg.kg^{-1}$. All samples were kept in plastic bags and transferred in a special cool box to the laboratory for analysis.

**Number of samples analyzed**

To determine the residual amounts of hormonal growth stimulants, progesterone, testosterone and estradiol, and antibiotics of tetracycline, chloramphenicol, and streptomycin, total number 105 chicken meat samples were examined, including 42 samples of local brands and 63 samples of imported chicken meat (four brands from each country).

## Statistical Analysis

The statistical analysis data were produced by Microsoft Excel. Limit detection of the RIDASCREEN TC test (in terms of standard substance) about 1.5 $\mu g.L^{-1}$ for tetracycline, about 28 $mcg.kg^{-1}$ for streptomycin, about 5 $ng.kg^{-1}$ for chloramphenicol, 50 $ng.kg^{-1}$ for testosterone, 500 $ng.kg^{-1}$ for estradiol-17β, 200 $ng.kg^{-1}$ for progesterone.

## RESULTS AND DISCUSSION

The results obtained were compared with the maximum permissible levels of hormones (MRLs), adopted by the Directives of the European Union (EU) and Russian Regulations. According to the results of the analysis of domestic meat, there is absolutely no residue of hormonal stimulants. A value of less than 0.6 absorbance units (A450 nm ≤0.6) for the zero standards. The zero standard is thus made equal to 100%, and absorbance values are quoted in percentages. The authors' findings showed that out of 64 analyzed samples of imported chicken meat, the amount of at least one of the identified anabolic hormones was determined in 11 forms, which is 18.3%.

The excess of the maximum permissible level of testosterone over the indicators is seen in 7 samples of poultry meat (11.6%) produced in the United States. The level of testosterone in the studied samples of birds increased from the maximum permissible level to 1.0 – 1.9 times. For the estradiol-17β, 11 samples were found to exceed the permissible level, which was 18.3% of the total number of detected hormones. Excess of MRLs was detected in 15 samples (4 brands) of chicken meat produced in the United States.

Table 1 provides a comparative analysis of the results of determining hormone residues in meat from different countries. The minimum traces of testosterone in all local brands of 0.000 $mg.kg^{-1}$and 0.005 $mg.kg^{-1}$in Russia do not reach the MRLs and acceptable daily intakes (ADI). Maximum testosterone traces exceeding MRLs brands of the USA are 0.029 $mg.kg^{-1}$ and Ukraine 0.018 $mg.kg^{-1}$. According to FAO and who, the ADI of testosterone should not exceed 0.002 $mg.kg^{-1}$ per day. If the US samples exceed the MRLs of testosterone by 93% and ADI by 45%, the results of the Ukrainian samples exceed 20%. Estradiol ADI of 0.00005 $mg.kg^{-1}$. The lowest level of estradiol exceeding the ADI and MRLs in the samples of local production 0.000 $mg.kg^{-1}$, and Russian 0.002 $mg.kg^{-1}$. Indicators of meat brands in Ukraine are slightly less than MRLs by 20%. The largest indicator of the US brand 0.022 exceeds the MRLs four times. Progesterone in both local and imported brands does not exceed the MRLs, although the results exceed the ADI of 0.03 $mg.kg^{-1}$, the highest indicator of the US brand is four times less than the MRLs. MRLs of these hormones in meat according to FAO and WHO is undesirable Joint FAO/WHO Expert Committee on Food Additives **(JECFA, 2000)**. Indicators of testosterone brands in the USA and Ukraine exceeded the ADI, the expected effect is the androgenic effects when receiving a regular dose of testosterone **(Kim et al., 2021)**.

After analyzing the results for the number of excess hormone residues in the meat of different areas, the authors analyzed the accumulation of residues in parts of the poultry carcass. In Table 2, if you look at the results of an enzyme-linked analysis of US meat, you can see the largest accumulation of testosterone residues in chicken legs 0.029 $mg.kg^{-1}$, then 61% less in wings and brisket 0.018 – 0.02 $mg.kg^{-1}$. The remainder of the hormone testosterone in Ukrainian meat brands is the largest amount in only one brand 0.018 $mg.kg^{-1}$in chicken legs, and 12% less in other parts of meat. Although Russian meat remains more than local, as well as local does not exceed the MRLs for all hormones and this minimum amount is evenly distributed in all parts of the meat **(Sokolova et al., 2015)**.

**Table 1** The content of the residual amount of hormone in meat.

| | Brand | Local | USA | Russia | Ukraine | MRLs |
|---|---|---|---|---|---|---|
| Testosterone | 1 | 0.000 | 0.029 | 0.005 | 0.018 | 0.015 $mg.kg^{-1}$ |
| | 2 | 0.000 | 0.027 | 0.002 | 0.016 | |
| | 3 | 0.000 | 0.022 | 0.001 | 0.005 | |
| | 4 | 0.000 | 0.021 | 0.000 | 0.002 | |
| Estradiol-17β | 1 | 0.000 | 0.022 | 0.002 | 0.004 | 0.005 $mg.kg^{-1}$ |
| | 2 | 0.000 | 0.0099 | 0.001 | 0.002 | |
| | 3 | 0.000 | 0.0047 | 0.001 | 0.0018 | |
| | 4 | 0.000 | 0.0044 | 0.001 | 0.0016 | |
| Progesterone | 1 | 0.000 | 0.005 | 0.001 | 0.002 | 0.015 $mg.kg^{-1}$ |
| | 2 | 0.000 | 0.005 | 0.001 | 0.002 | |
| | 3 | 0.000 | 0.005 | 0.001 | 0.002 | |
| | 4 | 0.000 | 0.005 | 0.001 | 0.002 | |

**Table 2** Content of the residual amount of the hormone in imported meat samples.

| Type of meat | | USA | | | | Russia | | | | Ukraine | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Testosterone | Leg | 0.029 | 0.022 | 0.027 | 0.021 | 0.005 | 0.002 | 0.001 | 0.000 | 0.018 | 0.016 | 0.005 | 0.002 |
| | Wings | 0.018 | 0.02 | 0.018 | 0.018 | 0.005 | 0.002 | 0.001 | 0.000 | 0.016 | 0.01 | 0.002 | 0.01 |
| | Brisket | 0.02 | 0.021 | 0.018 | 0.02 | 0.005 | 0.002 | 0.001 | 0.000 | 0.016 | 0.01 | 0.03 | 0.02 |
| Estradiol-17β | Leg | 0.022 | 0.0099 | 0.0047 | 0.0044 | 0.002 | 0.001 | 0.001 | 0.001 | 0.004 | 0.0022 | 0.0018 | 0.0016 |
| | Wings | 0.004 | 0.004 | 0.0044 | 0.004 | 0.002 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.0016 | 0.001 |
| | Brisket | 0.0047 | 0.003 | 0.0045 | 0.004 | 0.002 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.0016 | 0.0016 |
| Progesterone | Leg | 0.005 | 0.005 | 0.005 | 0.005 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.002 | 0.002 | 0.002 |
| | Wings | 0.003 | 0.005 | 0.003 | 0.005 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.001 |
| | Brisket | 0.005 | 0.002 | 0.005 | 0.005 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.002 | 0.001 | 0.002 |

**Table 3** Antibiotic residues in raw chicken meat from local and imported samples, which exceeded the maximum allowable level.

| Sample | Brand | Tetracycline | Chloramphenicol | Streptomycin |
|---|---|---|---|---|
| | MRLs | 0.01 mg.kg$^{-1}$ | 0.01 mg.kg$^{-1}$ | 0.5 mg.kg$^{-1}$ |
| Local samples | 1 | 0.002 ±0.002$^{cdC}$ | 0.007 ±0.002 $^{cdB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 2 | 0.003 ±0.002$^{cB}$ | 0.005 ±0.001 $^{dB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 3 | 0.003 ±0.002 $^{cdC}$ | 0.007 ±0.002 $^{cdB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 4 | 0.002 ±0.002 $^{cdB}$ | 0.002 ±0.002 $^{eB}$ | 0.000025 ±0.000 $^{aA}$ |
| Russia | 1 | 0.001 ±0.001 $^{dC}$ | 0.007 ±0.001 $^{cdB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 2 | 0.001 ±0.001 $^{dC}$ | 0.008 ±0.001 $^{cB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 3 | 0.001 ±0.001 $^{dC}$ | 0.007 ±0.001 $^{cdB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 4 | 0.001 ±0.001 $^{dC}$ | 0.007 ±0.002 $^{cdB}$ | 0.000025 ±0.000 $^{aA}$ |
| Ukraine | 1 | 0.011 ±0.000 $^{bC}$ | 0.018 ±0.001 $^{bB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 2 | 0.011 ±0.000 $^{bC}$ | 0.017 ±0.001 $^{bB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 3 | 0.011 ±0.000 $^{bC}$ | 0.017 ±0.001 $^{bB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 4 | 0.011 ±0.000 $^{bC}$ | 0.016 ±0.001 $^{bB}$ | 0.000025 ±0.000 $^{aA}$ |
| USA | 1 | 0.018 ±0.001 $^{aC}$ | 0.022 ±0.001 $^{aB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 2 | 0.018 ±0.001 $^{aB}$ | 0.022 ±0.002 $^{aA}$ | 0.000025 ±0.000 $^{aA}$ |
| | 3 | 0.018 ±0.001 $^{aC}$ | 0.021 ±0.001 $^{aB}$ | 0.000025 ±0.000 $^{aA}$ |
| | 4 | 0.018 ±0.001 $^{aC}$ | 0.021 ±0.000 $^{aB}$ | 0.000025 ±0.000 $^{aA}$ |

*Note: data are expressed as mean ± SD of triplicates.*

Estradiol residues in chicken legs are 0.022 mg.kg$^{-1}$, 0.0099 mg.kg$^{-1}$, which is four times higher than residues in other parts of meat of the same brand. Hormone-like progesterone that does not exceed the MRLs **(Sánchez-Salazar et al., 2019; Kadim et al., 2020)** in all samples are distributed evenly in all parts of the bird. Table 3 shows the results of an enzyme immunoassay for three antibiotics. In the first column, the tetracycline residue in local meat does not exceed the MRLs level and is 0.003 mg.kg$^{-1}$, and does not exceed the ADI of 0.03 mg.kg$^{-1}$. Traces of tetracycline residues are less than the MRLs level and are 0.001 mg.kg$^{-1}$ in Russian brands. In Ukrainian brands, tetracycline is 0.011 mg.kg$^{-1}$, which exceeds the MRLs by 10%. The remaining tetracycline in US meat is 0.018 mg.kg$^{-1}$ and exceeds the MRLs level by 80%. In local meat brands, even the highest value of 0.007 mg.kg$^{-1}$of chloramphenicol is less than the MRLs. The Russian results for chloramphenicol as well as local ones do not exceed the MRLs and the result is 0.008 mg.kg$^{-1}$. The level of chloramphenicol residue 0.016-0.018 mg.kg$^{-1}$, in all brands exceeds the MRLs by 60-80%. The highest amount of chloramphenicol in US meat brands is 0.022 mg.kg$^{-1}$ and exceeds the MRLs twice. In all samples, the level of streptomycin is less than MRLs and is 0.000025 mg.kg$^{-1}$. Table 4 shows the results of both hormones and antibiotics. In the United States, the most polluted brands were found in the US brand No. 1 for the hormones testosterone, estradiol, and the antibiotics tetracycline and chloramphenicol **(Khan et al., 2015; Yang et al., 2019; Kim et al., 2021)**. US Brands No. 2, No. 3, and No. 4 also exceed these indicators by a difference of 10 – 20%. No. 1 brand of Ukraine for testosterone, tetracycline, and chloramphenicol exceeds MRLs and ADI.

In all samples, the levels of progesterone and streptomycin do not exceed MRLs and ADI. Small concentrations of antibiotics were found in local samples. Additionally, when processing the data obtained, the authors compared each peak of the standard mixture of antibiotics regulated by EU standards with the peaks obtained during the analysis of meat samples to determine whether the analyzed samples contain antibiotics with concentrations higher than those declared by EU standards. From the data presented in Table 4, it is clear that out of 12 brands of imported meat, the highest content of tetracycline was observed in all meat brands from the United States and Ukraine.

**Table 4** The content of the residual amount of hormone and antibiotic that exceeded the maximum allowable level

| | Brand | Local sample | USA | Russia | Ukraine | MRLs |
|---|---|---|---|---|---|---|
| Testosterone | 1 | 0.000 | 0.029 | 0.005 | 0.018 | 0.015 mg.kg$^{-1}$ |
| | 2 | 0.000 | 0.027 | 0.002 | 0.016 | |
| | 3 | 0.000 | 0.022 | 0.001 | 0.005 | |
| | 4 | 0.000 | 0.021 | 0.000 | 0.002 | |
| Estradiol-17β | 1 | 0.000 | 0.022 | 0.002 | 0.004 | 0.005 mg.kg$^{-1}$ |
| | 2 | 0.000 | 0.0099 | 0.001 | 0.0022 | |
| | 3 | 0.000 | 0.0047 | 0.001 | 0.0018 | |
| | 4 | 0.000 | 0.0044 | 0.001 | 0.0016 | |
| Progesterone | 1 | 0.000 | 0.005 | 0.001 | 0.002 | 0.015 mg.kg$^{-1}$ |
| | 2 | 0.000 | 0.005 | 0.001 | 0.002 | |
| | 3 | 0.000 | 0.005 | 0.001 | 0.002 | |
| | 4 | 0.000 | 0.005 | 0.001 | 0.002 | |
| Tetracycline | 1 | 0.002 | 0.018 | 0.001 | 0.011 | 0.01 mg.kg$^{-1}$ |
| | 2 | 0.003 | 0.018 | 0.001 | 0.011 | |
| | 3 | 0.003 | 0.018 | 0.001 | 0.011 | |
| | 4 | 0.002 | 0.018 | 0.001 | 0.011 | |
| Streptomycin | 1 | 0.000025 | 0.000025 | 0.000025 | 0.000025 | 0.5 mg.kg$^{-1}$ |
| | 2 | 0.000025 | 0.000025 | 0.000025 | 0.000025 | |
| | 3 | 0.000025 | 0.000025 | 0.000025 | 0.000025 | |
| | 4 | 0.000025 | 0.000025 | 0.000025 | 0.000025 | |
| Chloramphenicol | 1 | 0.007 | 0.022 | 0.007 | 0.018 | 0.01 mg.kg$^{-1}$ |
| | 2 | 0.005 | 0.022 | 0.008 | 0.017 | |
| | 3 | 0.007 | 0.021 | 0.007 | 0.017 | |
| | 4 | 0.002 | 0.021 | 0.007 | 0.016 | |

Exceeding the MRLs tetracycline, chloramphenicol, streptomycin was not observed in the samples from Kazakhstan and Russia (Figure 1).



**Figure 1** The study tested 105 chicken meat samples from Kazakhstan, Russia, Ukraine, and the USA.

The level of tetracycline in the studied meat samples from the USA (80%) and Ukraine (10%) exceeded MRLs. The lowest concentration of the antibiotic was observed as a result of the determination of streptomycin in poultry samples and averaged 0.000025 mg.kg$^{-1}$ (25.10$^{-6}$) mg.kg$^{-1}$ with a norm of 0.5 mg.kg$^{-1}$. Thus, the concentration of streptomycin in the samples did not exceed the maximum permissible value. Following the technical regulations of the Customs Union (TR CU) 021/2011 "on food safety" **(Technical Regulations of the Customs Union, 2011)** and TR CU 034/2013 "on the safety of meat and meat products" **(Technical Regulations of the Customs Union, 2013)**, the content of tetracycline's in food products should not exceed 0.01 mg.kg$^{-1}$ **(Law of the Republic of Kazakhstan, 2019)**. Antimicrobials are used as growth promoters, especially in poultry farming. To increase the efficiency of fattening, feeding is practiced in relatively small doses of antibiotics over a long period **(Qiu et al., 2021)**. Antibiotics used in feeding birds have a stimulating effect on their growth, productivity, and reproduction, which leads to an average of 4 – 5% increase in live weight gain animals compared with control groups, feed costs per unit of growth are reduced by 5 – 8 %, the body resistance is activated, the period of the feeding of birds is reduced **(Robert et al., 2015; Saksrithai and King, 2018)**. Residual amounts of antibiotic substances are capable of the pass into the meat and eggs of birds and have a toxic effect on the body of man **(Sang-Hee et al., 2010)**.

Inhibiting substances in raw meat are contained in larger quantities than in meat, subject to heat treatment **(Sweileh, 2021)**. Heat treatment does not reduce antibiotic activity, but it is extracted from tissues and organs into the broth, while the antibiotic substance contained in the broth in amounts comparable to its level in poultry meat **(Zaugolnikova and Vistovskaya, 2016)**. The results of monitoring showed that in the Almaty region annually wholesale deliveries of meat products from the USA, Belarus, Ukraine, and Russia are carried out regularly. On average in the region from 2016 to 2019, the specific weight meat imports averaged 47.9% of total consumption **(Sokolova et al., 2015; Mamyshev, 2019)**. The main part of imported meat consumption is poultry – chicken legs.

The largest supplier of poultry meat to the Almaty market is the USA **(Tukusheva, 2014; Kaidarova et al., 2020)**. In particular, the poultry industry has been associated with the regular use of antimicrobials **(Gelaude et al., 2014; Van Boeckel et al., 2015; Yang et al., 2019)**. However, since a clear association has been established between the extent of antimicrobial use (AMU) in livestock and the development of antimicrobial resistance (AMR) **(Persoons et al., 2011; Chantziaras et al., 2014; Sánchez-Salazar et al., 2019)**, increased awareness is pushing the poultry sector towards reduced and rational use of antimicrobials **(Laxminarayan et al., 2013)**.

## CONCLUSION

As a result of this work, a higher content of tetracycline and chloramphenicol was revealed compared to other antibiotics: the concentration of tetracycline in the samples is on average 0.018 $mg.kg^{-1}$at a rate of 0.01 $mg.kg^{-1}$, which is almost twice the norm; the concentration of chloramphenicol in the samples is 0.022 $mg.kg^{-1}$at a rate of 0.01 $mg.kg^{-1}$, which is more than twice the norm. According to the results of the study, imported meat from the United States for the remnants of hormonal stimulants and antibiotics showed the most excess of the MRLs 10 – 20% less than the US exceeds the permissible level of antibiotics in Ukraine's brand. Antibiotic streptomycin and the hormone progesterone in all samples do not exceed the norm. Kazakh and Russian samples of poultry meat meet the acceptable standards and the results show that it contains only traces of antibiotics chloramphenicol and tetracycline. The results of research on imported products indicate that the fact that problem of meat entering our country with increased content of hormonal growth stimulants and antibiotics exists. A total of 105 samples were examined by us between 2019 and 2020, including 63-import meat and 42 samples of the meat of local production. A significant share of contaminated meat, 62.7%, is produced in the United States and 31% in Ukraine. Exceeding the maximum, the acceptable level in the studied samples of meat was: testosterone from 1.0 to 2 times, estradiol from 1 to 4 times, antibiotics tetracycline from 1 to 1.8 times, chloramphenicol from 1.0 to 2.2 times. Due to the particular importance of this problem, the focus of our goal is to conduct monitoring studies on imported and domestic meat for the residual content of hormones and antibiotics in poultry meat.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# VETERINARY AND SANITARY ASSESSMENT AND DISINFECTION OF REFRIGERATOR CHAMBERS OF MEAT PROCESSING ENTERPRISES

***Kateryna Rodionova, Anatoliy Paliy, Mariia Khimych***

## ABSTRACT

The results of microbiological studies of air samples of refrigerating chambers of meat processing enterprises are presented. The quantitative composition of the air microbiota of the chambers of the refrigerating shop was studied. It has been established that the technological regimes for cooling meat in cooled chambers (t = +4 °C) and deep freezing chambers (t = -18 °C and -22 °C) have no bacteriostatic effect on the life activity of mold fungi. The developed disinfecting preparation (hydrogen peroxide (8.0 – 10%), acetic acid (10%), peracetic acid (5.0 – 7.0%), stabilizing additives, water) ensures the destruction of sanitary-indicative microorganisms in cold rooms meat processing plants when applied at a concentration of 0.05% – 60 minutes, 0.1% – 30 minutes, 0.15% – 10 minutes and does not have a toxic effect on meat raw materials that are stored in chambers of the refrigeration shop after disinfection.

**Keywords:** refrigerating chambers; microorganisms; disinfectant; concentration; exposure

## INTRODUCTION

Today, refrigeration is the most common and effective method of preserving meat products. Ensuring the quality and safety of raw meat during long-term storage at low temperatures primarily depends on the creation of a continuous refrigeration chain throughout the production of the product – from production to consumer **(Ryabchenko, 2016; Göranssona, Jevingerb and Nilssonc, 2018; Ishevskiy and Davydov, 2017)**.

During refrigerated storage, the properties of products change due to the processes that occur in them (physical, biochemical, microbiological, etc.). In some cases, they improve the consumer properties of products, and in others – cause their spoilage **(Bruckner et al., 2012; Stonehouse and Evans, 2015; Bermúdez-Aguirre and Welti-Chanes, 2016)**. Therefore, the purpose of refrigeration and storage of products is to ensure the favorable passage of the first (meat autolysis, etc.) and minimize other (drying, microbiological changes, oxidation, etc.) processes **(Kaalea et al., 2011)**.

It should be noted that maintaining the proper quality of products during their long-term storage also depends on the general sanitary and hygienic conditions of refrigerators. Without timely washing and disinfection, pathogenic bacteria actively multiply in refrigerators, mold develops, and as a result, an unpleasant odor appears. This directly affects the quality of meat products, which begin to spoil and become completely unfit for consumption and dangerous to human health **(Bogatko and Sakhniuk, 2013)**.

Low temperature does not destroy microorganisms or enzymes, but only inhibits their activity. According to temperature resistance, microorganisms are divided into three groups: thermophiles (develop at a temperature of 20 – 80 °C, optimally at 50 – 75 °C), mesophiles (5 – 57 °C), and psychrophiles (-10 – 10 °C). Psychrophiles are the most interesting for refrigeration technology. They are divided into facultative, which can live in the same conditions as mesophiles, and obligate, which can reproduce only at low temperatures. Psychrophilic bacteria actively multiply on products with low acidity–meat, fish, non-acidic dairy products at temperatures of -5 – -8 °C. Most molds are psychrophilic and actively develop on frozen foods. They stop their reproduction at -2 – -3 °C (certain types of molds - only at -8 – -10 °C). Separate colonies of molds appear on the surface of frozen meat stored at a temperature higher than -8 °C **(Berk, 2018)**.

The mycelium of the fungus penetrates the thickness of the product, forming spores (for fungi and yeast, they are not only a means of protection against adverse external factors but also a means of reproduction). Mold begins to multiply, forming white, grey, and black spots on the surface of the product, as a result of which the products of mold activity accumulate in meat products, an unpleasant odor appears **(Mohapatra et al., 2017)**.

Ways of contamination of raw materials, semi-finished products, and finished products by microorganisms are extremely diverse **(Dukhnytskyi et al., 2011; Dudchik et al., 2013; Paliy, Rodionova and Palii, 2017)**. However, the most important source of additional microbial contamination of meat and meat products during refrigerated storage is the air of the refrigerator. Microorganisms, regardless of the pathogenicity that

accumulates in the air, on the walls and technological equipment, and their products (especially microscopic fungi) in case of contact with raw meat can pose a danger to human health due to infection or food poisoning, so sanitary-microbiological control of air in the chambers of the refrigeration shop has an important veterinary-sanitary significance **(Bogatko and Sakhniuk, 2013; Prokopenko, 2013)**.

In the food industry, in production shops, and in places of storage of products the level of microbic air pollution depends on a way of cleaning of rooms, the organization of production process, application and efficiency of work of ventilation and other conditions **(Iliashenko et al., 2008)**.

Today, despite the large arsenal of modern disinfectants, the search for new effective drugs is continuing, both through the synthesis of new chemical compounds and through the creation of compositions from existing substances. In composite means, it is possible to supplement the functional properties of each of the components, as well as to achieve the mutual strengthening of the activity of components (synergism) **(Glazova, 2007; Paliy and Rodionova, 2017)**.

Analysis of the nomenclature and active substances of the market of veterinary disinfectants registered in Ukraine shows that the main active ingredients of disinfectants are Quaternary Ammonium Compounds (QAC), aldehydes, oxygen-containing compounds, organochlorines, guanidines, and their combinations. The share of means of domestic production is only 45.5% **(Dymko, Pushkova and Solomon, 2015)**. In addition, it should be noted that about 50% of the modes of use of disinfectants specified by the manufacturer are not able to provide effective disinfection of facilities. In the instructions for use of these tools, the concentrations of working solutions and exposures are artificially underestimated in the tens or even hundreds of times from those that can cause the death of microorganisms **(Kanishchev and Eremeeva, 2016)**.

Oxygen compounds have become widely used in world veterinary practice. They show a wide range of bactericidal activity, can dissolve blood and many other biological substrates, coagulate protein well, have no odor, rapidly decompose in the environment into non-toxic products **(Paliy, 2018; Rodionova and Paliy, 2017)**. Drugs of this group are strong oxidants, the main action of which is the formation of free radicals that disrupt lipid metabolism in the cell membrane, DNA, and other important components of the microbial cell. Despite the reproduction by many microorganisms of catalase, which protects the cell from the effects of peroxides by decomposing them into water and oxygen, the concentrations used during disinfection allow, in most cases, to overcome this mechanism of resistance. The main disadvantages of the drugs of this group are high tissue toxicity (class 2) with local irritant and resorptive effects. At the same time, they can cause corrosion of metals and discolor tissues. It is noted that from the group of oxidants promising is the use of drugs containing peracetic acid and hydrogen peroxide **(Kovalenko and Nedosekov, 2011; Zavgorodniy et al., 2013; Rodionova, 2017)**.

Promising are also compositions that are based on hydrogen peroxide with the addition of organic acid. The peracids, which are formed in this composition, significantly increase the bactericidal action of the disinfectant. Drugs 'Nu-Cidex' (England), 'Clindezin-oxy' (USA), 'Anioxide-1000' (France) were obtained based on hydrogen peroxide and peracetic acid. Drugs 'Vofasteril' and 'Persteril' are used in medical practice for disinfection abroad **(Kuchma, 2004)**.

Along with this, it should be noted that today the range of oxygen disinfectants for use in food technology is quite limited, which led to the relevance of our work on the development of modern import-substituting antimicrobials.

## Scientific hypothesis

Compliance with sanitary and hygienic requirements in meat processing plants during the production of food with good manufacturing practices (GMP), good hygiene practices (GHP), and the implementation of the HACCP system is the key to the production of safe and competitive meat products in each relevant enterprise. The study of bacterial contamination of the air and the sanitary condition of refrigerators is an urgent task of practical veterinary medicine because the improper organization of veterinary and sanitary control causes the risk of contamination of meat with environmental microflora.

We investigate several hypotheses in our study:

1. The low temperature in the deep-freeze chambers does not destroy microorganisms, but only inhibits their activity.
2. Developed disinfectant (hydrogen peroxide (8.0 – 10%), acetic acid (10%), peracetic acid (5.0 – 7.0%), stabilizing additives, water) provides destruction of sanitary- indicative microorganisms and colonies of molds in refrigerators meat processing plants.

## MATERIAL AND METHODOLOGY

### Samples

The experimental part of the work was carried out based on meat processing enterprises of Kharkiv and Volyn oblasts of Ukraine, the laboratory of veterinary sanitation and parasitology of the National Research Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv), and the Department of Veterinary Hygiene, Sanitation, and Expertise of Odesa State Agrarian University, Odesa). During the sanitary and hygienic study of the air, the samples were taken at five different points of the room, not lower than 0.5 m above the floor level by the sedimentation method **(Golovko and Rublenko, 2010)**.

### Chemicals

Before placing the raw meat in the cooling and storage chamber, it was thoroughly washed and disinfected using 'Anti-Jerm SR S 25' detergent (Vetsintez LLC, Ukraine) (2.0% – 30 min) and disinfectant 'CD-256' (IPAX Cleanogel, Inc., USA) (0.3% – 1 hour).

To design the formulation of a new disinfectant, we identified the chemical components and selected their optimal ratio. Hydrogen peroxide, acetic, and peracetic acid were used as active substances.

Acetic acid — organic acid, monobasic carboxylic acid, chemical formula $CH_3COOH$. Danger class - 3, moderately toxic substances **(GOST 12.1.007-76, 1977)**.

Peracetic acid — organic peracid (peroxyacetic acid), chemical formula is $CH_3CO_3H$. According to the parameters of acute toxicity belongs to the 3rd class of danger **(GOST 12.1.007-76, 1977)** when brining into the stomach, to the 4th class of danger when it is applied on the

skin, to the 2nd class of danger according to the classification of inhalation danger. Hydrogen peroxide (Hydrogen peroxide $H_2O_2$, Latin Hydrogenii peroxydum) belongs to peroxy compounds. Danger class – 3 **(GOST 12.1.007-76, 1977)**.

**Animals and Biological Material:**

To determine the bactericidal properties of the developed disinfectant, use test cultures of microorganisms stored in the collection of NSC 'IECVM': *Escherichia coli* (strain *K 99*), *Salmonella Dublin* (strain 41*), Staphylococcus aureus* (strain *209*).

The cultures were incubated at a temperature of 37.5 ±0.5 °C on the meat peptone agar (MPA) and meat peptone broth (MPB) for 24 hours.

**Instruments**

The air of the chamber was rehabilitated using a portable vertical irradiator-recirculator of closed type ORUB-01-'KRONT' (CJSC 'KRONT-M', Russia) for 30 min to achieve air sterility, which was confirmed by microbiological studies.

Before preventive disinfection, the walls, ceiling, and floor of the refrigeration chambers were mechanically cleaned, washed, and degreased with detergents using a KÄRCHER machine.

In front of the entrance to the cell, a sanitary pass SPD 08.04 of the checkpoint type (INOX TIME, Ukraine) was placed to exclude secondary contamination of raw meat during contact with the working staff. Only members of the control and expert group approved by the Order of the enterprise had access to raw meat.

Lamps TUV-30W (Philips, Netherlands) are the source of ultraviolet radiation in laminar boxes ozone-free.

Colonies grown both on the surface and in the depth of the MPB were counted using a colony meter 'SYNBIOSIS aCOLyte' (Synbiosis, USA).

The optical microscope Axioskop 40 (Zeiss, Germany) was used for smear microscopy.

To determine lead, cadmium, copper, zinc, mercury the Atomic Adsorption spectrometer 'Agilent AA 240 FS' with a graphite-thermal add-in-device for atomization 'GTA 120' (Agilent, USA) has been used. Arsenic was determined using a spectrophotometer ULAB 102 UV (ULAB, Ukraine).

Pesticides were determined with a gas chromatograph 'Agilent 7890 A GS System' (Agilent, USA). Organochlorine pesticides were determined with an electron capture detector (ECD) and Agilent HP-5 capillary column (30 m × 0.32 mm × 0.10 mm), organophosphorus pesticides nitrogen were determined with a phosphorus detector (NPD) and capillary Agilent HP-5 MSA column (30 m × 0.25 mm × 0.10 mm).

To determine mycotoxin (aflatoxin B1), a liquid chromatograph Agilent 1260 (Agilent, USA) with fluorometric detector (FLD) series G1321B and a capillary column Agilent Zorbax SB-C 18 (4.6 m × 150 mm × 5 mkm) were used.

**Laboratory Methods**

The experiments were performed to meet the requirements for food safety set out in the documents: **ISO 22000 (2005)** Food safety management systems. Requirements for any food chain organizations; **ISO 22002-1 (2009)** Prerequisites for food safety; CAC/RCP 1-1969, Rev.4-2001 Recommended International Code of General Principles of Food Hygiene.

The total number of microorganisms (TNM) and the number of mold colonies in 1 $m^3$ of air were determined. Petri dishes with nutrient medium (NA) were used to determine (TNM) The cultures were incubated in a thermostat at a temperature of 30 ±0.5 °C for 72 hours **(DSTU ISO 4833, 2006)**. Accounting for crop growth was carried out by the method of counting colonies per 1 $m^3$ of air by the formula (1):

$$x = \frac{y \times 10000}{78}, \quad (1)$$

Where:

x – is the total number of microorganisms, $m^3$; $y$ – the average number of colonies grown on five Petri dishes; 10000 – constant value; 78 – the area of the Petri dish, $cm^2$.

Saburo agar with glucose was used to detect mold fungi. The cultures were incubated in a thermostat at a temperature of 22.5 ±0.5 °C for 5 days. The result was the average number of colonies grown on five Petri dishes.

Bactericidal properties of the disinfectant in the laboratory were determined according to the handbook **Kovalenko, Nychik and Mandyga (2014)**. Cleaning and disinfection and quality control were carried out following the guidelines **Paliy and Rodionova (2016)**.

**Description of the Experiment**

Nutrient media following (State Standard) **DSTU ISO/TS 11133-1 (2005)**, laboratory glassware, and laboratory equipment under (State Standard) **DSTU ISO 1042 (2005)** were used for research. The manufacture of solutions of reagents, paints, indicators, which were used for microbiological analysis, was carried out under (State Standard) **DSTU 5093 (2008)**.

Samples for the determination of heavy metals were prepared by the method of dry mineralization in accordance with **DSTU 7670 (2014)**. Lead, cadmium, copper, zinc, mercury were determined in accordance with **GOST 30178 (1996)** 'Raw material and food-stuffs. Atomic absorption method for determination of toxic elements'. Arsenic was determined according to **GOST 26930-86 (1987)** 'Raw material and food-stuffs. Method for determination of arsenic'.

Pesticides were determined by thin-layer chromatography approach (TLC): HCH (gamma isomer), DDT, DDD, DDE - according to MV No 2142-80 § 2.5-2.6 'Methodological instructions for the determination of organochlorine pesticides in water, food, feed, and tobacco products with thin layer chromatography', bazudin, carbophos, chlorophos, DDVP – in accordance with MV No 3222-85 Unified method for the determination of organophosphorus pesticides in the product of vegetable and animal origin, medicinal plants, water, soil by chromatographic methods'.

Determination of aflatoxin B1 was carried out according to MV No 4082-86 'Methodological instructions for detection: identification and determination of aflatoxins in food raw materials and food products by high-performance liquid chromatography'.

**Sample preparation:** The final determination of the bactericidal action of the disinfectant was performed on sterile test objects contaminated with microorganisms: strips of lawn fabric measuring 1 cm × 2 cm, wooden bars,

ceramic, metal, plastic, glass plates measuring 12 cm × 12 cm × 2 cm, which used in experiments repeatedly after sterilization in an autoclave (132 °C, 2 atm., 60 min). Sterile test items were placed in metal enameled cuvettes placed in a sterile box. A mixture of experimental test culture suspension and sterile bovine serum was applied to the test items. per 1 $cm^3$ of two billion suspension of microorganisms – 0.5 $cm^3$ of serum. The 1.5 $cm^3$ mixture is evenly distributed on the surface of each test object. The test culture is dried on test objects at room temperature for 24 hours.

**Number of samples analyzed:** The air of the chamber – 94.

**Number of repeated analyses:** 5

**Number of experiment replication:** 3

### Statistical Analysis

Statistical processing of the obtained data was carried out on a computer system using spreadsheets Microsoft Excel XP Professional and STATISTICA 7.0 (Stat Soft, USA). It has been determined the arithmetic mean (M), the statistical error of the arithmetic mean (m). The probability of the difference between the arithmetic mean of the two variation series was determined by the reliability criterion and Student's tables.

## RESULTS AND DISCUSSION

### Analysis of the sanitary-microbiological composition of air in refrigerators

At the first stage of our research, we analyzed the sanitary-microbiological composition of the air of refrigeration chambers in the conditions of basic meat processing enterprises. During the study, the total number of microorganisms in the air (TNM) was determined. The results of the bacteriological examination are shown in Figure 1.

According to the results of research presented in Figure 1, it was found that the average number of microorganisms in the air of the cooling chambers (4 °C) varies from (12.92 ±0.4) × $10^2$ CFU.$m^{-3}$ on the 5$^{th}$ day of storage of raw meat to (31.92 ±0.2) × $10^2$ CFU.$m^{-3}$ at the end of the storage period (15 days). The obtained results indicate a proportional increase of TNM in cooling chambers during the storage of raw meat. On the 15$^{th}$ day of storage, this Figure is 2.5 times higher than on the 5$^{th}$ day.

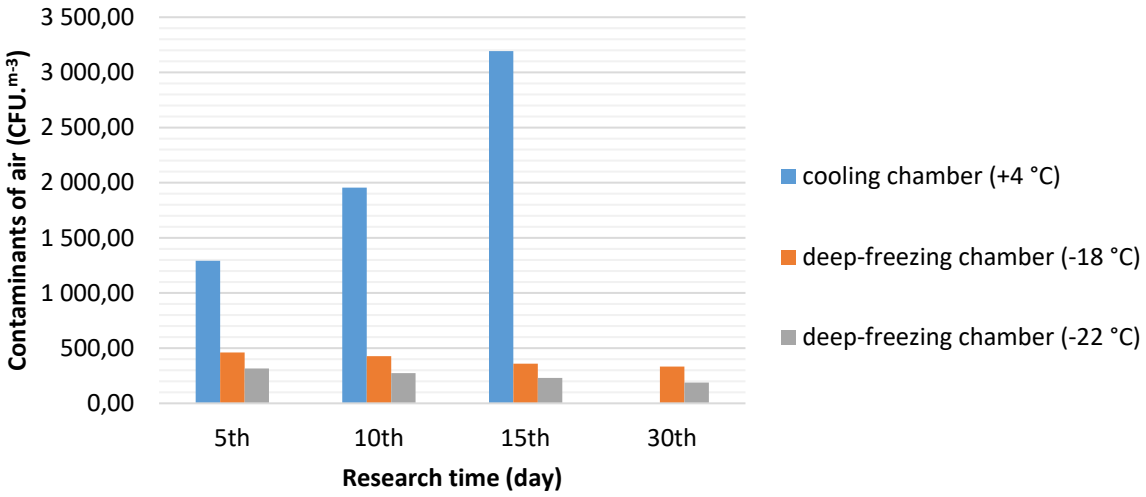


**Figure 1** The total number of microorganisms in refrigerators, CFU.$m^{-3}$ (n = 5).

**Table 1** Results of sanitary-microbiological research of air of refrigerating chambers (n = 5, M ±m)

| Air temperature °C | Research period | Results of bacteriological research of air, CFU.$m^{-3}$ | | | | |
|---|---|---|---|---|---|---|
| | | Number of mold colonies on 5 Petri dishes | | | | The average number of colonies on 1 cup |
| | | Average number | including | | | |
| | | | *Cladospores* | *Tamnidum* | *Penicillium* | |
| +4 | 5$^{th}$ day | 10.6 ±1.3 | 0.4 ±0.2* | 0 | 0.4 ±0.2* | 2.1 ±0.3 |
| –18 | | 11.8 ±1 | 0.4 ±0.2* | 0.4 ±0.2* | 2.0 ±0.5* | 2.4 ±0.2 |
| –22 | | 12.4 ±1 | 0.8 ±0.4* | 0.8 ±0.4* | 2.4 ±0.5* | 2.5 ±0.2 |
| +4 | 10$^{th}$ day | 20.6 ±1 | 3.6 ±0.9* | 1.4 ±0.4* | 7.0 ±0.3* | 4.12 ±0.2 |
| –18 | | 17.2 ±1 | 0.6 ±0.2* | 0.4 ±0.2* | 4.2 ±0.7* | 3.44 ±0.2 |
| –22 | | 19.8 ±0.7 | 0.8 ±0.4* | 0.8 ±0.4* | 5.0 ±0.3* | 4.0 ±0.1 |
| +4 | 15$^{th}$ day | 26.4 ±1.2 | 3.0 ±0.6* | 1.3 ±0.2* | 6.4 ±0.8* | 5.3 ±0.2 |
| –18 | | 31.2 ±1.4 | 3.0 ±0.6* | 1.2 ±0.5* | 2.6 ±0.4* | 6.2 ±0.3 |
| –22 | | 30.8 ±1.0 | 2.4 ±0.2* | 1.6 ± 0.2* | 6.0 ±0.3* | 6.2 ±0.2 |
| –18 | 30$^{th}$ day | 34.2 ±1.1 | 3.6 ±0.5* | 1.6 ± 0.2* | 3.0 ±0.7* | 6.8 ±0.2 |
| –22 | | 36.6 ±1.99 | 2.0 ±0.32* | 2.6 ±0.51* | 6.4 ±0.75* | 7.32 ±0.4 |

Notes: $p$ >0.999 comparison of the value of the total number of molds and by species.

Analyzing the sanitary condition of deep freezing chambers No 1 (-18 °C) and No 2 (-22 °C), we came to the conclusion that during the shelf life of raw meat TNM decreases proportionally on average from (3.9 ±0.7) × $10^2$ CFU.$m^{-3}$ ($5^{th}$ day of storage) to (2.6 ±0.7) × $10^2$ CFU.$m^{-3}$ at the end of the storage period ($30^{th}$ day), in other words by 1.5 times (33.3%). It should be noted that the results of the study show that the lower the temperature in the deep freezer, the greater the bacteriostatic effect it demonstrates. Comparing TNM in deep-frozen chambers with a temperature of -22 °C and TNM in chambers with a temperature of -18 °C, it was found that on the 5th day of storage of raw meat this Figure is lower in chambers with a temperature of -18 °C by 31.4%, on the $10^{th}$ day – by 36%, and by 43.6% – on the $30^{th}$ day (end of the storage period).

Thus, the results of microbiological studies indicate a high degree of contamination of the air in the chambers of the refrigeration premises by microorganisms, which are a source of bacterial contamination of raw meat.

Analyzing the results of the research, a high positive correlation (r) was found between the TNM in the air of the refrigeration chambers and the air temperature in the chamber. It was found that the correlation between TNM in the air of meat cooling chambers and TNM in the air of deep-freezing chambers (-18 °C) is equal to (r = 1), while between TNM in the cooling chamber of meat raw materials and TNM in the deep freezing chamber (-22 °C) – r = 0.643.

During the technological process of cooling and freezing meat, special attention should be paid to the presence of microscopic mold fungi in the air, especially cladospores. This is because cladospores can show their toxigenic properties at low temperatures.

To determine the level of contamination of refrigerators with mold fungi, a sanitary-microbiological study of the air of the refrigeration chambers with different storage temperatures of raw meat was conducted. The results of the experiment are presented in Table 1.

Analyzing the materials in Table 1, the conclusion that the low temperature of refrigerators (from +4 °C to -22 °C) does not adversely affect the activity of mold fungi could be made. Microbiological examination revealed the following species of molds: *Tamnidium*, *Penicillium*, and *Cladosporium.*

It was found that the number of mold colonies in the cooling chambers (+4 °C) during 15 days of meat storage increases almost by 2.5 times.

It should be noted that in the cooling chamber at the end of the storage period of meat ($15^{th}$ day) bacterial air pollution by mold fungi was 26.4 ±1.2 CFU.$m^{-3}$, among them: fungi of the species *Cladosporium* – 4.9%, *Tamnidium* – 20.15% and *Penicillium* – 24.2%.

During the microbiological examination of the air in the deep-freeze chambers ( -18 °C and -22 °C) during 30 days of storage of raw meat, the number of mold colonies increases almost by3 times, reaching 35.4 ±1.2 CFU.$m^{-3}$.

The most contaminated with mold fungi on the 30th day of meat storage was the air of deep-freezing chambers at a temperature of -22 °C, where the total number of colonies of mold fungi was 36.6 ±1.99 CFU.$m^{-3}$, which is 6.5% more, than in the chamber at a temperature of -18 °C. It should be noted that among the total number of mold fungi in deep-frozen chambers at a temperature of -18 °C most often recorded fungi of the species *Cladosporium* (10.5%), while in deep-frozen chambers at a temperature of -22 °C most often recorded fungi of the species Penicillium (17.5%).

It should also be noted that among the total number of identified colonies of molds (22.9 ±2.9 CFU.$m^{-3}$) in the chambers of the refrigeration shop, the percentage of fungi of the species *Cladosporium* was 8.3% (1.9 ±0.4 CFU.$m^{-3}$), *Penicillium* – 17.9% (4.1 ±0.7 CFU.$m^{-3}$), and *Tamnidium* – 4.8% (1.1 ±0.2 CFU.$m^{-3}$).

Our data correlate with the results of **Bogatko and Sakhniuk's studies (2013) who also** found that the technological modes of cooling meat in the cooling (t = -1 °C) and refrigeration (t = -12 °C) chambers do not have a bacteriostatic effect on the life of mold fungi. It was determined that the highest infestation of air fungi and plaster walls of the chambers is observed in their lower part, at a height of 0.5 m from the floor **(Bogatko and Sakhniuk, 2013)**.

The results of our research are confirmed by the results of **Kukhtyn et al. (2020a)**. They have experimentally substantiated the quantitative indicators of the content of psychrotrophic microorganisms on the surface of beef intended for storage in a cooled and frozen state. Proved that the storage of meat in the frozen state inhibits or completely stops the development of mesophilic microorganisms for 20 days **(Kukhtyn et al., 2020a; Kukhtyn et al., 2020b)**.

Thus, our research results indicate a satisfactory assessment of the sanitary condition of refrigeration chambers and deep-freeze chambers, and therefore require quality disinfection to remove mold colonies before the next laying of raw meat.

## Formulation development and testing of disinfectant

It should be noted that preventing the production, sale, and consumption of meat products, which are affected by pathogenic microorganisms and molds that can harm the health of the population, is one of the most important problems of meat processing plants. To address this issue, it is necessary to carry out systematic veterinary and sanitary control of refrigeration chambers, which includes sanitary and hygienic control of cleanliness and quality control of washing and disinfection.

Obligatory and timely cleaning and preventive disinfection of equipment and inventory used in the processing of raw meat are important elements of continuous industrial sanitary control, which includes sanitary and microbiological control of cleanliness, quality control of washing, and disinfection. Therefore, there is a need to find new universal, easy-to-use, safe disinfectants that, together with the availability and low cost, would be highly effective.

To sanitize the objects of veterinary control of meat processing enterprises, we have developed a recipe for a domestic disinfectant. The composition of the disinfectant includes hydrogen peroxide (8.0 – 10%), acetic acid (10%), peracetic acid (5.0 – 7.0%), stabilizing additives, water.

At the previous stage of the study, the spectrum of bactericidal action of the developed disinfectant against test cultures of microorganisms was studied (Table 2).

From the results presented inTable 2, it is seen that the developed disinfectant when used at a concentration of 0.01% at an exposure of 10 – 60 min, at a concentration of 0.03% at an exposure of 10 – 30 min and a concentration of 0.05% at an exposure of 10 min does not show bactericidal action to test cultures of *E. coli, S. Dublin* and *S. aureus* microorganisms. When using the drug at a concentration of 0.03% at an exposure of 60 min, at a concentration of 0.05% at an exposure of 30 min, and a concentration of 0.1% at an exposure of 10 min, it was found that it destroys test cultures of *E. coli* and *S. Dublin*, however, it acts bacteriostatically on *S. aureus*. The bactericidal action of this disinfectant was determined when used at a concentration of 0.05% at an exposure of 60 min, at a concentration of 0.1% at an exposure of 30 – 60 min, and at a concentration of 0.15% at an exposure of 10 min.

The obtained research results gave us a basis for further testing of the developed disinfectant. The next step was to conduct experiments involving test objects contaminated with test cultures of microorganisms. Sterile bovine serum was used as biological protection to determine the effect of organic contaminants on the bactericidal activity of the disinfectant. The results of the studies are presented in Table 3.

From the materials presented in Table 3, it is seen that the disinfectant, which has been developed, when it is used at a concentration of 0.03% at an exposure of 10 – 60 min does not show a disinfectant effect on these test objects.

**Table 2** The results of the study of bactericidal properties of the disinfectant (n = 3).

| Concentration, % | Exposure, min | Test culture of microorganisms | | |
|---|---|---|---|---|
| | | ***E. coli*** | ***S. Dublin*** | ***S. aureus*** |
| 0.01 | 10 | + | + | + |
| | 30 | + | + | + |
| | 60 | + | + | + |
| 0.03 | 10 | + | + | + |
| | 30 | + | + | + |
| | 60 | – | – | + |
| 0.05 | 10 | + | + | + |
| | 30 | – | – | + |
| | 60 | – | – | – |
| 0.10 | 10 | – | – | + |
| | 30 | – | – | – |
| | 60 | – | – | – |
| 0.15 | 10 | – | – | – |
| | 30 | – | – | – |
| | 60 | – | – | – |
| control | 10 | + | + | + |
| | 30 | + | + | + |
| | 60 | + | + | + |

Notes: «–» - growth of microorganisms is absent; "+" - the growth of microorganisms is available.

**Table 3** The results of the study of the disinfectant action of the disinfectant (n = 3).

| Test culture | Lawn | | | Wood | | | Tile | | | Metal | | | Plastic | | | Glass | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration, min | | | | | | | | | | | | | | | | | |
| | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 | 10 | 30 | 60 |
| concentration 0.03% | | | | | | | | | | | | | | | | | | |
| *E. coli* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *S. Dublin* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| *S. aureus* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| concentration 0.05% | | | | | | | | | | | | | | | | | | |
| *E. coli* | + | + | – | + | + | – | + | + | – | + | + | – | + | + | – | + | + | – |
| *S. Dublin* | + | + | – | + | + | – | + | + | – | + | + | – | + | + | – | + | + | – |
| *S. aureus* | + | + | – | + | + | – | + | + | – | + | + | – | + | + | – | + | + | – |
| concentration 0.10% | | | | | | | | | | | | | | | | | | |
| *E. coli* | + | – | – | + | – | – | + | – | – | + | – | – | + | – | – | + | – | – |
| *S. Dublin* | + | – | – | + | – | – | + | – | – | + | – | – | + | – | – | + | – | – |
| *S. aureus* | + | – | – | + | – | – | + | – | – | + | – | – | + | – | – | + | – | – |
| concentration 0.15% | | | | | | | | | | | | | | | | | | |
| *E. coli* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| *S. Dublin* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| *S. aureus* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

Notes: «–» - there is no growth of microorganisms; "+" - the growth of microorganisms is available.

**Table 4** Activity of disinfectant in production conditions (n = 3).

| Object disinfection | Regime application | Samples were investigated | | | % disinfection |
|---|---|---|---|---|---|
| | | total | including | | |
| | | | disinfected | not disinfected | |
| metal | 0.05%, 60 min | 60 | 60 | – | 100 |
| plastic | | 60 | 60 | – | 100 |
| glass | | 60 | 60 | – | 100 |
| metal | 0.1%, 30 min | 60 | 60 | – | 100 |
| plastic | | 60 | 60 | – | 100 |
| glass | | 60 | 60 | – | 100 |
| metal | 0.15%, 10 min | 80 | 80 | – | 100 |
| plastic | | 80 | 80 | – | 100 |
| glass | | 80 | 80 | – | 100 |

**Table 5** Results of toxicological studies of meat after storage.

| Name of indicator and unit of measurement | MDR according to regulatory documents | Research results * | | |
|---|---|---|---|---|
| | | cooling chamber (+4 °C) | deep-freezing chamber (-18 °C) | deep-freezing chamber (-22 °C) |
| **Toxic elements** | | | | |
| Mass fraction of lead, $mg.kg^{-1}$ | Not more 0.5 | 0.12456 | 0.32043 | 0.12452 |
| Mass fraction of cadmium, $mg.kg^{-1}$ | Not more 0.05 | 0.00652 | 0.00234 | 0.00186 |
| Mass fraction of copper, $mg.kg^{-1}$ | Not more 5.0 | 1.03450 | 0.87445 | 1.05602 |
| Mass fraction of zinc, $mg.kg^{-1}$ | Not more 70.0 | 10.81625 | 13.88760 | 10.62380 |
| Mass fraction of arsenic, $mg.kg^{-1}$ | Not more 0.1 | Not detected (less sensitivity of the method <0.02) | Not detected (less than the sensitivity of the method <0.014) | Not detected (less than the sensitivity of the method <0.014)) |
| Mass fraction of mercury, $mg.kg^{-1}$ | Not more 0.03 | Not detected (less sensitivity of the method <0.00155) | Not detected (less sensitivity of the method <0.00185) | Not detected (less sensitivity of the method <0.00255) |
| **Pesticides** | | | | |
| HCH (gamma isomer), $mg.kg^{-1}$ | Not more 0.1 | Not detected <0.05 | Not detected <0.05 | Not detected <0.05 |
| Mass fraction of bazudin | Not allowed | Not detected <0.2 | Not detected <0.2 | Not detected <0.2 |
| Mass fraction of carbophos | Not allowed | Not detected <0.2 | Not detected <0.2 | Not detected <0.2 |
| Mass fraction of chlorophos | Not allowed | Not detected <0.2 | Not detected <0.2 | Not detected <0.2 |
| Mass share of DDVP | Not allowed | Not detected <0.2 | Not detected < 0.2 | Not detected <0.2 |
| Mass fraction of DDT | Not more 0.1 | Not detected <0.05 | Not detected <0.05 | Not detected <0.05 |
| Mass share of DDD | Not more 0.1 | Not detected <0.05 | Not detected <0.05 | Not detected <0.05 |
| Mass fraction of DDE | Not more 0.1 | Not detected <0.05 | Not detected <0.05 | Not detected <0.05 |
| Mass fate of metaphor | Not allowed | Not detected <0.2 | Not detected <0.2 | Not detected <0.2 |
| **Mycotoxins** | | | | |
| Mass concentration of aflatoxin B1 | 0.005 | Not detected (less method sensitivity <0.001) | | |

Note: * – the competence of the laboratory for these methods is confirmed by NAAU in accordance with State standard **DSTU ISO/IEC 17025 (2006)**.

At the action of the drug at a concentration of 0.05% and 0.1% at exposures of 60 and 30 min, respectively, and a concentration of 0.15% at 10 min, it was found that it destroys test cultures of microorganisms, regardless of the treated test-object. These ways of application of the drug were tested in production conditions.

To assess the sanitary condition of the meat processing plant in the washings after disinfection, the presence of the amount of MAFAnM and *Escherichia coli* bacteria was determined. For this purpose, we used the method of direct seeding on nutrient media. The results of the experiment are presented in Table 4.

According to the results presented in Table 4, it was found that the developed disinfectant at a concentration of 0.05% (60 min), 0.1% (30 min) and 0.15% (10 min) completely disinfects surfaces made of different materials (metal, plastic, glass), and the use of the drug in these modes allowed 100% disinfection of objects of veterinary control of meat processing enterprises.

To study the effect of disinfectants on the safety of meat, a toxicological study of raw meat was performed 5 days after placing in the freezer. Product samples were taken following the Procedure for sampling products of animal, plant, and biotechnological origin for research, approved by

the Resolution of the Cabinet of Ministers of Ukraine of June 14, 2002, No 833 **(CMU, 2002)**.

According to the results of toxicological studies of frozen beef (Table 5) on the 5th day of storage in the refrigeration chambers for the presence of toxic elements (lead, cadmium, zinc, arsenic, mercury), pesticides, and mycotoxin (aflatoxin B1) deviations from the norm are not detected. The data obtained show that there is no effect of residual disinfectant on raw meat.

Disinfection is a major component of the general set of veterinary and sanitary measures that ensure a high level of sanitation in the meat processing industry **(Fagerlund et al., 2017; Coughlan et al., 2016; Møretrø, Langsrud and Heir, 2013; Larsen et al., 2014)**.

For this purpose, several disinfectants from different groups of chemical compounds are used **(Da Costa Luciano et al., 2016; Salata et al., 2018; Paliy et al., 2018)**. However, not all disinfectants are effective and efficient. This is due to the high organic contamination of the treated surfaces **(Hultman et al., 2015; Zwirzitz et al., 2020)**, the low bactericidal activity of disinfectants, the formation of resistance in microorganisms to chemical compounds **(Saá Ibusquiza, Herrera and Cabo, 2011; Ortiz et al., 2015; Møretrø et al., 2017)**.

To expand the range of antimicrobial agents, we have developed a formula of an innovative disinfectant, which includes environmentally friendly compounds such as hydrogen peroxide, acetic and peracetic acids. The use of these compounds for disinfection has become widespread in many countries around the world **(Hawley et al., 2018; Stopiglia et al., 2011)**.

The obtained results are expanding the range of highly active antimicrobial drugs that are promising during their use in the food industry. The proposed disinfectant is effective import-substituting development.

Decomposing only to oxygen and water, hydrogen peroxide is one of the environmentally safe and affordable chemicals **(Pędziwiatr et al., 2018)**. And organic acids are even used for food disinfection **(Wang et al., 2019)**. Therefore, these advantages of hydrogen peroxide and organic acids led to our choice to design a disinfectant formula for use in the meat industry. During testing, a disinfectant was developed in the laboratory and its modes of application were established to disinfect test objects (lawn, wood, tile, metal, plastic, glass) contaminated with test cultures of microorganisms (*E. coli*, *S. Dublin*, *S. aureus*). Our chosen methodological approaches to the assessment of disinfectants correlate with the reports of other researchers **(Tomasino, 2013; Tishyn et al., 2017; Rabenau et al., 2020)**. In assessing the disinfectant, it is necessary to model the production conditions **(Becker et al., 2019)**, which were taken into account by us. The established modes of application of disinfectant were tested in production conditions, which gave grounds to recommend this tool for widespread use in the processing industry.

## CONCLUSION

It was found that the average number of microorganisms in the air of the cooling chambers (+4 °C) increases proportionally during storage of raw meat from (12.92 ±0.4) × $10^2$ CFU.$m^{-3}$ on the 5th day of storage to (31.92 ±0.2) × $10^2$ CFU.$m^{-3}$ at the end of the storage period (15th day). The lower the temperature in the deep-freeze chambers, the greater the bacteriostatic effect on microorganisms.

Among the total number of identified colonies of molds (22.9 ±2.9 CFU.$m^{-3}$) in the chambers of the refrigeration shop, the percentage of fungi of the species C*ladosporium* was 8.3% (1.9 ±0.4 CFU.$m^{-3}$), *Penicillium* – 17.9% (4.1 ±0.7 CFU.$m^{-3}$), and *Tamnidium* – 4.8% (1.1 ±0.2 CFU.$m^{-3}$). It is proved that the low-temperature regime of refrigeration chambers (from +4 °C to -22 °C) does not have a detrimental effect on the activity of mold fungi.

Developed disinfectant (hydrogen peroxide (8.0 – 10%), acetic acid (10%), peracetic acid (5.0 – 7.0%), stabilizing additives, water) provides destruction of sanitary-indicative microorganisms in refrigerators meat processing plants when used in a concentration of 0.05% – 60 min, 0.1% – 30 min, 0.15% – 10 min and has no toxic effect on raw meat, which is stored in the chambers of the refrigeration plant after disinfection.

Strict observance of the requirements of technological processes of meat storage and processing and high-quality disinfection ensure satisfactory sanitary and hygienic conditions of cooling and refrigerating chambers.

Prospects for further research are to provide theoretical and experimental justification for the use of new detergents and disinfectants in the meat processing industry and their impact on the final product, as well as to establish the features of legal support of proper sanitary and hygienic requirements of the refrigeration chain in Ukraine and meat processing industry compared to the requirements of the European Union.

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This article does not contain any studies that would require an ethical statement.

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# GENETIC IDENTIFICATION OF THE CAUSATIVE AGENT OF BRUCELLOSIS

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## ABSTRACT

The development of animal husbandry suffers various kinds of losses due to the spread of infectious diseases among animals, in particular Brucellosis. A challenge faced by *Brucella* researchers has been the accurate identification of new isolates within the genus while preserving sufficient, and not excessive, biosafety and biosecurity requirements. The availability of discriminatory molecular typing tools to inform and assist conventional epidemiological approaches would be invaluable in controlling these infections, but efforts have been hampered by the genetic homogeneity of the genus. In this work, for better identification of infection, for control and monitor the source of outbreaks in prosperous areas was carried out identification of *Brucella* spp. strains which circulating in the Kostanay region. For this was used using multilocus analysis of a variable number of tandem repeats sequenced by 16 s – PNK on a genetic analyzer (sequencer). According to the results of a study of cattle, cultures of microorganisms were infected: No. 4, 5, 7, 8. Comparison of the obtained results with similar results of domestic and foreign works by A. Shevtsov, G. Borrello, P. Le Fleche, G. Garofolo suggest that the genotyping of local strains has an importance in the molecular epizootology of the Republic of Kazakhstan.

**Keywords:** amplification; sequencing; Brucella abortus; Brucella melitensis; strain

## INTRODUCTION

Brucellosis is a zoonotic disease of major public health, animal welfare, and economic significance worldwide. In humans, infection with *Brucella* can lead to a chronic debilitating infection; in domesticated animals, the main symptom is a reproductive failure. Among the 12 species currently proposed in this genus, *Brucella melitensis*, *Brucella abortus*, and *Brucella suis* are highly dangerous for humans and cause disease with severe complications and chronic processes **(Meyer, 1990; Godfroid et al., 2011)**. Rare cases of infection of people with other *Brucella* species are also recorded **(Ficht, 2010)**. Despite its low mortality rates, brucellosis is a very important public health problem in Kazakhstan. Brucellosis causes great economic damage to livestock. The disease is accompanied by mass abortions and barrenness of animals suffering from brucellosis, joint damage, inflammation of mucous bags (in horses at the withers and the nape of the neck), the sex glands, the appearance of abscesses (more often in pigs), a decrease in the viability of offspring, a decrease in animal productivity. At the same time, elimination of brucellosis requires great effort and cost **(Demirev, 2013; Whatmore et al., 2006; Whatmore, 2009)**. The development of discriminatory molecular tools for the identification and typing of *Brucella* has been problematic, reflecting the lack of genetic polymorphism in *Brucella*.

To address the issue of epizootology and understanding the pathogenesis of the disease, an accurate and timely diagnosis is necessary. The success of the fight against brucellosis depends on the rate of detection of all infected animals, on the effectiveness of diagnostic studies. The traditional system of identification and systematization of isolated field strains of *Brucella* is imperfect. None of the existing molecular tools provide adequate resolution to confidently permit epidemiological traceback in the case of accidental import or deliberate release. The phenotypic methods used allow differentiation of the causative agent of brucellosis to the species-specific level, but they are characterized by laboriousness, duration, and non-specificity of the analysis **(Whatmore, 2009; Alton et al., 1988; Le Flèche et al., 2006)**. These methods do not meet modern typing requirements. The solution to this problem is seen in the use of modern methods of molecular genetic typing of *Brucella* isolates.

Using molecular methods of analysis, genomic polymorphism is simply detected – quantitative changes in mini- and microsatellite DNA sequences (decrease or increase in the number of repeats), creating a series of unique alleles for each variable locus. These types of polymorphic loci are convenient markers. The phenomenon of polymorphism underlies the common method of identifying mutant DNA fragments – analysis of microsatellite markers **(Ayala and Kiger, 1980; Gershkovich, 1968; Al Dahouk et al., 2007, Kattar et al., 2008)**. In this regard, the use of information about the causative agent of brucellosis obtained in the analysis of multilocus provides high-quality, effective elimination and prevention of the infectious process.

### Scientific Hypothesis

Using multilocus analysis with a variable number of tandem to create a molecular genetic cluster in the 16S rRNA epizootic. Recursive multistep analysis of a tandem of a molecular genetic cluster in epizootology using 16S rRNA in the Kostanay region strain of genotyping. In addition, the data obtained can be used for epidemiological surveillance and rapid detection of foci of brucellosis in epidemic zones. Currently, the research of the genetic modification of *Brucella* through 16s rRNA is necessary for a better understanding of the spread and monitoring of the occurrence of this infection in these areas.

## MATERIAL AND METHODOLOGY

### Samples

An object of study – cultures of microorganisms: No. 4, 5, 7, 8. The subject of the study is the polymorphism of a fragment of ribosomal RNA (16S rRNA). The study material – samples of genomic DNA isolated from the obtained cultures of microorganisms.

### Instruments

Capillary genetic analyzer 8-16s rrna 3500 genetic Analyzer ("Applied Biosystems", USA) for genotyping and defining *Brucella* isolators is a worthy tool for identifying genotypes.

### Laboratory Methods

On the initial stages of the work, the following processes took place:

1. Isolation of DNA from microorganism cultures. For DNA extraction, the commercial CTAB Sample Kit was used (DNA-Technology, Russia). The work with the kit was carried out following the manufacturer's instructions.

2. Determination of DNA nucleotide sequence by the method of F. Sanger (enzymatic method)

3. Sequencing steps. Sequencing of amplification fragments was performed using the ABI PRISM Big Dye Terminator v.3.1 kit (Applied Biosystems, USA), according to the manufacturer's instructions using the Applied Biosystems 3500 Genetic Analyzer 8 capillary genetic analyzer (Applied Biosystems, USA). After conducting electrophoretic analysis of amplification 2 using 2% agarose, we placed micro schemes with amplification products as a tripod. Each row of gel tracks must have a DNA molecular weight marker **(Ferreira et al., 2012)**. Next on paraffin paper with a pipette dosing, we add 10 μL of amplification, 2 μL of 6X DNA Loading Dye, pipetting. 10 μL is poured into the well of the agarose gel. Electrophoresis results were registered using a QUANTUM Model 1100 SUPER Documentation System Gel, which has ultra-high sensitivity, including for fluorescence, is controlled via PC. One band is visible on the resulting image, which indicates the purity of the obtained cultures, the presence of two or more bands in the results indicates that the cultures are contaminated by other microorganisms **(Corbel, 1997; Gandara et al., 2001)**. In this case, it is impossible to conduct sequencing of this culture **(Whatmore et al., 2006, Gwida et al., 2012)**.

4. Clearing the amplification from residual reagents with CleanSweep reagent, mix composition. Incubated at 37 °C – 15 min, 80 °C – 15 min and left for storage.

### Sample preparation

Cultures were obtained in the Kostanay regional branch of RSE on REM "Republican veterinary laboratory". Microorganisms had been seeded on a specific nutrient medium for *Brucella* – erythritol agar had been grown for 20 – 30 days at 37 – 38 ℃. For microscopic examination, staining was performed according to Shuliak-Shin.

### Statistical Analysis

Sequence data were obtained by overlaying the sequenced sequences from the forward and reverse primers using the SeqMan program (Lasergene 6). Alignment was performed using the on-line BLAST program hosted on the NCBI website – National Center for Biotechnological Information, USA.

## RESULTS AND DISCUSSION

Since there were essentially no molecular tools that could provide useful discrimination beyond the level of biovar, the first aim of this study was to characterize highly discriminatory loci that would facilitate the development of tools to permit epidemiological traceback of *Brucella* to the source of infection The initial stages of the work focused on: 1. Isolation of DNA from microorganism cultures; 2. Determination of DNA nucleotide sequence; 3. Sequencing steps; 4. Cleared the amplification from residual reagents with CleanSweep reagent, mix composition (Table 1).

Then a sequencing reaction was performed. Each PCR product was pipetted into the 2 wells of a strip of 1 μL (the primers were excavated separately in the wells, to the first was added straight, to the second – the reverse) + the reaction mixture of the following composition (Table 2).

Put on the amplification according to the following program (Table 3). The rate of increase or decrease in temperature – 1 °C/sec.

Purification (precipitation) before sequencing (performed in a plate with cooling) was performed.

**Table 1** The composition of the mixture for the purification of amplification products.

| Mix component | Amount per reaction |
|---|---|
| CleanSweep | 2 μL |
| Amplification | 5 μL |

**Table 2** The reaction mixture for sequencing.

| Component of the reaction mixture | Amount per reaction |
|---|---|
| Primer 16S-F (ITS-F), either primer 16S-R (ITS-R) | 1 μL |
| Purified amplification | 1,5 μL |
| Big Dye Terminator 3.1 Ready Reaction Mix | 4 μL |
| Deionized water | 4 μL |

**Table 3** Amplification program.

| Temperature | Time | Number of cycles |
|---|---|---|
| 96°C | 1 min | |
| 96°C | 10 sec | |
| 50°C | 5 sec | 25 cycles |
| 60°C | 1,5 min | |
| 4°C | storage | |

45 μL Sam Solution and 10 μL Xterminator Solution had been added to the amplification obtained after the sequential reaction, shaking the reagent strongly, as it quickly precipitated. The resulting mixture was vortexed at 1800 rpm for 20 minutes using Digital Vortex-Genie 2. After that, the plate was centrifuged at 1000g for 2 minutes. 15 μL of the supernatant was collected and the plate was loaded into the sequencer. As a result of sequencing the cultures of microorganisms, the following results were obtained (Table 4, Figure 1).

**Table 4** Results of identification by the method of analysis of the nucleotide sequence of the gene 16S rRNA.

| The name of the strain | The sequence of the fragment 16S r RNA | Identification of nucleotide sequences in the international database (http://www.ncbi.nlm.nih.gov/) BLAST algorithm | |
|---|---|---|---|
| | | The name of the strain | matches |
| 1/8 | GTATCAAAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGACT TAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCGAACAACG CTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGG CTTCTTCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACA ACCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCG CCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 2/8 | TCCGGGGTTGAGCCCCGGGATTTCACCCCTGACTTAAAAGTCCGCCTA CGTGCGCTTTACGCCCAGTAAATCCGAACAACGCTAGCCCCCTTCGTA TTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGGTTA CCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCCTTC ATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCAATA TTCCCCACTGCTGCCTCCCGTA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 4/8 | AGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGACTTAAAAGTCCGC CTACGTGCGCTTTACGCCCAGTAAATCCGAACAACGCTAGCCCCCTTC GTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTTCTCCGG TTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCTAGGGCC TTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCATTGTCCA ATATTCCCCACTGCTGCCTC | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 5/5м | ACCTCTACACTCGGAATTCCACTCACCTCTACCATACTCAAGACTTCC AGTATCAAAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGA CTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCGAACAAC GCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGG GCTTCTTCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTAC AACCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGC GCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 6/5 | GGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGGCG GACTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGC CTTTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGA GTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAA GGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 7/5 | AGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGG CGGACTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACT GCCTTTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCC GAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCG AAGGCGGCTCACTGGACCATTACTGACGCTGAGGTGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 8/5 | CTTCCAGTATCAAAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCC CTGACTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCGAA CAACGCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGC CGGGGCTTCTTCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGC TTTACAACCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGG CTTGCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 9/7 | ACCTCTACACTCGGAATTCCACTCACCTCTACCATACTCAAGACTTCC AGTATCAAAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGA CTTAAAAGTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCGAACAAC GCTAGCCCCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGG GCTTCTTCTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTAC AACCCTAGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGC GCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |
| 10/4 | AAGGCAGTTCCGGGGTTGAGCCCCGGGATTTCACCCCTGACTTAAAA GTCCGCCTACGTGCGCTTTACGCCCAGTAAATCCGAACAACGCTAGCC CCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGGGCTTCTT CTCCGGTTACCGTCATTATCTTCACCGGTGAAAGAGCTTTACAACCCT AGGGCCTTCATCACTCACGCGGCATGGCTGGATCAGGCTTGCGCCCAT TGTCCAATATTCCCCACTGCTGCCTCCCGTAA | *Brucella abortus* | 100% |
| | | *Brucella melitensis* | 100% |
| | | *Brucella canis* | 100% |

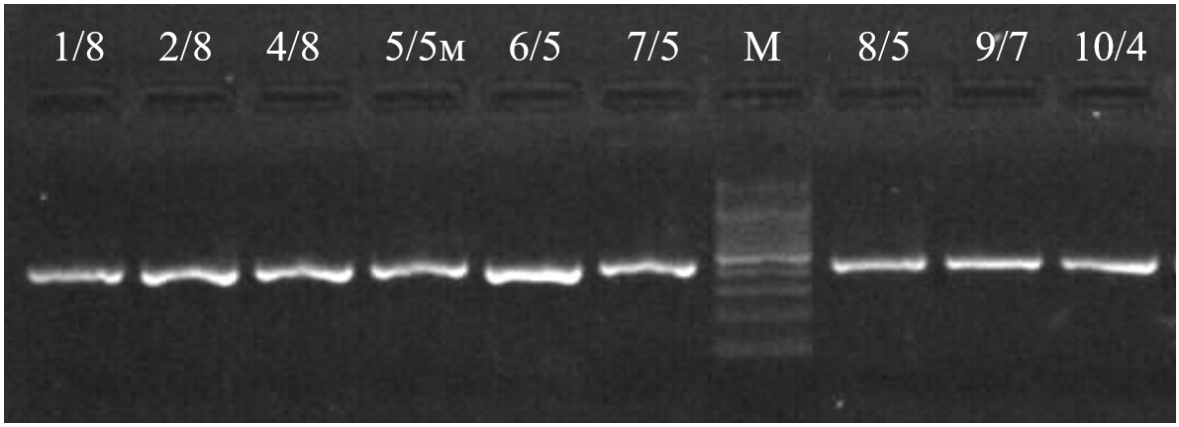


**Figure 1** Electrophoregram of PCR products with primers for 16S rRNA (M – molecular weight marker 100 bp.

The success of brucellosis control depends on the speed of detection of all infected animals and the effectiveness of diagnostic tests **(Alton et al., 1988; Meyer, 1990; Yagupsky, 1999)**. Up-to-date disease detection helps to take steps to isolate the infected animal early as a source of infection **(Bricker and Ewalt, 2005; Ridler et al., 2005)**. The following serological tests for animal brucellosis are officially recognized in Kazakhstan: RBS, AR, CBD, and ELISA. The rate of serological reactions in brucellosis is unstable **(Ficht, 2010; Scholz and Vergnaud, 2013)**. While each method is complex with other methods, it does not isolate all sick animals. Traditional phenotypic differentiation methods make it difficult to standardize the situation, which is the reason for erroneous differentiation **(Pappas et al., 2006; Godfroid et al., 2011, Borriello et al., 2013)**. It is known that the phenotypic characteristics of bacteria can vary depending on the growing conditions and the allelic state responsible for gene expression. The most conservative structure that characterizes the type of microorganism in its gene **(Ayala and Kiger, 1980; Gershkovich, 1968; Al Dahouk et al., 2007, Kattar et al., 2008)**. In this regard, the most promising is the assessment based on the nature of the pathogen genome.

The solution of the problem made it possible to determine the genotype of the studied strains using cluster analysis and to start an investigation of the outbreak in the regions endemic for brucellosis **(Frachetti et al., 2017)**. Timely provision of information on the causative agent of brucellosis, obtained using multilocus analysis, for the use of scientific research results in veterinary practice, which ensures high-quality monitoring of the epidemic process, effective eradication, and prevention **(Le Flèche et al., 2006; Whatmore, 2009; Demirev, 2013)**. The expediency of the introduction of 16S rRNA is an accurate and timely diagnosis of brucellosis in animals since the timely detection of the disease will help to take measures for the early isolation of the animal as a source of infection and prevent its further development and spread **(Garofolo et al., 2013; Shevtsov et al., 2015)**.

## CONCLUSION

The region of the 16S rRNA gene sequence among the primers used helps to determine 100% affinity, but the specificity is not enough to identify the species. The obtained data are consistent with bacteriological data (colony shape – S, bluish-transparent in daylight; microscopy: round negative rods with rounded ends, located immediately; all characteristics correspond to *Brucella abortus*). It can be said that the *Brucella* family is present in the studied samples of microorganisms (no. 4, 5, 7, 8). It turned out that the study of the genetic diversity of brucellosis using *Brucella* spp. The genetic structure of the Kazakh population has not been studied enough.

Multiple-locus analysis genotyping constitutes a convenient medium-resolution classification assay for large-scale investigations. Follow-up studies based on the whole genome sequencing of selected strains will be necessary to more precisely decipher the dynamics of strain circulation both within Kazakhstan and with Kazakhstan's neighboring countries. Such studies will also permit to design of new low-cost genotyping assays tailored for Central Asia.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This article does not contain any studies that would require an ethical statement.

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# THE XANTHINE OXIDASE AND ITS ASSOCIATED ACTIVITIES IN THE OVINE MILK AND LIVER: DISTINCTIVE IN IMPACT OF *IN VIVO* MOLYBDENUM

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## ABSTRACT

Xanthine oxidase is molybdenum and iron-containing flavoprotein, catalyzing the final oxidation stage of purines and oxidative transformation of pterins and some aliphatic and aromatic aldehydes. Despite the importance of this enzyme, the distribution of xanthine oxidase in traditional household animal's milk and tissues is unknown. Formerly, we have found most of the xanthine oxidase molecules in animal milk are inactive because of a lack of molybdenum. Ovine milk was processed by inserting *in vivo* molybdenum (tungsten) into drinking water. We gave opposite dates in the presence of tungsten too. Heating the milk of animals at 80 °C for 5 minutes in the presence of molybdenum and cysteine led to a sharp increase of xanthine oxidase and its associated – nitrate reductase and nitrite reductase activities. The change of xanthine oxidase and its associated activities were examined by spectrophotometry after treatment. It was established that metal ions added in drinking water for animals have an impact on enzyme activities. The activity is formed in the ovine liver even in the absence of exogenous molybdenum in drinking water. The associated activities of liver enzymes in the presence of molybdenum in drinking water had slightly increased. Tungsten-containing water led to the loss of all activities of liver xanthine oxidase. It is proposed that the liver contains a special protein involving in the incorporation of molybdenum (or tungsten) into xanthine oxidase molecule, however, the milk or mammary gland compounds lack this protein.

**Keywords:** tungsten; reductase; protein; heat treatment; ovine milk

## INTRODUCTION

Xanthine oxidase (XO) is the enzyme that is responsible for the synthesis of uric acid in mammalian. Xanthine oxidase is widespread in mammalian tissues and is a major component of the membrane of milk fat globules that surround the fat globules in milk. Uric acid is the final major product of the metabolism of nitrogen-containing compounds in animals, and it functions as an antioxidant to reduce oxidative stress **(Matata and Elahi, 2007)**. Purines and other substrates react with xanthine oxidase at the site containing molybdenum, and the electron acceptors react at the FAD site **(Harrison, 2002; Schwarz, 2005)**. The protein part of the enzyme is rich in cysteine and contains 60 – 62 free sulfhydryl (-SH) groups. In the structure XO, some centers represent the 2Fe-2S complex **(Harrison, 2006)**. Molybdenum (Mo) is in the body of animals, and its concentrations were varying depending on tissue type. Molybdenum is an essential cofactor of animal molybdenum-containing enzymes (Mo-enzymes) such as xanthine oxidase, aldehyde oxidase, sulfite oxidase, and recently discovered mitochondrial amidexime-reducing protein (mARC) **(Hille et al., 2011; Santamaria-Araujo et al., 2012; Carroll et al., 2017)**. Two S-bonds connect it with the side chain of the cofactor molecule **(Bryan et al., 2009)**.

It was found earlier that homogenous xanthine oxidase purified from cow's milk reduces the nitrate ($NO_3^-$) to nitrite ($NO_2^-$) **(Alikulov, Lvov and Kretovich, 1980)**. However, it was unclear in what substances nitrite reduces. Later, scientists had established that the xanthine oxidase isolated from the liver of the cow reduces nitrite under anaerobic conditions and converts it into nitric oxide (NO) **(Millar et al., 1998)**.

At present, *in vivo* studies of milk xanthine oxidase in sheep liver have not been performed. But attempts to study milk xanthine oxidase activity in the liver for comparative analysis can be considered using the example of other animals. Research by **Grebennikova et al. (2013)** defines molybdenum as an important enzyme for the development of plant growth. The lack of molybdenum leads to a decrease in xanthine oxidase activity. The artful addition of molybdenum to camel and mare milk helped to remove nitrates and nitrites from it. According to research by **Ozturk et al. (2019)** on the effect of heat treatment on xanthine oxidase in cow's milk, it can be concluded that the higher the temperature, the faster the inactivation of

xanthine oxidase occurs, in contrast to raw milk, where high activity was not observed. By **Sharma et al. (2016)**, Everett compared the xanthine oxidase activity of bovine milk treated with an impulsive electric field and an increase in temperature. The denaturation of xanthine oxidase was less after electric field treatment compared to heat treatment. Thus, an increase in temperature promotes the decontamination of milk and an increase in xanthine oxidase in all cases.

Thus, xanthine oxidase, contained in the tissue and liquids in the animal body, is a unique tool for decontamination of nitrates and nitrites and the formation of important substances for the body nitric oxide. This study aimed to determine the effect of heat treatment of milk on the activity and biological functions of xanthine oxidase in sheep liver *in vivo* **(Sharma et al., 2016)**. To accomplish this goal, it is necessary to perform the following tasks: conducting an experiment on the activity of milk xanthine oxidase in the liver using the example of 3 control groups and determining their results, pointing out the differences, and conducting an experiment of xanthine oxidase activity during freezing and heating milk. Concluding what factors influence the increase in milk xanthine oxidase activity in the liver of sheep. Consequently, the data for the study on nitrates and nitrites reduction activity mechanisms has both scientific and practical value. So, the knowledge base will increase by research ovine liver and milk xanthine oxidase in this area.

## Scientific Hypothesis

Xanthine oxidase, contained in the tissue and liquids in the animal body is a unique tool not only for decontamination of nitrates and nitrites but also for the formation of important substances for the body – nitric oxide. Consequently, the data for the study on nitrates and nitrites reduction activity mechanisms has both scientific and practical value. So, the knowledge base will increase by research ovine liver and milk xanthine oxidase in this area.

## MATERIAL AND METHODOLOGY

### Samples

Ovine milk was obtained from healthy six sheep in the mid-lactation period (from May to June) based on a farm in the Almaty region (Kazakhstan). Liver tissues were obtained from six non-lactating animals.

### Chemicals

The experiments carried out using molybdenum (M = 241.95), sodium tungstate (M = 329.9), L-cysteine (M = 157.6), sulfanilamide (M = 172.21) from Sigma-Aldrich Chemical Co., N-(1-naphthyl)-ethylenediamindigidro-chloride (M = 259.18) from AppliChem (Germany).

### Instruments

Preparation of the milk for the detection of the various enzymatic activities. Before treatment in the milk of domestic animals added 10 μM ethylenediaminetetraacetic acid (EDTA) to bind heavy metals. For boiling fresh milk is poured into the narrow conical tubes in a volume of 2 mL. Then, for further determination of the enzymatic activity the tubes are placed in the water bath at 35 °C temperature and kept for 10 min, then using the special reagents different activities of XO are determined.

### Laboratory Methods

It is known that the smallest concentration of molybdenum in water can prevent the inclusion of tungsten in xanthine oxidase. Therefore, tungsten is given in high concentrations. 100 g liver slices of slaughtered cattle were also frozen at -20 °C. The frozen samples were defrosted after 30 days. The xanthine oxidase (XO), nitrate reductase (NR), and nitrite reductase (NiR) activities were simultaneously determined in milk and liver samples. The liver homogenate was obtained by carefully rubbing the mixture in a porcelain mortar. Samples of liver were mixed in a 1:5 ratio with a cold 0.1 M sodium phosphate buffer containing 10 μm EDTA and 10 μm phenylmethylsulfonyl fluoride for protease inhibition. The supernatant was obtained by centrifugation of liver homogenate and milk aliquot at 15000g for 20 minutes **(Harrison, 2002)**.

Sodium phosphate buffer solution was added to milk to a final concentration of 100 mM, pH 6.5, 10 μM EDTA solution, 2 mM sodium molybdate ($Na_2MoO_4$), or sodium tungstate ($Na_2WO_4$), and 2 mM cysteine solution. Then, the milk and liver of the supernatant are heated at 80 °C for 5 minutes. After cooling to determine the enzyme activity, 100 mL aliquots of milk and liver supernatant were used. Each activity was determined in triplicate (n = 3, *SD* ±) **(Godber et al., 2000)**.

To determine the steps carried out, the xanthine oxidase activity is described in the developed method **(Alikulov, Bespaev and Yakupbaev, 1999)**. Activation of the milk and liver samples using metal ions and thiols.

The sodium phosphate buffer solution was added to the milk in final concentrations of 100 mM, and pH is 6.5; 10 μm EDTA solution, 2 mM sodium molybdenum ($Na_2MoO_4$) or sodium tungstate ($Na_2WO_4$), and 2 mM cysteine solution. The liver supernatant has the same concentration of sodium molybdenum or tungstate and cysteine solutions. After the milk aliquot and liver, the supernatant warmed up at a temperature of 80 °C for 5 min. After cooling, 100 μL aliquots of milk and liver supernatant were used to determine enzymatic activities. The determination of each activity was carried out in three repetitions.

Nitrate reductase activity (NR) was determined by the formation of nitrites, and nitrite reductase activity (NiR) – by the disappearance of nitrites in the reaction mixture after incubation. The reaction mixture in the volume of 500 mL (do not take into account milk or liver supernatant) contained 20 mM sodium nitrate ($NaNO_3$) or 80 μM sodium nitrite ($NaNO_2$), 20 mM methyl- or benzyl viologen reduced by dithionite ($Na_2S_2O_4$). The mixture was incubated at 37 °C for 15 minutes. Then to the reaction mixture, 500 mL of sulfanilamide solution and 500 mL of N-(1-naphthyl)-etilendiamine (NEDA) was added. These reagents were prepared in the following steps: 6 g of sulfanilamide was dissolved in 1 liter of 20% HCl; 1.23 g NEDA – in 1 L of water distilled by milliQ. At pH 2-2.5, sulfanilamide nitrite forms a red diazonium compound.

### Sample preparation

The live weight of animals at the beginning of the experiments was 40 to 45 kg. Animals were fed with freshly

**Table 1** Dynamics of changes in the associated activities of XO of fresh ovine milk obtained daily during the month.

| Days of milking | Activities | | | | | |
|---|---|---|---|---|---|---|
| | XO activity* | | $NO_3$-reductase activity** | | $NO_2$-reductase activity*** | |
| | Milk + Mo | Milk + Mo +heating | Milk + Mo | Milk + Mo + heating | Milk + Mo | Milk + Mo + heating |
| 0 | >0.2 | ~3.2 | ~1.4 | 136.8 ±13.7 | ~2.3 | 243.7 ±28.3 |
| 4 | >0.2 | ~3.2 | ~1.4 | 136.7 ±20.1 | ~2.3 | 243.7 ±24.9 |
| 8 | >0.2 | ~3.0 | ~1.4 | 136.3 ±18.6 | ~2.2 | 243.1 ±19.8 |
| 12 | >0.2 | ~2.8 | ~1.3 | 132.6 ±12.3 | ~2.0 | 240.2 ±22.3 |
| 16 | >0.2 | ~2.8 | ~1.3 | 132.5 ±14.8 | ~1.8 | 240.2 ±27.6 |
| 20 | >0.2 | ~2.8 | ~1.2 | 130.7 ±21.6 | ~1.8 | 238.3 ±21.4 |
| 24 | >0.2 | ~2.6 | ~1.1 | 128.8 ±23.5 | ~1.8 | 236.4 ±18.7 |
| 28 | >0.2 | ~2.6 | ~1.1 | 128.6 ±18.9 | ~1.7 | 236.4 ±21.8 |
| 32 | >0.2 | ~2.6 | ~1.0 | 128.4 ±10.3 | ~1.7 | 236.2 ±22.4 |

Note: XO-activity*: nanomoles of uric acid formed/100 µL milk/min; NR** activity: nanomoles of $NO_2$- formed/100 µL milk/min); NiR*** activity: nanomoles of $NO_2$- disappeared/100 µL milk/min.

**Table 2** Influence of exogenous Mo on the dynamics of changes in the associated activities of sheep milk.

| Days milking | Activities | | | | | | Mo* |
|---|---|---|---|---|---|---|---|
| | XO activity | | $NO_3$-reductase | | $NO_2$-reductase activity | | |
| | Milk + Mo | Milk + Mo + heating | Milk + Mo | Milk + Mo + heating | Milk + Mo | Milk + Mo + heating | |
| 0 | >0.2 | 3.2 ±0.4 | ~1.4 | 136.8 ±24.6 | ~2.3 | 243.7 ±41.6 | >2 |
| 4 | >0.2 | 3.2 ±0.3 | ~1.4 | 136.8 ±22.4 | ~2.3 | 243.7 ±42.3 | >2 |
| 8 | ~0.3 | 3.2 ±0.4 | ~1.5 | 142.7 ±28.6 | ~2.5 | 249.7 ±43.7 | 12.6 ±2.1 |
| 12 | ~0.4 | 3.4 ±0.5 | ~1.5 | 149.8 ±19.4 | ~2.6 | 249.5 ±51.6 | 42.7 ±7.2 |
| 16 | ~0.4 | 3.4 ±0.4 | ~1.6 | 152.9 ±12.6 | ~2.5 | 252.3 ±32.4 | 48.5 ±6.3 |
| 20 | ~0.4 | 3.2 ±0.5 | ~1.5 | 150.7 ±13.2 | ~2.5 | 252.2 ±28.6 | 51.3 ±8.4 |
| 24 | ~0.4 | 3.2 ±0.3 | ~1.4 | 148.9 ±24.3 | ~2.4 | 248.5 ±35.4 | 51.3 ±7.8 |
| 28 | ~0.4 | 3.0 ±0.3 | ~1.4 | 148.6 ±13.8 | ~2.3 | 246.7 ±28.3 | 51.4 ±9.4 |
| 32 | ~0.4 | 2.9 ±0.4 | ~1.3 | 148.3 ±12.8 | ~2.2 | 246.8 ±32.6 | 51.5 ±11.3 |

Note: *molybdenum content in milk in nanograms in milliliter.

cut green plants (Adlib feeding). For the experiment, three control groups were defined. To study exogenous molybdenum, ammonium molybdate $(NH_4)_2MoO_4$ was added to the drinking water of animals per 10 kg of animal weight. During the reporting period, the animals were watered twice – the first time, the volume of water with molybdenum was three liters, the second time, they gave the same portion, but without molybdenum. Animals were weighed weekly for correcting the dose of molybdenum. The second pair of animals was fed similarly to the first group, but they were given tungsten water once a day – one liter of such water contained 100 mg of sodium tungstate. Control third group animals were fed daily with freshly cut grass. They drank natural water without the addition of molybdenum or tungsten. Once a day, sheep were milked. Every four days, 100 mL portions of fresh milk were immediately frozen at a temperature of -20 °C. Thus, 18 samples of each pair of sheep (about 1.8 L) were frozen (5.4 L were obtained from six animals) **(NIIP, 2017)**.

A parallel experiment was conducted to study the effect of molybdenum or tungsten on sheep liver xanthine oxidase activity. Since the slaughter of six non-lactating animals was planned, one pair of animals was kept as a control, i.e., they were fed with green grass, freshly cut in a certain field watered with natural water for 30 days before the slaughter. The second pair of animals received the same plant food and drank the same water, but containing 10 $mg.mL^{-1}$ of ammonium molybdenum. Instead of molybdenum, the third pair received 10 times the tungsten concentration in the form of 100 $mg.mL^{-1}$ of sodium tungstate compared to molybdenum.

## Statistical Analysis

The absorbance obtained color solution was measured photometrically at a wavelength of 548 nm in spectrophotometer ("Specol-2000", Germany). The calibration curve is constructed by using increasing concentrations of nitrite ($NaNO_2$) colored with sulfanilamide and NEDA. The advantage of the photometric method is its high sensitivity. The amount of molybdenum was determined using the method developed by our research group. The heat treatment of molybdenum led to determine the total amount of molybdenum **(Alikulov, Mukhamejanova and Kultaeva, 2017)**. The statistical analysis data were produced by Microsoft Excel.

## RESULTS AND DISCUSSION

According to the results, sheep that were drunk with molybdenum-contain water for a month accumulated molybdenum in their milk. The concentration of which reached a maximum (51 $nanograms.mL^{-1}$) on the $20^{th}$ day. Just such an amount of molybdenum in milk did not lead to the demonstration of all associated XO activities after heat treatment in the presence of cysteine (but without exogenous molybdenum). In this case, only exogenous

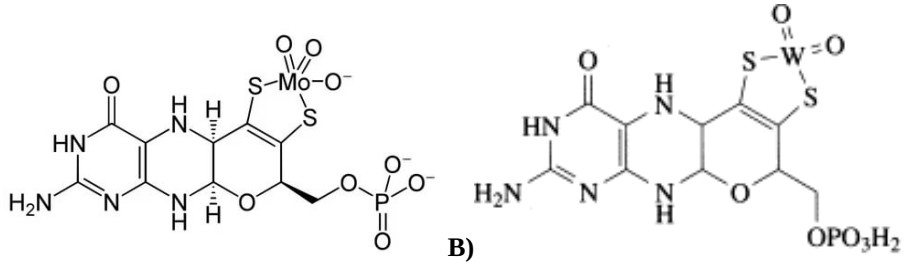


**Figure 1** The structure of the molybdenum-factor and its relation to the molybdenum (A) and tungsten (B) atoms in the active center of xanthine oxidas.

**Table 3** Influence of tungsten on the dynamics of the changes in the associated activities of sheep milk.

| Days of milking | Activities | | | | | |
|---|---|---|---|---|---|---|
| | XO activity | | $NO_3$-reductase | | $NO_2$-reductase activity | |
| | Milk + Mo | Milk + Mo + t° | Milk + Mo | Milk + Mo + t° | Milk + Mo | Milk + Mo + t° |
| 0 | 0.0 | 3.2 ±0.4 | 0.0 | 136.7 ±21.6 | 0.0 | 243.6 ±23.6 |
| 4 | 0.0 | 3.2 ±0.3 | 0.0 | 136.8 ±22.2 | 0.0 | 243.5 ±20.3 |
| 8 | 0.0 | 3.1 ±0.4 | 0.0 | 135.5 ±24.7 | 0.0 | 242.3 ±22.3 |
| 12 | 0.0 | 3.1 ±0.5 | 0.0 | 135.5 ±19.1 | 0.0 | 240.4 ±28.2 |
| 16 | 0.0 | 2.9 ±0.4 | 0.0 | 134.7 ±15.8 | 0.0 | 239.3 ±31.4 |
| 20 | 0.0 | 2.9 ±0.5 | 0.0 | 134.6 ±21.6 | 0.0 | 238.7 ±27.5 |
| 24 | 0.0 | 2.8 ±0.4 | 0.0 | 131.6 ±15.2 | 0.0 | 237.5 ±26.3 |
| 28 | 0.0 | 2.8 ±0.4 | 0.0 | 130.4 ±17.3 | 0.0 | 237.8 ±23.2 |
| 32 | 0.0 | 2.7 ±0.4 | 0.0 | 129.3 ±20.3 | 0.0 | 236.6 ±21.9 |

**Table 4** Effect of molybdenum and tungsten on associated liver xanthine oxidase activity.

| Variants | Activities | | | | | |
|---|---|---|---|---|---|---|
| | XO activity | | $NO_3$-reductase activity | | $NO_2$-reductase activity | |
| | Control | + M | Control | + Mo | Control | + Mo |
| Control | 2.9 ±0.3 | 3.5 ±0.4 | 118.7 ±14.6 | 132.7 ±18.6 | 197.6 ±25.9 | 218.6 ±23.6 |
| + molybdenum | 3.2 ±0.3 | 3.3 ±0.4 | 130.9 ±16.3 | 132.9 ±21.6 | 217.6 ±22.3 | 219.6 ±20.3 |
| + tungsten | 0.0 | 3.1 ±0.2 | 0.0 | 128.9 ±25.4 | 0.0 | 216.3 ±24.2 |

molybdenum activated XO activity after heat treatment in the presence of cysteine (Table 1).

It can be assumed that before embedding molybdenum in the active center (or before binding to the MPT in the active center), newly synthesized XO molecules are involved in the formation of the inner membrane of the milk fat globule (MFGM) **(Cebo et al., 2012; Saadaoui et al., 2013; Mendel, 2013)**. XO is located in the inner MFGM, as indicated in the research **(Pisanu et al., 2011; Lu et al., 2005)**. It is no longer available for molybdenum *in vivo*.

However, in the heat treatment of ovine milk in the presence of molybdenum and cysteine, XO was activated and showed high associated activity (Table 2), comparable to the version of molybdenum-containing water. Cysteine only slightly increased these liver XO activities of sheep. The steady presence of tungsten for a month in drinking water leads to the inactivation of liver xanthine oxidase and its associated activities, i.e., inactive molecules of this enzyme are formed. As it is known, tungsten, as a chemical analog of molybdenum, is widely used to identify new molybdenum enzymes **(Harrison, 2002; Schwarz, 2005; Sharma et al., 2016)**. Tungsten is very close to molybdenum in atomic and ionic radius, as well as chemical properties. Therefore, in the absence of molybdenum, tungsten is easily embedded in the active center of enzymes, instead of molybdenum **(Brondino et al., 2006)** (Figure 1).

In the active center of the enzymes, one molybdenum atom binds to two sulfhydryl groups of the cofactor. However, the relationship between the cofactor and molybdenum is not strong, i.e., when a cofactor is isolated from an apoenzyme or when an enzyme molecule is denatured, molybdenum is easily separated from the cofactor. Moreover, the cofactor is extremely sensitive to oxygen since it is instantly inactivated in the air (two SH-groups oxidize to form disulfide-S-S). As a result, the presence of thiols in experiments with a cofactor is necessary. This is most likely a non-physiological effect of the "mass action" of molybdenum *in vivo*. This is most likely a non-physiological effect of the "mass action" of molybdenum *in vivo*. Consequently, the presence of excess oxygen does not need in molybdenum or a chemical analog – tungsten for the synthesis of XO in milk and its inclusion in the MFGM. The results of this experiment are presented in Table 3.

The results show that natural drinking water contains enough molybdenum for the normal xanthine oxidase activity, the normal xanthine oxidase activity in the liver. Increasing the content of molybdenum in drinking water only slightly increased the liver xanthine oxidase-associated activity. As can be seen from Table 4, liver XO shows high associated activity without heat treatment and warming up at 80 °C in the presence of molybdenum. Determination of the associated activities of xanthine oxidase (NR and NiR



**Figure 2** Kumys, a fermented dairy product traditionally made from mare's milk.

activity) in fresh milk and after storage at -20 °C showed that storing frozen milk preserves the activities of the milk.

Results show that in fresh sheep milk obtained during the month, none of the associated XO activities were detected as one of the associated XO activities.

However, after the heat treatment of milk at 80 °C for 5 minutes (in Table 4 shown as + Mo + to) in the presence of exogenous molybdenum and cysteine, all associated activities of this enzyme appear (Table 4). Among other natural and artificial thiols, glutathione and mercaptoethanol were active in restoring these activities by exogenous molybdenum. The absence of molybdenum or cysteine during heat treatment did not lead to the appearance of these activities.

The results presented in the table show that the associated XO activity up to the 10th day in milk. It slightly increased first and decreased then (Tables 1, 2, and 4). There is an assumption that the relatively high activity of XO at the beginning of lactation is associated with the anti-pathogenic property of this enzyme. However, our results show that milk XO does not contain molybdenum. Accordingly, XO is inactive. Perhaps there is another explanation that the superoxide-producing center does not contain molybdenum but contains FAD **(Godber et al., 2000; Maia et al., 2007)**. The results show that in fresh sheep milk, xanthine oxidase does not contain molybdenum. XO, located in the fat globule micelles (MFGM), is not available for exogenous molybdenum. During heat treatment at 80 °C, the milk globules are destroyed, then the molecule is denatured. As a result, the access of molybdenum to the MPT (molybdopterin or metal-binding Pterinene-1.2-diThiolate) – containing active center increases. Our and other numerous studies have shown that MPT is extremely sensitive to oxygen. Therefore, the presence of antioxidant-cysteine protects sulfhydryl groups of MPT from oxygen. Cysteine forming temporary disulfide bonds with MPT protects it from oxidation. The active MPT is easily displaced by cysteine and associated with him in the XO active site from the active MPT in exogenous molybdenum **(Hille et al., 2011; Santamaria-Araujo et al., 2012; Carroll et al., 2017)**.

An experiment was conducted to determine the effect of elevated dietary Mo and duration of feeding on its concentrations in internal organs (Figure 2). Forty lambs were fed diets containing different concentrations of added Mo as sodium molybdate for 14 or 28 days. The concentration of Mo increased linearly in internal organs as dietary Mo increased. Liver Mo concentration increased more rapidly at 14 days than at 28 days, but other organ Mo increased to a greater extent in lambs fed 28 days **(Pott et al., 1999)**. The molybdenum absorption in the gastrointestinal tract depends on its chemical nature. Molybdenum and its compounds penetrate directly to the gastrointestinal tract. For instance, water-soluble molybdate, thiomolybdate, and oxothiomolybdate are absorbed from 75 % to 90 % in the gastrointestinal tract and after that completely excreted in the form of molybdenum, mainly by the urine. The minimum toxic concentrations of molybdenum in drinking water for calves varied between 10 and 50 ppm **(National Toxicological program, 1997)**.

The defined protein includes metal ions in molybdopterin (molybdcofactor) at relatively low concentrations of molybdenum concentrations in liver tissues. For example, the bacterial MogA (or animal protein – gefirin) protein exhibits an affinity for molybdenum. It acts as a molybdenum chelatase, which includes molybdenum in the molecule of the molybdenum cofactor **(Liu et al., 2000; Harrison, 2002; Harrison, 2006; Dutta et al., 2013)**. This protein likewise incorporates this tungsten into the molecule of xanthine oxidase at high concentrations of it. There is no

protein in the milk or the mammary gland. Therefore heat treatment at 80 °C of milk in the presence of exogenous molybdenum and cysteine led to activate xanthine oxidase in sheep milk **(Dyusembayev et al., 2016)**; liver xanthine oxidase shows high associated activity without heat treatment, and absence of exogenous molybdenum in drinking water shows normal associated activities in sheep liver than in milk **(Nine et al., 1971; Johnson et al., 1974; Raina et al., 2015)**; there are distinctive in the effect of *in vivo* molybdenum in sheep milk and liver **(Kincaid, 1980; de Araújo et al., 2017)**.

## CONCLUSION

The paper analyzes the effect of molybdenum and tungsten on the activity of conversions of xanthine oxidase in milk and liver *in vivo*. Xanthine oxidase is present in almost all tissues of the animal body. It has the highest specific activity in the liver, and milk serves as an object for its excretion. Determined that the activity of xanthine oxidase directly depends on the content of molybdenum in the body. It is important to note that xanthine oxidase activity largely depends on the intake of exogenous molybdenum into the body. One way to study the *in vivo* effect of exogenous molybdenum on the activity of xanthine oxidase is by adding a salt of the metal in the drinking water of domestic animals.

It was determined that the xanthine oxidase of the milk of the sheep is inactive in its natural state. However, heat treatment of milk in the presence of exogenous sodium molybdate and thiols (cysteine) led to the appearance of associated xanthine oxidase activities. Because at high temperatures, the enzyme molecule is partially denatured and, as a result, the active site is available for exogenous molybdenum and cysteine. Natural drinking water contains enough molybdenum for normal xanthine oxidase activity, oxidase activity in the liver without thermal treatment. Tungsten replaces molybdenum in the active center of the enzyme, which leads to its irreversible inactivation.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This research was approved by the Ethics Committee of the Astana Medical University following the legislation of the Republic of Kazakhstan (Project identification code: 1253/GF4, Date of approval: April 17, 2020. Ethics Committee of the Astana Medical University).

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# THE STUDY OF THE PRODUCTIVITY POTENTIAL OF GRAPE VARIETIES ACCORDING TO THE INDICATORS OF FUNCTIONAL ACTIVITY OF LEAVES

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## ABSTRACT

Photosynthetic activity in the leaf of ten grape varieties was studied in the conditions of the northern part of Forest-Steppe of Ukraine. From 2019 to 2020 meteorological data were recorded by the "Meteotrek" meteorological station. The influence of weather conditions of the spring-summer period at the beginning of the vegetation phases, which in 2019 took place on average 12 days earlier than in 2020, was noted. Analysis of changes in the induction of chlorophyll fluorescence (ICF) in leaves revealed the more efficient use of quantum energy of absorbed light by $F_0$ in the leaves of most varieties was noted in the phase "the beginning of berry ripening". During the three periods of the study, the varieties Aromatnyj, Zagadka, Ilichevskij rannij, and Kardishah showed a lower content of chlorophyll molecules, which do not have a functional connection with the reaction centers (RC). According to the *Fp* index the highest intensity of photosynthesis on average for all varieties was observed in the phase "the beginning of ovary growth", the lowest – "the inflorescence is visible". The potential productivity of grape plants determined by the ICF coefficient for all varieties was at a very high level and had a weak or moderate correlation with weather conditions during the growing season. The hydrothermal coefficient and Σ act t ≥10 °C had a weak effect on the efficiency of the light phase of photosynthesis (*Ki*) and a noticeable one (correlation coefficient r = 0.50 – 0.69) on the efficiency of dark photochemical processes (*Rfd*) in the leaves of most grape varieties.

**Keywords:** grape variety; chlorophyll fluorescence; phenology; photosynthesis; potential productivity

## INTRODUCTION

The specificity of viticulture industry development is determined by its close connection with the weather and climatic conditions. Due to global climate change, there is a need to assess the response of grape plants to possible changes in agro-climatic growing conditions to use the resources of a particular region more efficiently and increase gross harvest and crop quality **(Kovalyshyna et al., 2020a)**.

Grapes are in demand in many countries around the world. The share of fertile grape plantations in the world is over 3.1%, which is about 12.5 thousand hectares. Most of these plantations are concentrated in the Middle East and European countries **(Zheplinska et al., 2019)**. Ukraine ranks in the world in terms of grape production. According to the FAO, the world grape market is underfunded. According to scientifically sound standards, the annual consumption of grapes per capita should be 2 **(Zheplinska et al., 2021)**. World grape production should reach 14 million tons to ensure such volumes.

The weather has a significant impact on the formation of the grape harvest. Weather conditions change from year to year, which determines the variability of crop values **(Kovalyshyna et al., 2020b; Kolyanovska et al., 2019)**. The fundamental process, during which the formation of the crop takes place, is photosynthesis **(Smetanska et al., 2021)**. The intensity of photosynthesis in fruit and grape plants is most often investigated by assessing the functional state of their photosynthetic apparatus using the method of induction of chlorophyll fluorescence (ICF). This method determines the sensitivity of the process of photosynthesis to various stress factors, such as temperature, light intensity, the action of toxins, heavy metals, air pollution, and changes in vertical zonation, precipitation, etc. **(Mamonova et al., 2018)**. Studies using the method of ICF and correlation analysis make it possible to determine the potential productivity of grape varieties and establish the level of influence of weather conditions on them. Therefore, this work aimed to determine the level of functional activity of leaves of different grape varieties during the growing season and to establish the influence of weather conditions on the processes of photosynthesis in plants grown in vineyards in the northern part of Ukraine.

## Scientific Hypothesis

Determining the potential productivity of grape plants in new soil and climatic conditions based on the influence of weather conditions during the growing season on the efficiency of photosynthesis in the leaves will assess the adaptive properties and productivity of plants in the northern forest-steppe of Ukraine for further introduction of the best grape varieties into industrial crops.

## MATERIAL AND METHODOLOGY

### Samples

Studies to determine the functional state of the leaves of grape plants were conducted in the Laboratory of Plant Physiology and Microbiology of the Institute of Horticulture of the National Academy of Agrarian Sciences of Ukraine (IH NAAS). The objects of the study were ten varieties of grapes selected by NSC "Institute of viticulture and wine-making after V. Ye. Tairov". Plants were planted in the spring of 2017 on rootstock 101-14 '*Riparia* × *Rupestris*' on the territory of the training laboratory (TL) "Fruit and Vegetable Garden" of the National University of Life and Environmental Sciences of Ukraine (Kyiv) according to the scheme 3.0 × 1.5 m. The soil of the experimental plot is sod-podzolic, light loam with optimal pH (6.47 – 6.81), and high content of nitrogen and phosphorus. The soil retention system is black fallow; the plot is irrigated. Grape culture is covered. Meteorological indices for the period 2019 – 2020 were recorded by the "Meteotrek" meteorological station, which is located at a distance of 80 m from the experimental vineyard, and processed in Excel.

### Chemicals

No chemicals were used.

### Biological Material:

The study used green fully formed leaves of ten varieties of grapes which were on the same side of the row and the leaf blade was evenly lit.

### Instruments

A portable chronofluorometer "Floratest" developed at the Institute of Cybernetics. V. M. Glushkova NAS of Ukraine. The device online registers the fluorescence of the plant leaf. Information in the form of a chlorophyll fluorescence induction curve is displayed on the device displays. This curve reflects the physiological state of the entire chain of photosynthesis and the kinetics of its various links. The shape of this curve and its sections can be used to assess and predict the degree of influence on the plant of both the main environmental factors and endogenous ones.

### Laboratory Methods

Diagnosis of the functional activity of grape leaves was performed with a portable chronofluorometer "Floratest", which monitors the work of Photosystems II (PSII) and chloroplasts (Figure 1). The device registers the "Kautsky curve" (induction changes in fluorescence), the shape of which fully reflects the course of photosynthesis in the chloroplasts of leaves **(Brajon et al., 2000)**. Kautsky effect (induction of chlorophyll fluorescence) is a phenomenon of change in the glow of chlorophyll, which occurs during prolonged illumination of a plant leaf previously adapted to darkness.

During the assessment of the functional state of the photosynthetic apparatus by inductive changes in chlorophyll fluorescence, a set of parameters was used, which allowed us to analyze the changes in photosynthetic processes in the leaves, namely:




**Figure 1** Functional activity research of grape leaves using a portable chronofluorometer "Floratest".

$F_0$ – "background" level of fluorescence; depends on the loss of excitation energy during the migration of the pigment matrix, as well as on the content of chlorophyll molecules that have no functional connection with the reaction centers (RC). At the initial moment ($F_0$) all the channels of photosynthetic electron transfer are open and the maximum energy of the excited electrons goes to the photosynthetic process.

*Fp* – the maximum value of fluorescence, which characterizes the highest level of fluorescence of chlorophyll *a* (*Chla*) and is displayed as a maximum on the induction curve.

*Ft* – a stationary level, which is characterized by a dynamic equilibrium between the processes that cause an increase in fluorescence and the processes that lead to its decrease.

The following indicators were calculated by the analytical method:

*Ki* – coefficient of induction of chlorophyll fluorescence shows the share of chlorophylls involved in photosynthesis, from their total number and determines the efficiency of the light phase of photosynthesis: $(Fp - F_0)/Fp$;

*Rfd* – coefficient of efficiency of dark photochemical processes or the coefficient of decline fluorescence, which characterizes quantum efficiency of photosynthesis (viability index): $(Fp - Ft) / Ft$ **(Havryliuk et al., 2019)**.

**Description of the Experiment**

For integrated assessment of the state of the photosynthetic apparatus by ICF, a green, fully formed, pre-adapted to the darkness grape leaf was placed between the plates of the remote optical sensor of the device "Floratest" and changes in chlorophyll fluorescence were recorded for 4 min. Plant samples (each in five repetitions) – leaves 30 – 35 days of age were analyzed in three phases of grape development: the first – the inflorescence is visible; the second is the beginning of ovary growth; the third is the beginning of berry ripening.

**Sample preparation:** leaves of grape plants were selected from the middle part of the vine, in the morning from 8 to 9 o'clock. Which are on one side of the row, well lit, and not shaded by other leaves. They were placed in dark plastic bags, without access to light, and after 2 – 3 hours analyzed in the laboratory.

**Number of samples analyzed:** 300 leaves for 2 years.
**Number of repeated analyses:** leaves of 10 grape varieties in five repetitions.
**Number of experiment replication:** three times during the growing season, for two years.

## Statistical Analysis

Data from the device was transferred to a PC, where it was interpreted by the program "Floratest" in Microsoft Excel 2016.

Using correlation analysis, the strength of the connection between meteorological elements for the years of the field experiment and indices of photosynthesis efficiency in the leaves of the studied grape varieties was calculated. The influence of the factor by the correlation coefficient is weak ≤0.29, moderate: 0.30 – 0.49, noticeable: 0.50 – 0.69, high: 0.70 – 0.89, very high: 0.90 – 0.99. Statistical processing was performed in Microsoft Excel 2016 in combination with XLSTAT.

## RESULTS AND DISCUSSION

According to agro-climatic zoning, the study area according to the average long-term data belongs to the northern part of Forest-Steppe (HTI 1.0 – 1.3, the sum of active temperatures of 10 °C and more is 2700 °C). Several scientists from Ukraine and Europe were engaged in similar scientific researches, they carried out the scientific work, both in the open and in the closed ground in various climatic zones **(Conradie et al., 2019; Malu, Sharma and Pearce, 2017; Schwerz et al., 2017; Alameldin, 2017)**. Over the years of the field experiment during the growing season, various weather conditions developed, which allowed assessing the degree of their influence on the functional activity of the leaves of grape plants in certain phases of development of the latter. In 2019, during the growing season, the hydrothermal coefficient fluctuated in the range of 0.8 – 1.2, the lowest being at the end of summer. The sum of active temperatures of 10 °C and more (Σ act t ≥10 °C) during the growing season was 3317 °C. The weather of 2020 differed from 2019 by less heat accumulation in the spring and summer and more precipitation (HTI 1.6 – 3.6). According to Σ act t ≥10 °C the terms of the study were almost the same (2020 – 3370 °C), which is on average 644 °C more than the climatic norm.

In 2019, from the beginning of the growing season to the phase "the inflorescence is visible" Σ act t ≥10 °C was 577 °C, in 2020 – 522 °C, but its accumulation occurred 12 days later, compared with 2019. In the phase "the beginning of ovary growth" Σ act t ≥10 °C was 1300 °C, which in 2019 was recorded 11 days earlier compared to 2020 (1308 °C). In 2019, from the beginning of the vegetation to the phase "the beginning of berry ripening" Σ act t ≥10 °C was equal to 1906 °C and occurred 12 days earlier than in 2020 (1944 °C).

A comparative analysis of precipitation in the years of the study showed that in 2020 in all three terms of measuring the amount of precipitation was higher by 64, 53, and 48%, respectively. In May 2020, precipitation was 3.2 times more than in 2019 and 3.1 times more than the climatic norm. June precipitation of 2019 and 2020 accounted for only 62% of the long-term norm, for July the situation with humidity was also different: in 2020 precipitation was almost the norm – 92%, and in 2019 only half of the climatic norm. In August, the amount of precipitation in 2019 was 75%, in 2020 – 45% of long-term values.

After the onset of light influence on the previously adapted to the dark leaf of the plant, the fluorescence intensity of chlorophyll changes significantly during the measurement period. The time dependence of ICF has the characteristic appearance of a curve with several maxima. The shape of this curve reflects the changes that occur in the photosynthetic apparatus of the plant during its adaptation to different environmental conditions.

According to **Kornyeyev (2002)**, the level of chlorophyll fluorescence emitted by PS II complexes with "open" reaction centers depends on the loss of excitation energy during its migration through the pigment matrix of light-harvesting complexes.

**Table 1** A characteristic of the functional state of grape leaves according to $F_0$ and $F_P$ (c. u.) NULES of Ukraine, 2019 – 2020.

| Varieties | $F_0$ | | | $F_p$ | | |
|---|---|---|---|---|---|---|
| | according to phenological phases | | | | | |
| | inflorescence is visible | beginning of ovary growth | beginning of berry ripening | inflorescence is visible | beginning of ovary growth | beginning of berry ripening |
| Aromatnyj | 264ab | 238 a | 226 a | 1506a | 987 a | 1309 a |
| Zagadka | 243ab | 270ab | 245ab | 1520a | 1166ab | 1480ab |
| Ilichevskij rannij | 256ab | 243ab | 226 a | 1662ab | 1208ab | 1477ab |
| Kardishah | 253ab | 269ab | 230 a | 1600ab | 1202ab | 1333ab |
| Kishmish tairovskij | 269ab | 282 b | 274 b | 1694ab | 1379 b | 1558 b |
| Kometa | 274b | 293bc | 270b | 1763ab | 1302 b | 1606 b |
| Muskat odesskij | 235a | 240 a | 261b | 1523 a | 1141ab | 1474ab |
| Persej | 286 b | 272 b | 251ab | 1768 b | 1310 b | 1541 b |
| Shkoda | 270ab | 318 c | 253ab | 1669ab | 1347 b | 1469ab |
| Yarilo | 272 b | 274 b | 254ab | 1653ab | 1222 b | 1461ab |

Note: Different letters indicate values that differ significantly according to the 5% level of significance (for all tables).

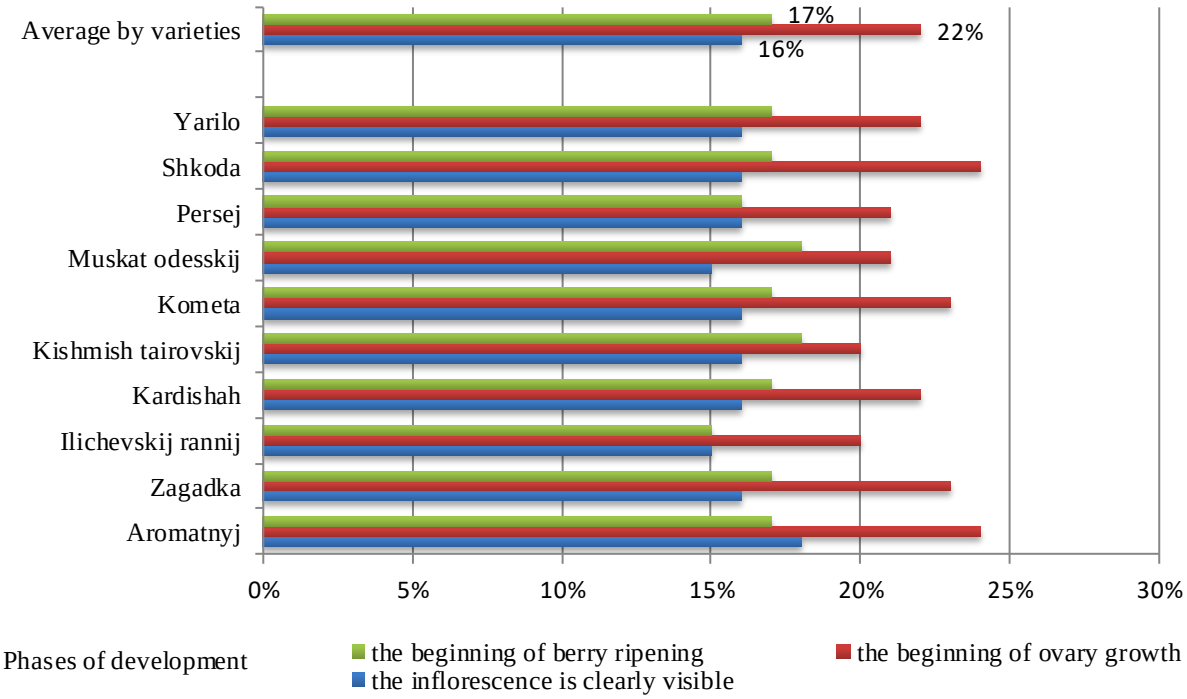


**Figure 2** The level of energy use for the synthesis of organic matter ($F_0/Fp$) at different phases of plant development of the studied grape varieties.

In active photosynthesis, when all reaction centers are in the open operating state, in low light conditions, about 3% of the absorbed light energy is not used in the process of photosynthesis. But the authors of the following scientific papers argue that in active photosynthesis when all reaction centers are in normal light, about 10% of absorbed light energy is not used in photosynthesis, which can have a significant impact on the yield and quality of grapes **(Palamarchuk et al., 2020; Drenjančević et al., 2017)**. As a rule, in conditions of cultivation favorable for the plant, the value of $F_0$ is insignificant which indicates the active use of energy of absorbed light by cells. At different phases of development of grape bushes, the background level of chlorophyll fluorescence in leaves varied in the range of 235 – 286, 238 – 318, and 226 – 274 c. u. (Table 1).

The more efficient use of quantum energy of absorbed light by $F_0$ in the leaves of most varieties was noted in the phase "the beginning of berry ripening". During the three periods of the study, the varieties Aromatnyj, Zagadka, Ilichevskij rannij, and Kardishah showed a lower content of chlorophyll molecules, which do not have a functional connection with the reaction centers (RC) **(Pascale et al., 2017; Martínez et al., 2018)**.

**Table 2** Characteristics of the functional state of grape leaves according to *Ki* and *Rfd* (c. u.). NULES of Ukraine, 2019 – 2020.

| Varieties | *Ki* | | | *Rfd* | | |
|---|---|---|---|---|---|---|
| | according to phenological phases | | | | | |
| | inflorescence is visible | beginning of ovary growth | beginning of berry ripening | inflorescence is visible | beginning of ovary growth | beginning of berry ripening |
| Aromatnyj | 0.822 a | 0.740 a | 0.825ab | 1.94 a | 1.80 a | 1.83 b |
| Zagadka | 0.836 a | 0.761ab | 0.834ab | 2.64 b | 1.79 a | 2.09bc |
| Ilichevskij rannij | 0.844 a | 0.792 b | 0.842 b | 2.61 b | 1.93 b | 1.94bc |
| Kardishah | 0.838 a | 0.771ab | 0.824 a | 2.73 b | 1.84ab | 2.01bc |
| Kishmish tairovskij | 0.841 a | 0,792 b | 0.823 a | 2.52 b | 2.01 b | 1.87bc |
| Kometa | 0.843 a | 0.771ab | 0.829ab | 2.77 b | 2.18ab | 2.21bc |
| Muskat odesskij | 0.845 a | 0.784 b | 0.823 a | 2.18ab | 1.50 a | 1.52 a |
| Persej | 0.833 a | 0.788 b | 0.835ab | 2.77 b | 2.02 b | 2.13 c |
| Shkoda | 0.837 a | 0.756ab | 0.825ab | 2.50 b | 2.04 b | 1.79ab |
| Yarilo | 0.831 a | 0.770ab | 0.824 a | 2.23ab | 1.79 a | 2.19 c |

**Table 3** Correlation between meteorological elements and intensity of photosynthesis of grape plants (according to *Ki* and *Rfd* indices). NULES of Ukraine, 2019 – 2020.

| Varieties | *Ki* | | | *Rfd* | | |
|---|---|---|---|---|---|---|
| | Meteorological elements | | | | | |
| | HTI | Σ act t ≥10 | Σ precipitation | HTI | Σ act t ≥10 | Σ precipitation |
| Aromatnyj | 0.19 | 0.22 | 0.46 | 0.53 | -0.12 | 0.30 |
| Zagadka | 0.16 | 0.21 | 0.38 | 0.37 | -0.49 | -0.19 |
| Ilichevskij rannij | 0.16 | 0.19 | 0.36 | 0.52 | -0.55 | -0.07 |
| Kardishah | 0.29 | 0.03 | 0.35 | 0.74 | -0.54 | 0.04 |
| Kishmish tairovskij | 0.38 | -0.17 | 0.23 | 0.41 | -0.63 | -0.36 |
| Kometa | 0.27 | 0.01 | 0.28 | 0.30 | -0.55 | -0.28 |
| Muskat odesskij | 0.36 | -0.14 | 0.25 | 0.50 | -0.60 | -0.29 |
| Persej | 0.03 | 0.36 | 0.35 | 0.51 | -0.51 | -0.18 |
| Shkoda | 0.18 | 0.01 | 0.14 | 0.50 | -0.59 | -0.09 |
| Yarilo | 0.23 | 0.14 | 0.42 | 0.42 | -0.07 | 0.17 |

Adaptive changes in the structure of the pigment complex to the lighting conditions because the *Fp* index is the most variable. The values of the maximum value of fluorescence depend on the intensity of the excited radiation and they are proportional to the total number of chlorophylls and inversely proportional to the density of the reaction centers. It is believed that at the point *Fp* at the maximum level of chlorophyll fluorescence photosynthesis is at a minimum one **(Kytaiev et al., 2005)**. Thus, the highest intensity of photosynthesis on average for all varieties was observed in the phase "the beginning of ovary growth", the lowest – "the inflorescence is visible". Analyzing in detail the *Fp* index by varieties, it was found that the intensity of photosynthesis was significantly higher in the second measurement period compared to the first, namely: for the variety Aromatnyj – by 34%; Zagadka – 23%; Ilichevskij rannij – 27%; Kardishah – 25%; Kishmish tairovskij – 18%; Kometa – 26%; Muskat odesskij – 25%; Persej – 26%; Shkoda – 19%; Yarilo – 26%, which in our opinion is due to insufficient insolation in the first measurement period. The process of photosynthesis under such conditions is limited by the intensity of light entering the plant **(Lukatkin et al., 2011)**.

According to long-term studies of the Laboratory of Plant Physiology of the IH NAAS, the optimal level of background fluorescence is not more than 20 – 25% of the maximum value of fluorescence (*Fp*). We found that for all studied varieties of grapes pigment complex functions actively in all phases of bush development (Figure 2).

In the phases "the inflorescence is visible" and "the beginning of berry ripening" the index $F_0/Fp$ fluctuates on the average on grades in the range – 16 – 17%, in a phase "the beginning of ovary growth" – 22%. We believe that this is due to the large amount of energy expended on the intensive growth of the fruit (ovary).

An effective way to monitor the impact of environmental stress on the plant is a calculated indicator – the fluorescence induction coefficient of chlorophyll (*Ki*), which shows the share of chlorophylls involved in photosynthesis, their total number and determines the efficiency of the light phase of photosynthesis. According to the obtained data (Table 2), the *Ki* index in the leaves of grape plants in the first and third measurement periods ranged in the same range of 0.822 – 0.845 and 0.823 –

0.842 c. u. respectively, in the second – 0.740 – 0.792 c. u. Studies performed by **Lukatkin et al., (2011)**, indicate a high level of efficiency of photophysical processes near the reaction. Several scientists **(Hu et al., 2019; Conde et al., 2018; Kernaghan, Mayerhofer and Griffin, 2017)** have been engaged in similar scientific researches and found that the *Ki* index in the leaves of grape plants in the first and third measurement periods ranged in the same range of 0.810 – 0.838 and 0.813 – 0.822 s. u. respectively, in the second – 0.720 – 0.762 s. u.

The value of *Ki* is associated with changes in the efficiency of the process of photochemical quenching of chlorophyll fluorescence. This index of dark-adapted plants reflects the potential quantum efficiency of PS II, used as an indicator of photosynthesis productivity, the optimal value of which for most plant species, under saturating intensity of exciting light, does not exceed 0.83 **(Ivanova et al., 2021)**.

An increase of this index diagnoses the phenomenon of photoinhibition and testifies to the influence of stress factors on plants **(He et al., 1996; Sukhenko et al., 2019)**. At the phase "the inflorescence is visible" for grape leaves Ilichevskij rannij, Kishmish tairovskij, Kometa, Muskat odesskij indicator *Ki* was higher than the optimal value, but among varieties as to the value of *Ki* a significant difference was not found. At the phase of "the beginning of ovary growth," variety Aromatnyj as to this index differed significantly from most varieties. In the third measurement period, a significant difference in the value of *Ki* was among varieties Muskat odesskij, Kishmish tairovskij, Yarilo, Kardishah compared to the variety Ilichevskij rannij.

Thus, according to the fluorescence induction coefficient of chlorophyll (*Ki*), the potential productivity of grape plants of all varieties was at a very high level compared with data obtained by other analysis **(Zelenianska, 2009; Havryliuk et al., 2019; He et al., 2018; Bober et al., 2020)**.

The coefficient of adaptability or "viability index" (*Rfd*) we used to assess the impact of growing conditions on the state of the plant. Reduction of the calculated *Rfd* to 1.47 – 1.91 c. u. indicates the negative impact of growth conditions or the possible effect of stress on the efficiency of the Calvin cycle **(Lysenko et al., 2013; El Sayed et al., 2019; Shanina et al., 2019)**. In grape plants at the development phase, "the inflorescence is visible", the average viability index among varieties was 2.49 c. u., at the phase of "the beginning of ovary growth" there was a decrease to 1.89; at the phase "the beginning of berry ripening" – up to 1.96 c. u. Plants were cultivated on a well-lit area, on a vertical trellis, the access of the sun to all parts of the grape bush is very good, so, in our opinion, the efficiency of photosynthetic processes is generally high.

The efficiency of photosynthetic processes by the coefficient of adaptability of the variety Muskat odesskij indicates a low intensity of photochemical processes in the second and third measurement period; the decrease in *Rfd* was 30.3 – 31.2%. In our opinion, it is associated with a negative effect of anthracnose on the plant; Muskat odesskij variety is more susceptible than other varieties. The smallest changes in the viability index (5.7 – 7.2%) were determined for plants of the variety Aromatnyj.

To establish the influence of weather conditions during the growing season on the processes of photosynthesis in grape leaves, we used the indices of hydrothermal coefficient, the sum of active temperatures, the amount of precipitation (*Ki* index), except for Kishmish tairovskij, Muskat odesskij and Persej, for which a greater influence (correlation coefficient r = 0.36 – 0.38, moderate influence) was noted in comparison with other grape varieties (Table 2).

In general, the potential productivity of grape plants (according to *Ki* index) was weakly and moderately dependent on weather conditions during the growing season. Among the meteorological elements, the greatest influence on the efficiency of the light phase of photosynthesis in the leaves of the studied grape plants was the amount of precipitation (moderate).

The influence of weather conditions on the value of the coefficient of adaptability (*Rfd*) was greater than the value of *Ki.* The high influence of HTI on the efficiency of dark photochemical processes in the leaves of the variety Kardishah, for most varieties of grapes it was noticeable (correlation coefficient r = 0.50 – 0.69), only for varieties Zagadka, Kishmish tairovskij, Kometa and Yarilo it was moderate (correlation coefficient r = 0.30 – 0.49). The efficiency of dark photochemical processes in the leaves of most grape varieties significantly (r = 0.50 – 0.69) depended on Σ act t ≥10 °C during the growing season, the least dependence on these factors was observed among the varieties Aromatnyj and Yarilo. The amount of precipitation had a weak effect on the quantum efficiency of photosynthesis in the leaves of most grape varieties, but it had a moderate effect on the efficiency of photosynthesis in the leaves of Aromatnyj and Kishmish tayirovskij varieties.

## CONCLUSION

Analysis of changes in the induction of chlorophyll fluorescence (ICF) of leaves revealed the more efficient use of quantum energy of absorbed light by $F_0$ in the leaves of most varieties was noted in the phase "the beginning of berry ripening". During the three periods of the study, the varieties Aromatnyj, Zagadka, Ilichevskij rannij, and Kardishah showed a lower content of chlorophyll molecules, which do not have a functional connection with the reaction centers (RC). According to the *Fp* index the highest intensity of photosynthesis on average for all varieties was observed in the phase "the beginning of ovary growth", the lowest one was at "the inflorescence is visible" phase. The potential productivity of grape plants by the coefficient of ICF for all varieties was at a very high level and had a weak and moderate correlation with weather conditions during the growing season. The hydrothermal coefficient and Σ act t ≥10 °C had a weak effect on the efficiency of the light phase of photosynthesis and a noticeable one (correlation coefficient r = 0.50 – 0.69) on the efficiency of dark photochemical processes in the leaves of most grape varieties. Weather conditions during the vegetation in 2019 – 2020 differed from the average long-term data by a greater accumulation of heat (on average by 644 °C). The influence of meteorological elements of the spring-summer

period on the onset of the vegetation phases, which in 2019 took place on average 12 days earlier than in 2020, was noted.

These data show that despite the differences in the onset of the vegetation phases and weather conditions during 2019 and 2020, the processes of photosynthesis in grape leaves are at a high level. That indicates a high potential for plant adaptation to the conditions of the northern part of the Forest-Steppe of Ukraine.

In conditions of local and global climate change, it is important to study the adaptive properties of plants to new soil and climatic conditions. In the future, it will allow expanding and changing the areas of cultivation of grapes plants. So that to provide with enough grapes, the population of Ukraine and the world.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# CHARACTERISTICS OF GELATIN FROM SKIN AND BONE OF SNAKEHEAD (*CHANNA STRIATA*) EXTRACTED WITH DIFFERENT TEMPERATURE AND TIME

***Rosmawati, Abu Bakar Tawali, Muhammmad Irfan Said, Wahidu Zzaman, Rovina Kobun, Nurul Huda***

## ABSTRACT

This study aimed to determine the physicochemical properties of the skin and bone of snakehead fish as a potential source of gelatin through extraction at different temperatures and times compared to commercial gelatin. Extraction of skin and bones of wild snakehead fish (*Channa striata*) at different temperatures (50, 60, 70 ºC) and time (12,18, 24 hours). The pre-treatment process used a 0.1 M Ca (OH) 2 (1:6 w/v) immersion solution for 1 h and continued with 0.05 M citric acid (1:6 b/v) for 5 h. Before pre-treatment, the minerals of bones were degreased with 3% HCL solution for 24 hours. The results of the analysis showed that the differences in raw materials, temperature, and extraction time had a significant effect ($p$ <0.05), as well as interactions among treatments ($p$ <0.05) on the yield and gel strength. The yield of skin and bone tended to increase with extending extraction temperature and time, while the highest gelatin strength was found at 60 °C for 12 hours on the skin and 24 hours for the bone. The best gelatin was accomplished based on the highest performance of gel strength on skin and bones and compared to bovine commercial gelatin. The amino acids of the three types of gelatin showed higher levels of glycine and proline than other types of amino acids. Based on the total residues of each amino acid, skin gelatin and bone gelatin showed more dominant hydrophobic properties than hydrophilic properties, in contrast to bovine commercial gelatin. The three types of gelatin showed diverse chemical compositions, emulsion activity index, emulsion stability, water resistance, and fat binding capacity, which was reflected to be closely related to the source of the raw material and its amino acid content. The FTIR results showed that the extracted snakehead fish skin and bones have the potentiality to be used as gelatin equivalent to a commercial one.

**Keywords:** skin gelatin; bone gelatin; gel strength; snakehead gelatin; amino acid; FTIR

## INTRODUCTION

Collagen is one of the important proteins that make up the connective tissue in animal bodies, which is found in skin, bones, teeth, scales, and tendons **(Lv et al., 2019; Schmidt et al., 2016)**. Each of the collagen characteristics is relatively different, depending on the cross-linking structure and the triple helix molecule. Collagen is the raw material for gelatin, through a partial hydrolysis process **(Montero and Gómez-Guillén 2000; Huda et al., 2013a)**. The very wide application of gelatin has prompted the search for sources of collagen for the raw material for gelatin, mainly from pigs and cows, and to a lesser extent from other animals such as chicken, duck, goat, and fish **(Ahmad and Benjakul, 2011; Nurkhoeriyati, Huda and Ahmad, 2011; Huda et al., 2013b; Kuan et al., 2017)**. Changes in social behavior, religious awareness, and understanding the importance of health become selective qualifications for the use of collagen raw materials for gelatin production.

Recently, the global market for halal gelatin has been steadily in demand. One source of raw materials that many researchers are currently exploring is the skin and bones of fish **(Karim and Bhat, 2009; Herpandi, Huda and Frederick, 2011)** because it contains high collagen so that it has the potential to be used as a source of gelatin **(Abd, Mirghani and Adam, 2013; Mirzapour, Moosavi-Nasab and Aminlari, 2018)**. Fish collagen is a potential byproduct of the fisheries industry and is allegedly able to accommodate the needs of gelatin for all ethnicities **(Gomez-Guillen et al., 2011)**. According to **Le Thi et al. (2020)**, the advantages of fish gelatin over mammalian gelatin are cheaper production costs, water solubility, and better thermal stability.

Fish skin and bones which are the remains of the fishing industry **(Cho et al., 2006; Herpandi, Huda and Frederick, 2011)** estimated at 30 to 41.5% of the total fish weight **(Gómez-Guillén et al., 2002; Stevens et al., 2018)**. The remaining products of the fish industry that have not been used optimally are the skin and bones of snakehead fish **(Rosmawati et al. 2018a)**. Snakehead fish are carnivores in freshwater and are an alternative source of animal protein in most parts of Indonesia which have a muddy area typology. Various studies have shown the potential of snakehead fish as a source of albumin which is proven to have a healing effect while restoring body health **(Haniffa et al., 2014)**. This is an opportunity for more intensive fish farming. However, high production has an impact on increasing waste products as a result of large-scale fish utilization. On the other hand, directing the potential of the skin and bones of snakehead fish as valuable materials is important so that they are not wasted. Thus, these efforts can increase the economic value of the skin and bone of snakehead fish.

Several types of solutions have been used in pretreatment for processing collagen extraction into gelatin. The alkaline solution usually helps to release non-collagen proteins, while the acid solution causes the process of breaking or weakens the bonds between the collagen molecule and other protein molecules, thereby facilitating the extraction process. Apart from solution, temperature and extraction time can affect the quality and quantity of gelatin produced **(Kittiphattanabawon et al., 2010; Kittiphattanabawon et al., 2012; Kołodziejska et al., 2008; Montero and Gómez-Guillén, 2000; Sanaei et al., 2013)**.

This study aimed to determine the physicochemical characteristics of the skin and bones of snakehead fish as potential sources of gelatin through extraction at different temperatures and times compared to commercial gelatin.

## Scientific hypothesis

The study hypothesizes that different temperatures and times will affect the physicochemical characteristics of gelatine extracted from the skin and bones of snakehead fish.

## MATERIAL AND METHODOLOGY

### Samples

Wild Snakehead (*Channa striata*) fish of between 600 to 700 g with a length of approximately 42.07 ±0.75 cm were obtained from Bili Bili Dam, Gowa South Sulawesi, Indonesia (Figure 1).



**Figure 1** Wild snakehead fish (Channa striata) used in the experiment.

**Chemicals**

The analytical grade chemicals $Ca(OH)_2$, Citric acid, HCl, KBr (Merck, Germany) were used in the experiments. The commercial gelatin used was bone bovine gelatin, 150 g gel strength (Green Valley, Global Capsule Ltd. Dhaka 1000-Bangladesh).

**Instruments**

The instrument used in this study were oven (Memmert UM 400, Germany), dry blender (Miyako 152PF, PT. Kencana, Indonesia), weighted (Mettler Toledo, J81603-C/FACT, Germany), water-bath (Memmert W350, Germany), TAXT2 Texture Analyzer (Stable Micro System, UK), ultra-performance liquid chromatography (UPLC, ACQUITY UPLC-H Class, Waters, USA), vortex (IKA Labortechnik VF2, Germany), homogenizer (IKA T25 digital-ULTRA TURRAX, Germany), spectrophotometer (Thermo Fisher Scientific 4001/4, Genesys VF2, Germany), centrifuged (High speed refrigerated micro centrifuge, Tomy Mx-305) and Fourier Transform Infrared Spectrophotometry (FTIR) (IR Prestige-21, FTIR-8400, Shimadzu, Japan).

**Description of Experiments**

***Extracting Gelatin***

*Process for Pretreatment*

The pre-treatment process refers to **Saida et al. (2011)**. The skin or bone was soaked with 0.1 M $Ca(OH)_2$ (1:6 b/v) for 1 h in the early stages of the extraction process, washed vigorously with tap water three times, and then drained with a plastic filter. The next step was to soak the skin or bone with 0.05 M citric acid (1:6 b/v) for 5 h and then washed it with tap water three times again and rinsed it with a plastic filter.

***Method of Extraction and Analysis***

Using distilled water (1:6 b/v) at various temperatures and times, each 50, 60, and 70 °C for 12, 18, and 24 h in a water-bath on water-bath (Memmert W350, Germany), the snakehead skin or bone that had been processed by pretreatment was then extracted. Using four-folded cheesecloths, the extraction results were filtered. The liquid gelatin was dried in an oven (Memmert UM 400, Germany) for 48 h at 60 °C. To assess the yield of gelatin, the gelatin sheet was finely ground with a dry blender (Miyako 152PF, PT. Kencana, Indonesia) and weighted (Mettler Toledo, J81603-C/FACT, Germany), then stored in plastic Polyethylene at room temperature for further analysis.

The best method was used to obtain snakehead fish gelatin for the skin and bone at varying temperatures and extraction times. As the finest measure of gelatin consistency, the authors assign gel strength parameters. The selected skin and bone gelatin will be further characterized and compared to bovine commercial gelatin (BCG) gel strength. All analyses were conducted in triplicate.

*Gelatin Yield*

The gelatin yield (based on the wet weight base) was determined as the ratio between the dry weight of the gelatin and the total weight of the wet skin/bone base **(See et al. 2010)**.

*Gel Strength of Gelatin*

With some changes, the gel strength determination was based on **GMIA (2012)**. By mixing 7.5 g of gelatin and 105 mL of distilled water, a 6.67% (b/v) gelatin solution was prepared. To allow the gelatin to absorb water and swell, the mixture was left at room temperature for 30 minutes. The mixture was then heated in the water bath at 60 °C for 30 min to accomplish the gelatin solubility to obtain gelatin solution, which was then stored for 17 ±1 h at refrigerator temperature (4 ±1 °C). The strength of gel was determined using a TAXT2 Texture Analyzer (Stable Micro System, UK) with a 12.7 mm diameter plunger. The velocity of the plunger penetrates the gel at 10 $mm.min^{-1}$ at a maximum force of 0.5 $mm.sec^{-1}$ at a penetration depth of 4 mm. The result of the maximum force reading provided on the gel by the plunger was expressed in grams. Measurements were carried out three times.

*Proximate Analysis*

Using the methods of **AOAC (2005)**, the proximate composition of snakehead gelatin and bovine commercial gelatin was determined. Using the gravimetric method, moisture content was determined. For the determination of crude protein content (conversion factor of 5.55 × N), the Kjeldahl method was used. By using the Soxhlet method, lipid content was determined. The ash content of 16 h at 550 °C was determined by incineration.

*Amino Acid Composition*

According to **Nollet (2004)**, the amino acid composition was determined. Utilizing ultra-performance liquid chromatography (UPLC, ACQUITY UPLC-H Class, Waters, USA), amino acids were analyzed. The sample (0.1 g) was hydrolyzed with 5 mL of 6 N HCl and heated for 22 h at 100 °C. The prepared solution contained 500 µL filtrate, 40 µL AABQ, and 460 µL distilled water. The solution (10 µL) was incubated at 550 °C for 10 minutes after the addition of AccQ-Fluor Borate and 20 µL of Flour-A reagent and then injected into the UPLC testing method.

*Emulsifying Properties Determination*

A turbidimetric method based on **Pearce and Kinsella (1978)** with minor modification was used to determine the emulsion activity index (EAI) and emulsion stability index (ESI) of gelatin. A solution of 6 mL of gelatin with a concentration of 2% (w/v), an oil solution, and a gelatin solution combined with a vortex (IKA Labortechnik VF2, Germany) for 10 s was added with soy oil (2 mL). The mixture was then homogenized using a homogenizer (IKA T25 digital-ULTRA TURRAX, Germany) at 20,000 rpm for 1 min at room temperature (±28 °C) to prepare the emulsion. The emulsion, to acquire 1/100 dilution, was diluted with 1 $g.L^{-1}$ SDS. Using a spectrophotometer (Thermo Fisher Scientific 4001/4, Genesys VF2, Germany), absorption was measured against 1 $g.L^{-1}$ SDS at 500 nm. After emulsification, the absorbance was measured immediately at (A0) and 10 minutes (A10). The equations of **Ahmad and Benjakul (2011)** were used to measure EAI and ESI, as follows:

IAE ($m^2.g^{-1}$) = TDF / løC
ESI (min) = A0 / ΔA × Δt
Where, T = 2 × 2.303 × A500 × DF; DF = Dilution factor; l = long cuvette (m); ø = fat dispersion (0.25); C = gelatin concentration ($g.mL^{-1}$); A500 = wavelength; A0 = Wavelength at 0 min; ΔA = A0 - A10; Δt = 10 min.

*Water Holding and Fat Binding Capacity*

According to **Cho et al. (2006)**, water holding capacity (WHC) and fat binding capacity (FBC) are measured with partially modified input. Half a gram of gelatin was inserted and weighed (tube and gelatin) into a disposal centrifuge tube. A total of 10 mL of water or 10 mL of sunflower oil was applied to the tube, then left for 1 h at room temperature to swell and dissolve the gelatin in the water/oil. At 20,000 rpm for 20 min, the gelatin solution was then centrifuged (High speed refrigerated micro centrifuge, Tomy Mx-305). The upper step was removed and the centrifuge tube on the filter paper dried for 30 minutes. Based on **Shyni et al. (2014)**, the WHC and FBC gelatin values were measured, i.e. the difference between the initial volume of distilled water/sunflower oil applied to the gelatin sample and the supernatant volume was determined and the results recorded were absorbed as ml of water/oil per gram of gelatin sample.

*FTIR*

Fourier Transform Infrared Spectrophotometry (FTIR) (IR Prestige-21, FTIR-8400, Shimadzu, Japan) with a wavenumber of 4000 to 650 $cm^{-1}$ is used in the analysis process. A pellet type preparation process, KBr (Potassium Bromide), was the tool used. Snippets (0.1 – 2% by weight) were pounded together with KBr, then pressed to achieve a pellet form at a pressure of 8-20 tons. Under an infrared lamp, the KBr was established in a dry state to prevent condensation of vapors from the atmosphere and then to discharge the water. Pellet samples were then inserted on an infrared spectrophotometer into the sample site, and the results of the spectrum would be read via a computer monitor.

**Sample preparation:** The processing of fish refers to the **See et al. (2010)** procedure. About 58 of Snakehead fish have been manually scaled and all body parts have been removed. The skin (6 kg) and bone (10 kg) of fish separately were both gathered and washed, then covered with a plastic bag and stored at -20 °C for a maximum of 2 months. The skin and bones of the thawed snakehead fish were prepared before the pre-treatment process in triplicate experiments. Small pieces of fish skin measuring approximately 2 to 3 cm in length and width were taken. With tap water, the skin was washed three times and then drained using a plastic filter. To promote the release of meat and fat still attached to the bone, the thawed snakehead bone was boiled (1:3 w/v) at 60 °C for 30 minutes. The bone was washed, and then the joints were separated using pliers. The process of bone mineralization was accomplished to release the potent inorganic elements bound with collagen in 3% (1:6 w/v) HCL for 24 h by soaking the bone fragments.

**Number of samples analyzed:** Number of samples analyzed were 54 samples which consist of 27 samples of skin gelatin, 27 samples of bone gelatin.

**Number of repeated analyses:** All analyses were conducted in triplicate.

**Number of experiment replication:** Sample of skin and bones were prepared in triplicate experiments.

## Statistical analysis

The experimental data were tested using one-way analysis of variance (ANOVA) based on a completely randomized factorial design, namely yield, gel strength, proximate composition, amino acid composition, emulsifying properties, water holding and fat binding capacity of the skin and bone gelatin. The validity of diversity was checked using the Duncan test. The data was running using SPSS software (IBM Corporation, US).

# RESULTS AND DISCUSSION

## Snakehead gelatin yield

The yields were an important factor that was always considered in the conversion of collagen to gelatin because it was related to the effectiveness of the process applied and the economic benefits. The yield produced in the process of transforming collagen from the skin and bone of snakehead fish as raw material into gelatin at different extraction temperatures and times showed significant differences ($p$ <0.05), as shown in Table 1.

The yield of snakehead gelatin was influenced by the type of raw material, temperature, and extraction time. According to **Karim and Bhat (2009)**; **Kołodziejska et al. (2008)**, that the conversion of collagen to gelatin can be influenced by processing parameters, such as the nature of the raw material and the method of extraction, including the pre-treatment conditions, temperature, and time. The higher the extraction temperature, the yield of SSG and SBG significantly increased. Likewise, the longer the extraction time, the yield of the two types of gelatin also significantly increased. Gelatin yield was influenced by differences in the type of raw material, temperature, and extraction time.

However, extraction at a temperature of 50 °C with different extraction times had not obtained an increase in the SBG yield ($p$ >0.05). **Sims and Bailey (1992)** explained that skin collagen from immature animals contained an intermediate crosslink of dehydroxylysinonorleucine and when it reached adulthood it was converted into trivalent cross-linked of histidinohydroxylysinonorleucine which was more stable, while collagen in bones contained hydroxylysinocetonorleucine which was then converted to pyridinoline. According to **Bailey, Paul and Knott (1998)**, pyridinoline was more stable to heating. This possible phenomenon was one of the factors that cause the SBG yield to be lower than SSG due to the slower conversion process of collagen in bones compared to skin.

There were significant differences due to the interaction of raw materials and time; and the interaction of raw materials and temperature, as well as the interaction between temperature and time; and the interaction of raw materials, temperature, and time on gelatin yield ($p$ <0.05). Table 1 showed that SSG was significantly higher than

SBG at the same temperature and extraction time. During the extraction process, temperature affects the solubility rate of collagen, where high temperatures caused the collagen bond structure to open and result in the breaking of the bonds from the collagen protein molecule. Extraction time was the most significant factor affecting gelatin yield **(Ahmad and Benjakul, 2011; Hanjabam et al., 2015)**. The higher temperatures used the gelatin yield tended to increase. During the extraction process in the hydrogen bonds, the heating process in a water bath which stabilized the triple helix of the parent collagen is damaged, causing a transition from helix to coil resulting in the conversion of collagen into gelatin solution **(Benjakul, Kittiphattanabawon and Regenstein, 2012)**.

In general, the yield of SBG was relatively lower than SSG, either due to a single factor of raw material, temperature or time, or the interaction between treatments. The skin was composed of organic material that can easily change its character due to chemical treatment rather than bone. This was because the bone structure consists of organic and inorganic composites **(Szpak, 2011)**. The demineralization process of bone to ossein at the beginning of the pre-treatment process causes the release of several minerals, as suggested by **Muyonga, Cole and Duodu (2004a)** and **Muyonga, Cole and Duodu (2004b)**. The demineralization process causes the release of mineral components that fill the spaces between collagen molecules in the bone matrix, allowing easier conversion of collagen to gelatin. The yields of SSG and SBG were 18.25 ±0.26% and 8.19 ±0.17%, respectively, higher than the results obtained by **Muyonga, Cole and Duodu (2004b)** on the skin and bones of *Lates niloticus* fish as much as 12.3 ±2.1% to 16.0 ±0.3% and 1.3 ±1.0 to 2.4 ±0.7%, respectively.

## Snakehead Gelatin Gel Strength

The main parameter of gelatin was gel strength, which was used as a method for measuring the impact of production factors and optimizing the quality of the final product. According to **Karim and Bhat (2009)**, raw material characteristics, temperature, and extraction time play an important role in gelatin gel strength. There was a difference in gel strength between the skin and snakehead fish bones extracted at different temperatures and times ($p$ <0.05). The effect of the gel strength gelatin of skin and bone snakehead fish on the temperature treatment and extraction time were presented in Table 1.

The interaction of treatment combinations between raw materials and temperature; raw materials and time; and between raw materials, temperature and time had different effects on the gel strength of snakehead fish gelatin ($p$ <0.05). The existence of this interaction showed that there was a different gel strength on SSG and SBG. The longer the extraction time, the gel strength of SSG tends to decrease but vice versa in SBG was tending to increase.

**Table 1** The characteristics of skin and bone of gelatin snakehead using different temperatures and extraction times.

| Item | Yield (%) | Gel Strength (g) |
|---|---|---|
| SB1 (50 ºC, 12 h) | 2.57 ±0.26$^{a}$ | 41.37 ±7.42$^{a}$ |
| SB2 (50 ºC, 18 h) | 2.63 ±0.37$^{a}$ | 55.37 ±5.12$^{a}$ |
| SB3 (50 ºC, 24 h) | 3.19 ±0.32$^{a}$ | 64.67 ±4.68$^{ab}$ |
| SB4 (60 ºC, 12 h) | 3.78 ±0.17$^{ab}$ | 48.00 ±5.32$^{a}$ |
| SB5 (60 ºC, 18 h) | 4.79 ±0.12$^{b}$ | 144.77 ±20.02$^{cd}$ |
| SB6 (60 ºC, 24 h) | 6.46 ±0.32$^{c}$ | 239.53 ±20.69$^{gh}$ |
| SB7 (70 ºC, 12 h) | 8.19 ±0.17$^{d}$ | 104.23 ±16.35$^{bc}$ |
| SB8 (70 ºC, 18 h) | 8.91 ±0.23$^{d}$ | 189.83 ±28.83$^{ef}$ |
| SB9 (70 ºC, 24 h) | 8.90 ±0.10$^{d}$ | 173.80 ±27.77$^{de}$ |
| SS1 (50 ºC, 12 h) | 14.22 ±0.18$^{e}$ | 225.33 ±7.67$^{fg}$ |
| SS2 (50 ºC, 18 h) | 16.06 ±1.09$^{f}$ | 126.40 ±13.22$^{c}$ |
| SS3 (50 ºC, 24 h) | 16.30 ±0.32$^{f}$ | 109.63 ±23.58$^{c}$ |
| SS4 (60 ºC, 12 h) | 14.33 ±0.07$^{e}$ | 278.37 ±22.28$^{h}$ |
| SS5 (60 ºC, 18 h) | 16.64 ±0.07$^{f}$ | 253.77 ±51.66$^{gh}$ |
| SS6 (60 ºC, 24 h) | 16.70 ±0.94$^{f}$ | 108.23 ±27.10$^{c}$ |
| SS7 (70 ºC, 12 h) | 16.83 ±2.16$^{f}$ | 246.13 ±47.22$^{gh}$ |
| SS8 (70 ºC, 18 h) | 18.25 ±0.26$^{g}$ | 173.23 ±16.16$^{de}$ |
| SS9 (70 ºC, 24 h) | 18.70 ±0.50$^{g}$ | 108.00 ±21.40$^{c}$ |

Note: Different superscripts in the same column indicate a significant difference ($p$ <0.05). Values are presented as mean ±*SD* (n = 3). SB = snakehead bone; SS = snakehead skin.

**Table 2** The chemical composition of snakehead skin gelatin (SSG) and snakehead bone gelatin (SBG) from the extraction process used the best temperature and extraction time compared to bovine commercial gelatin (BCG).

| | Moisture | Protein | Fat | Ash |
|---|---|---|---|---|
| SSG | 7.46 ±0.28$^{b}$ | 83.41 ±0.56$^{b}$ | 0.31 ±0.01$^{b}$ | 1.14 ±0.04$^{a}$ |
| SBG | 3.50 ±0.20$^{a}$ | 93.12 ±0.50$^{c}$ | 1.05 ±0.05$^{c}$ | 1.33 ±0.07$^{b}$ |
| BCG | 13.70 ±0.40$^{c}$ | 76.99 ±0.93$^{a}$ | 0.03 ±0.00$^{a}$ | 1.93 ±0.04$^{c}$ |

Note: Different superscripts in the same column indicate a significant difference ($p$ <0.05). Values are presented as mean ±*SD* (n=3); SSG = Snakehead skin gelatin; SBG = snakehead bone gelatin; BCG = Bovine commercial gelatin.

The same properties were found in the gel strength of *Aluterus monoceros* skin gelatin **(Ahmad and Benjakul, 2011)** which tended to decrease with increasing extraction time. There was an increase in gel strength when the SSG was heated between 50 °C and 60 °C but decreased when the extraction temperature was increased by 10 degrees, while the gel strength of SBG tended to increase as the extraction temperature increased. It seems that the snakehead bone takes a relatively long extraction time to match the gel strength of snakehead skin. Compared to snakehead skin, the relatively long extraction process on the bones was thought to be related to the different chemical compositions of the two materials.

The skin consists of organic material and very little inorganic material, but bones contain organic material with a high proportion of inorganic material **(Olszta et al., 2007; Rosmawati et al., 2018a; Szpak, 2011)**. Demineralization of bone before pretreatment and extraction was an effective method for releasing inorganic materials **(Muyonga, Cole and Duodu, 2004a; Muyonga, Cole and Duodu, 2004b; Ranasinghe et al., 2020)**.

Based on Table 1 above, the shown that SSG and SBG have relatively the same gel strength when each extracted at different temperature and time combinations, SS4 (60 °C, 12 h) vs SB6 (60 °C, 24 h). Skin and bone have structural differences and cross-linkages **(Sims and Bailey, 1992)** so that different gel strengths can occur under the same extraction conditions. The extraction process allows the chain to break, the shorter the chain, the lower the gel strength. The temperature and extraction time contribute to the breaking of this chain. This is inseparable from the influence of the structure, sequence, and configuration of amino acids **(Kittiphattanabawon et al., 2012)**. The best gel strength was found in SSG extracted at 60 °C for 12 hours, while SBG at the same temperature with 24 hours extraction time was 278.37 ±22.23 g and 239.53 ±20.68 g, respectively. The gel strength of SSG and SBG was higher than BCG, namely 150 g (according to product label).

## Chemical Composition

The chemical composition of SSG and SBG compared with commercial bovine gelatin (BCG) was shown in Table 2. Analysis of variance showed the type of gelatin had a significant effect ($p$ <0.05) on moisture, protein, fat, and ash content.

The water content of the three types of gelatin showed significant differences ($p$ <0.05), where the water content of SBG was lower than SSG and BCG. The difference in gelatin water content was influenced by differences in raw materials and extraction processes used. The maximum level of gelatin water content based on the **Indonesian National Standard (SNI, 1995)** was 16%, while the **British Standard Institution (BSI, 1975)** regulates maximum water content between 13 to 14%. Based on this, the snakehead gelatin has fulfilled the standard regulations imposed.

The protein content of SSG was lower than SBG and BCG ($p$ <0.05). This difference may be related to the water content which tends to be low on SBG. Protein levels according to **Pranoto, Marseno and Rahmawati (2011)** can be used as a benchmark for evaluating the purity of gelatin. Protein levels in gelatin do not have a standard, but the levels can vary depending on the source of the raw material used. Different methods of extraction and pretreatment will affect the protein content obtained. The protein content of SBG was higher than SSG, in contrast to the results of research by **(Muyonga, Cole and Duodu, 2004a)** on the skin and bone (*Lates niloticus*) which produced a higher protein range in the skin (87.4 ±5.6 to 88.7 ±2.4) than bone (82.0 ±2.1 to 82.9 ±4.3) as was the case with **Wulandari, Suptijah and Tarman (2015)** and **Ratnasari et al. (2013)** in snakehead skin, were 96.21% and 87.27 ±0.78, respectively. Although **See et al. (2010)** were also only able to produce snakehead skin gelatin with lower protein levels, namely 75.63 ±1.05. Different contents of protein can be influenced by factors such as raw materials, pretreatment processes, and extraction methods **(Gomez-Guillen et al., 2011; Jamilah and Harvinder, 2002; Nagarajan et al., 2012)**.

The other important chemical compositions of gelatin were fat and ash. The highest SBG fat content was 1.05 ±0.05%. The relatively high-fat content in SBG compared to SSG can be attributed to the fat content of raw materials, where the fat of the snakehead bone was higher than the skin **(Rosmawati et al., 2018a)**. There was no definite standard for the minimum level of gelatin fat, but may be high levels of fat that can affect the quality of gelatin, including the shelf life. The fat content of commercial bovine skin gelatin was 0.23% **(Pranoto, Marseno and Rahmawati, 2011)**. This value was lower than the fat content of SSG but relatively higher than the fat content of BCG.

The gelatin ash content of the research results showed a fairly low value than **Indonesian National Standard (1995)**, which was less than 3.25%, whereas according to **GMIA (2012)** standard for type A gelatin which is about 0.3 to 2%. Ash content of SSG was lower than SBG and BCG, respectively ($p$ <0.05). The demineralization process before the extraction effectively has reduced the ash content of SBG.

The variation in chemical composition between gelatin occurs mainly due to differences in methods during the process of changing collagen to gelatin **(Gomez-Guillen et al., 2011)**, protein content in raw materials, including collagen levels, amount of dissolved components in the skin, initial treatment and washing process and extraction method used **(Abedinia et al., 2017; Jamilah and Harvinder, 2002)**. Meanwhile, according to **Irwandi et al. (2009)**, the chemical composition of gelatin produced can be related to the environment and type of fish feed.

## Amino Acid Composition

The amino acid composition of skin gelatin (SSG) and snakehead bone (SBG) compared to commercial gelatin (BCG) was presented in Figure 2. Glycine and proline were two types of the main amino acid **(Jamilah and Harvinder, 2002)** and were the highest in all types of gelatin were observed.

The characteristic feature of collagen and its derivatives was that the amino acid content of glycine and proline was higher than other amino acids **(Nagarajan et al., 2012)**, and glycine is the main amino acid **(Irwandi et al., 2009)**. However, specifically, the presence of proline and hydroxyproline were the main determinants of gelatin quality related to the gel strength, unfortunately,

hydroxyproline gelatin was not analyzed, and so it was rather difficult to predict gel strength really due to the presence of proline alone. It can be seen in Figure 2 that the highest proline levels of BCG then SSG and SBG, whereas the strength of the BCG gel was the lowest compared to SSG and SBG. The content of amino acids between fish and mammals was different, but its presence determines the stability of the gelatin conformation during the gel formation process **(Haug, Draget and Smidsrød, 2004)**. As **Gautieri et al. (2009)** emphasized that glycine was the most important amino acid in stabilizing collagen protein tertiary molecular structure whereas **Pranoto, Marseno and Rahmawati (2011)** suggested that the percentage of glycine can affect the binding properties of gelatin by water and lead to high values of gel strength and viscosity. The higher the temperature and the longer the extraction time affects the gel strength, this is assumed because the amino acids undergo cleavage in the peptide chain, and at the same time degradation occurs during the acid hydrolysis process **(Darragh et al., 1996)**.

Glutamate acid and alanine show levels that were not very different, and it seems that the levels of these two amino acids were higher in succession in the SSG than SBG and BCG. Arginine was also found to be quite abundant, respectively SBG, SSG, and BCG. The high levels of three amino acids may be linked to the source of the raw material of gelatin which was rich in these amino acids **(Rosmawati et al. 2018b)**. The other amino acids such as serine, aspartate acid, phenylalanine, threonine, leucine, isoleucine, methionine, tyrosine, valine, and histidine show an average of fewer than 5 residues/100 residues. Cysteine and tryptophan were not found in gelatin **(Haug and Draget, 2009)**, apart from the fact that both amino acids were present in sufficiently small amounts in their raw materials, these amino acids were thought to be susceptible to damage during the hydrolysis process. Amino acids were organic materials, that were bipolar by the presence of amine groups ($-NH_2$) and carboxyl (-COOH-), and side-chain groups (-R) that were specific and determine the level of reactivity of amino acids. Differences in side-chains cause differences in the physicochemical properties of proteins **(Aftabuddin and Kundu, 2007)**. Amino acids were grouped according to the type of side chain based on their solubility in water, hydrophilic, and hydrophobic amino acid **(Barrett and Elmore, 1998)**. Amino acids of gelatin with side chains were hydrophobic are alanine, leucine, valine, isoleucine, methionine, and phenylalanine, and hydrophilic side chains namely glycine, glutamate acid, lysine, serine, threonine, tyrosine, histidine, proline, arginine, and aspartate. The total percentage of amino acid groups based on their solubility in water was presented in Figure 3.

The hydrophilic amino acid of BCG was slightly higher than in SSG and SBG, and vice versa in the hydrophobic amino acids SSG and SBG were relatively slightly higher than the hydrophilic amino acids.

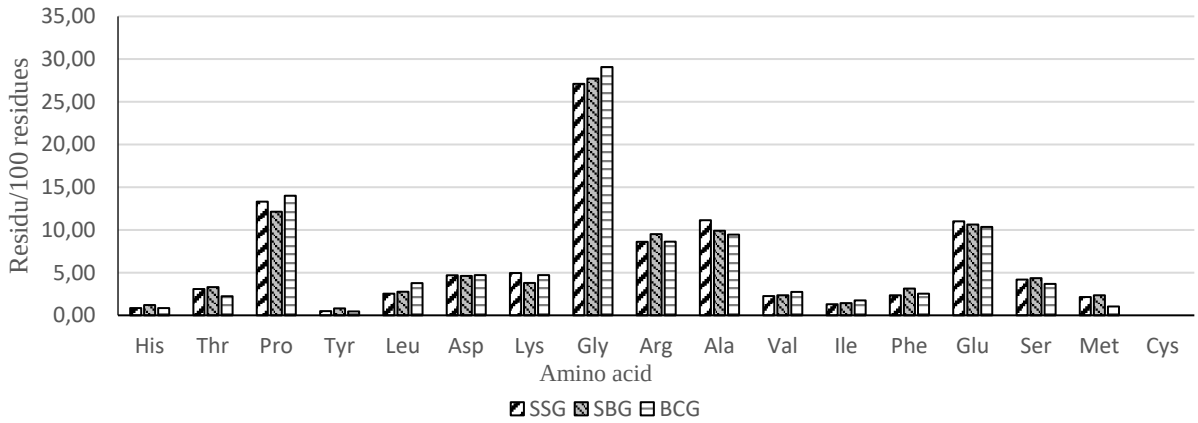


**Figure 2** The amino acids composition of snakehead skin gelatin (SSG), snakehead bones gelatin (SGB), and bovine commercial gelatin (BCG).

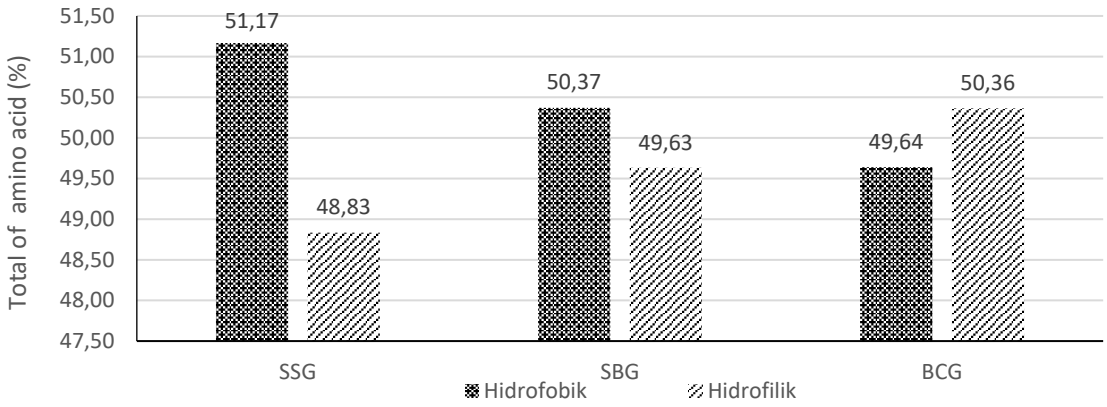


**Figure 3** Comparison of total hydrophilic and hydrophobic amino acids (%) from snakehead skin gelatin (SSG), snakehead bone gelatin (SBG), and bovine commercial gelatin (BCG).

**Table 3** Index of emulsion activity (IAE) and Index of stability emulsion (ISE) of snakehead skin gelatin (SSG) and snakehead bone gelatin (SBG) through the extraction process use the best extraction temperature and time and compared with bovine commercial gelatin (BCG).

| | IAE ($m^2.g^{-1}$) | ISE (min) |
|---|---|---|
| SSG | 32.49 ±0.28$^b$ | 26.25 ±0.19$^c$ |
| SBG | 28.47 ±0.36$^a$ | 19.16 ±0.18$^a$ |
| BCG | 28.92 ±0.09$^a$ | 25.42 ±0.16$^b$ |

Note: Different superscripts in the same column indicate a significant difference ($p$ <0.05). Values are presented as mean ±*SD* (n = 3).

**Tabel 4** Water holding capacity (WHC) and fat binding capacity (FBC) of snakehead skin gelatin (SSG) and snakehead bone gelatin (SBG) from the extraction process used the best temperature and extraction time and compared with bovine commercial gelatin (BCG).

| | Water holding capacity (%) | Fat binding capacity (%) |
|---|---|---|
| SSG | 192.53 ±4.76$^b$ | 223.97 ±4.80$^a$ |
| SBG | 166.48 ±2.87$^a$ | 227.67 ±3.28$^a$ |
| BCG | 298.85 ±4.30$^c$ | 219.10 ±3.14$^a$ |

Note: Different superscripts in the same column indicate a significant difference ($p$ <0.05). Values are presented as mean ±*SD* (n = 3).

**Aftabuddin and Kundu (2007)** stated that the average level of the hydrophobic node tends to be greater than the hydrophilic node. Consequently, the ability of these amino acids to bind to fat greater than their ability to attract water. Different from BCG which has a hydrophilic amino acid that was relatively larger than its hydrophobic amino acids, it will have an impact on the ability to attract water that was greater than its ability to bind fat.

Between SSG and SBG tends to show differences that were not so striking in the amino acid levels. But this relatively small difference still influences the functional characteristics of the two types of gelatin, as well as those reported in the gelatin of the skin and bones of *Lates niloticus* **(Muyonga, Cole and Duodu, 2004a; Muyonga, Cole and Duodu, 2004b)**. Extraction methods and different types of raw materials can result in differences in amino acid composition between the three types of gelatin observed.

## Index of Emulsion Activity (IAE) and Stability of Emulsion (ISE)

Gelatin is one type of hydrocolloid that has been widely used as an emulsifier in the industry, including food, pharmaceuticals, medicines, and other technical applications because it has surface-active properties **(Jellouli et al., 2011)**. This was a function of the ability of a surfactant to be hydrophilic and lipophilic at once, to reduce the surface tension caused by mixing oil and water. Gelatin was a type of natural surfactant, as a function of amino acids. The emulsion activity index and stability index of SSG, SBG, and BCG emulsion were presented in Table 3.

The index of emulsion activity of the three types of gelatin differed from one another ($p$ <0.05).

The index of emulsion activity (IAE) of each type of gelatin was relatively different. The index of emulsion activity of SSG was higher than BCG and SBG. The value of IAE reported by **Jellouli et al. (2011)** in the gelatin of gray triggerfish (*Balistes capriscus*) skin was lower (21.44 ±0.09), and as was reported by **(Jridi et al., 2013)** in gelatin cuttlefish skin (*Sepia officinalis*) were 25.97 ±1.05, 25.42 ±0.95, 27.64 ±1.26 and 27.65 ±1.35 respectively. The difference of IAE of gelatin can be caused by differences in sources of raw material **(Jellouli et al., 2011; Jridi et al., 2013; Zhang et al., 2012)** and extraction processes applied **(Ahmad and Benjakul, 2011; Kaewruang et al., 2013)**, which has implications for the intrinsic character of gelatin **(Khiari et al., 2013)**. The important intrinsic factor that influences the activity of gelatin emulsion was the contribution of amino acids making up the gelatin protein. The amino acid of gelatin with an active side chain gives the gelatin the ability to be hydrophilic (polar charge) and hydrophobic (non-polar charge). The peptide chain of hydrophobic amino acids contributes to the emulsifying properties of gelatin, because of the presence of surface-active properties, where the greater the content of hydrophobic amino acids, the more emulsion activity tends to be higher **(Karim and Bhat, 2009)**. **Ahmad and Benjakul (2011)** further explained that surface activity in the interface of water and oil in gelatin gave the ability to facilitate the formation and stabilization of droplets during and after the emulsion process. The emulsion activity index was related to the area between surfaces, where the higher the emulsion activity index value, the smaller the size of the fat globule. The emulsion activity index shows the ability of proteins to stabilize oil and water interfaces.

In Table 3, there was a significant effect ($p$ <0.05) of the gelatin type on the index of emulsion stability (ISE). The index of emulsion stability of SBG was smaller than BCG and SSG. Among the three types of gelatin, SSG appeared to show a better level of emulsion stability than BCG and SBG. However, the ISE of SBG was better than BCG, and in the same emulsion activity, SBG can achieve its stability in a shorter period. The same properties were reported by **Jellouli et al. (2011)** in gray triggerfish (*Balistes capriscus*) which had better ISE than bovine gelatin.

The emulsifying properties of gelatin were described as emulsion capacity or emulsion activity, which showed the ability of gelatin to aids the formation and stabilization of newly created emulsions and their ability to give emulsion

strength for the resistance of pressure. The index of emulsion activity was an interface measurement area. It was stabilized over a unit of protein weight ($m^2.g^{-1}$), and it was related to the ability of proteins to coat an interface **(Pearce and Kinsella, 1978)**. The emulsion activity index was influenced by the intrinsic factor of gelatin protein. Adding gelatin to an oil-water emulsion solution affects the oil-water interface. Emulsifying properties were produced due to the presence of hydrophobic regions in the peptide chain **(Karim and Bhat, 2009)**. The hydrophobic nature of the side chains of gelatin amino acid peptides reduces surface tension so that emulsion stability can be achieved. However, this depends on how large the ability of hydrophobicity of gelatin. The higher level of hydrophobic amino acid, the greater the IAE, and the shorter the ISE to achieve its stability. According to **Ahmad and Benjakul (2011)**; **Jellouli et al. (2011)**, the molecular weight can also influence the dispersion ability between oil and water, where the level of stability of emulsion from gelatin at high molecular weights results in better levels of stability than gelatin from lower molecular weights.

## Water Holding and Fat Binding Capacity of Gelatin

Water holding and fat binding capacity were functional properties that were closely related to the texture of food products as interactions between components such as water, oil, and other components **(Ismail et al., 2010; Rawdkuen, Thitipramote and Benjakul, 2013)**. The results of the analysis in Table 4 showed that the type of gelatin has a significant effect ($p$ <0.05) on the water holding capacity (WHC). Snakehead bone gelatin (SBG) has the lowest WHC and was highest in BCG and then SSG. The Differences in extraction methods, raw materials, and animal species were factors that influence the differences in the characteristics of gelatin. This difference also causes the WHC of the three types of gelatin to tend to be different.

Water holding capacity as in Table 4 show the percentage of BCG was relatively higher than the two types of gelatin from snakehead, but the percentage of both is still relatively higher than the WHC of cobia fish skin gelatin (97.03 ±1.53) **(Amiza et al., 2015)**. A study was carried out by **Balti et al. (2011)** in cuttlefish (*Sepia officinalis*) showed a relatively similar percentage with WHC on SSG and SBG, which was about 150 to 200%. The high WHC can be attributed to the hydrophilic amino acid content of gelatin **(Nurul and Sarbon, 2015)** and the main chemical component of gelatin, namely hydroxyproline **(Ninan, Jose and Abubacker, 2011; Zarai et al., 2012)**. Allegedly, if the hydroxyproline level was high then the ability to hold water of gelatin would also follow **(Jeya et al., 2012)**.

Water holding was one of the important functional properties in the processing process because of its function in improving the texture and compactness of food products. This trait according to **Zarai et al. (2012)** refers to the ability of proteins to absorb water and maintain it against the gravitational force in the protein matrix. Furthermore, **Zarai et al. (2012)** that its capacity to holding water makes it suitable and convenient to be applied to various frozen meat and fish products to reduce drip loss during the thawing or cooking process which impact protein denaturation resulting in partial loss of capacity to hold of water.

**Table 5** The amide absorption area of snakehead skin gelatin (SSG), snakehead bone gelatin (SBG), and bovine commercial gelatin (BCG) were based on the FTIR spectrum.

| Amide | Absorption peak area ($cm^{-1}$)* | Wavenumber ($cm^{-1}$) | | |
|---|---|---|---|---|
| | | SSG | SBG | BCG |
| A | 3600 – 3200 | 3388.93 | 3383.14 | 3442.94 |
| B | 3100 – 2300 | 3080.32 | 3076.46 | - |
| | | 2935.66 | 2929.87 | 2956.87 |
| | | 2881.65 | 2879.72 | 2927.94 |
| | | 2360.87 | 2360.87 | 2360.87 |
| | | 2335.80 | 2335.80 | 2335.80 |
| I | 1656 – 1644 | 1653.00 | 1651.07 | 1653.00 |
| II | 1560 – 1335 | 1541.12 | 1521.84 | 1543.05 |
| | | - | - | 1517.98 |
| | | 1450.47 | - | 1454.33 |
| | | 1413.82 | 1411.89 | - |
| | | - | - | 1396.46 |
| | | - | - | 1335.67 |
| III | 1240 – 670 | 1234.44 | 1234.44 | 1238.30 |
| | | 1197.79 | 1197.79 | 1199.72 |
| | | - | 1166.93 | 1163.08 |
| | | 1076.28 | 1074.35 | 1078.21 |
| | | 1028.06 | 1028.06 | 1031.92 |
| | | 970.19 | 970.19 | - |
| | | 923.90 | 921.97 | 923.90 |
| | | 873.75 | 871.82 | 875.68 |
| | | - | - | 673.16 |

Note: * **Muyonga, Cole and Duodu (2004c)**; **Uriarte-Montoya et al. (2011)**; **Kong and Yu (2007)**.

The three types of gelatin observed did not show any significant difference ($p$ >0.05) on FBC. The fat binding capacity of SBG was relatively slightly higher than in SSG and BCG. Both showed lower percentages than FBC from red snapper bone gelatin (*Lutjanus campechanus*) and brown spotted grouper (*Epinephelus chlorostigma*) which were 493.90% ±4.20 and 429.57% ±4.73 respectively **(Jeya et al., 2012)**, but it is higher than the cobia skin gelatin (*Rachycentron canadum*) which was 164.0% **(Amiza et al., 2015)**. The difference in FBC from gelatin cannot be separated from the characteristics of the gelatin itself, which among others was influenced by raw materials and extraction methods so that it may result in differences in the composition of amino acids. The ability of gelatin to bind fat was mainly facilitated by hydrophobic amino acids **(Jellouli et al., 2011)**, the greater the ability to bind fat tends to be higher Hydrophobic amino acids include tyrosine, isoleucine, valine, leucine, proline, phenylalanine, methionine and alanine. **Ninan, Jose and Abubacker (2011)** state that high levels of tyrosine were thought to be responsible for the binding capacity of fat in gelatin.

## Functional Group of Gelatin

Analysis using Fourier transform infrared (FTIR) spectroscopy was carried out to identify changes in the functional group and secondary structure of gelatin **(Muyonga, Cole and Duodu, 2004b)** based on the vibration spectra produced by the compounds contained therein at certain wavelengths. The absorption value of snakehead fish gelatin (SSG and SBG) compared to BCG was presented in Table 5.

Amide A peaks from SSG, SBG, and BCG in the absorption area of 3388.93 $cm^{-1}$, 3383.14 $cm^{-1}$, and 3442.94 $cm^{-1}$, respectively. The smaller value absorption that appears in the spectra, indicates a lower free amine group, which means less degradation. Absorption values of BCG which were in the range above 3400 $cm^{-1}$ indicate that wavenumbers were in the vibration region of free NH stretch **(Ahmad and Benjakul, 2011; Kittiphattanabawon et al., 2015; Sinthusamran, Benjakul and Kishimura, 2014)**. The SSG and SBG absorption areas show a slightly lower wavenumber, that was in the absorption area of 3400 – 3200 $cm^{-1}$, which means there was a shift in wavenumbers to a lower frequency, this was because there were NH groups of peptides involved with hydrogen bonds **(Ahmad and Benjakul, 2011; Muyonga, Cole and Duodu, 2004c)**. A relatively high SSG wave number compared to SBG shows that the hydrolysis process of snakehead fish skin at a temperature of 60 °C for 12 hours causes relatively more hydrogen bonds to be released than hydrogen bonds in snakehead bone at the same temperature. This indicates that the type of raw material and the relative extraction process affect the secondary structure of the gelatin protein, besides this difference according to **Kittiphattanabawon et al. (2015)** may also be due to differences in amino acid composition and sequence.

The amide I band between SSG and BCG were at the same wavenumber, which was slightly higher than SBG, namely 1653.00 $cm^{-1}$, 1653.00 $cm^{-1}$, and 1651.07 $cm^{-1}$, respectively. The difference in the type of raw material and the extraction method used causes the wavenumbers of the two types of snakehead fish gelatin to be different. At the same temperature (60 °C) with different extraction times, the SBG wave number (24 hours of extraction) was slightly lower than SSG (12 hours of extraction) indicating that the level of C=O reactivity in the skin was able to open and act reactively more quickly on the chain- α compared to snakehead fish bones, so this situation is according to **Jridi et al. (2015)** has the potential to cause the loss of the triple helix due to excessive hydrolysis of the intermolecular cross-linking. Furthermore, this situation by **Yu et al. (2014)** is alleged to have caused the loss of amino acid reactivity, including lysine, hydroxylysine, and histidine, which function to maintain inter and intra-molecular cross-links in the telopeptide region.

In the amide band II, the peak wavenumbers between SSG, SBG, and BCG have shown the absorption values were 1541.12 $cm^{-1}$, 1521.84 $cm^{-1,}$ and 1543.05 $cm^{-1}$, respectively. The amide region II showed an abnormality in the molecular structure due to the transformation of α-helix into a random coil structure due to the partial hydrolysis process during extraction **(Muyonga. Cole and Duodu. 2004b)**, which was characterized by the occurrence of NH flexural vibrations coupled with CN stretching vibrations **(Yu et al., 2014)**. The temperature and length of extraction time can cause changes in the triple helix as a result of the denaturation of collagen to gelatin **(Muyonga, Cole and Duodu, 2004b)**. The smaller the wavenumber, the smaller the triple helix damage rate observed.

The SSG and SBG wavelengths in the peak region of the amide band III showed the same absorption value, namely 1234.44 $cm^{-1}$, while BCG was at 1238.30 $cm^{-1}$, indicating a greater disturbance in the molecule, and this according to **Sinthusamran, Benjakul and Kishimura (2014)** associated with the loss of the triple helical structure. Amide region III was a spectrum region of 1220 – 1320 $cm^{-1}$ and is associated with CN stretching and NH deformation as well as absorption arising from vibrations of CH2 groups from the glycine backbone and proline side chains **(Ahmad and Benjakul, 2011)**.

## CONCLUSIONS

The snakehead skin and bone gelatin had different characteristics, and this was greatly influenced by the temperature and time of extraction. The higher the temperature and extraction time, the yield of both gelatins tended to increase. The gel strength between both gelatins was more influenced by the extraction time, which was 60 °C 12 h for the snakehead skin, and 60 °C 24 h for the snakehead bones. The difference gel strength as a characteristic indicator of snakehead fish skin and bone gelatin parameters was compared with commercial bovine gelatin. The amino acids of the three types of gelatin showed higher levels of glycine and proline compared to other types of amino acids. Based on the total residues of each amino acid, both skin gelatin and bone gelatin showed more dominant hydrophobic properties than hydrophilic properties, however vice versa in commercial bovine gelatin. The three types of gelatin showed different differences in chemical composition, index of emulsion activity, the stability of the emulsion, water holding, and fat binding capacity, and this was thought to be closely

related to the source of the raw material and its amino acid content. The characteristics of the three types of gelatin were supported through FTIR analysis and showed snakehead fish skin and bones as potential gelatin equivalent to commercial gelatin.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# The effect of Sorghum Tempeh (*Sorghum bicolor L. Moench*) on low-density lipoprotein (LDL) and malondialdehyde (MDA) levels in atherogenic diet-induced rats

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## ABSTRACT
An atherogenic diet induces oxidative stress leading to hypercholesterolemia. This condition causes atherosclerosis followed by increased LDL and MDA. Sorghum tempeh contains fiber and antioxidants that can protectively improve LDL and MDA levels. Therefore, this research aims to determine the effect of sorghum tempeh on LDL and MDA levels in atherogenic diet-induced rats compared to sorghum flour. It used a randomized pre-post test with a control group design. The test subjects were 30 male *Sprague Dawley* rats, consisting of 6 normal conditioned rats (C1), and 24 that were induced by an atherogenic diet (C2, T1, T2, T3) for 2 weeks. Sorghum flour was administered at a dose of 4.095 g (T1) and the sorghum tempeh at 3.041 g (T2) and 6.081 g (T3) for 4 weeks. Furthermore, C2 was constantly induced through an atherogenic diet. Total cholesterol and LDL levels were then analyzed using the CHOD-PAP method, and MDA levels, using the ELISA method. Meanwhile, statistical analysis for these variables was carried out using IBM SPSS Statistics 21 software. The results showed that the administration of sorghum flour and tempeh significantly reduced total cholesterol, LDL, MDA levels in each group ($p = 0.001$). Furthermore, it showed that there was a significantly strong correlation between LDL and MDA levels before and after treatment ($r = 0.610$, $r = 0.805$, and $p = 0.001$). The administration of sorghum tempeh at a dose of 6.081 g caused the greatest reduction (Δ) in LDL levels at -44.19 ±2.58 mg.dL$^{-1}$, although, it was not the same as normal control. Meanwhile, sorghum flour at a dose of 4.095 g was the most influential in reducing MDA levels to the same as normal control with delta (Δ) at -7.67 ±0.37 ng.mL$^{-1}$. In conclusion, sorghum tempeh and flour were the most effective at reducing LDL and MDA levels, respectively.

**Keywords:** sorghum tempeh; sorghum flour; LDL; MDA; atherosclerosis

## INTRODUCTION
Atherosclerosis occurs due to a progressive plaque growth process and is characterized by hypercholesterolemia, abnormalities in the shape of the arterial walls, and increased inflammatory mediators such as IL-6, IL-10, and MCP-1 **(Estruch et al., 2016)**. Moreover, the induction of an inflammatory process and a simultaneous increase in LDL in the tunica intima causes the formation of *reactive oxygen species* (ROS) **(Salvayre, Negre-Salvayre and Camaré, 2016; Marchio et al., 2019)**. High intracellular levels of ROS are involved in the process of lipid peroxidation which produces secondary reactive products such as MDA **(Papac-Milicevic, Busch and Binder, 2016)**.

Increased reactivity of MDA causes endothelial damage and modifies LDL, which leads to changes in cellular response **(Papac-Milicevic, Busch, and Binder, 2016)**. Meanwhile, an increase in the levels of LDL in blood plasma causes the formation of atheroma plaque, which would develop into cardiovascular disease **(Ratnasari, Santosa and Rachmawati, 2018)**. Various studies have used mice induced by an atherogenic diet as models of atherosclerosis. These mice experience an increase in the accumulation of fat in their blood vessels leading to abnormal signal transduction in organs, oxidative stress, lipotoxicity, and organ dysfunction. This affects the activity of transcription factors in lipid metabolism in the heart **(Lee et al., 2017)**.

One of the treatments of atherosclerosis is through the provision of fermented food **(Şanlier, Gökcen and Sezgin, 2019)**. Moreover, Indonesia is known to produce tempeh, the fermented food made from soybeans **(Hartanti, Rahayu and Hidayat, 2015)**. Sorghum is a cereal that contains high fiber and antioxidants, however, it still has low food value **(Espitia-Hernández et al., 2020)**. Furthermore, this cereal has been used as a raw material for the production of tempeh which was tested in atherogenic diet-induced rats. Sorghum tempeh (100 g) contains 134.49% energy, 15.20% carbohydrates, 6.75% protein, 5.21% fat, 66.40% water, 0.15% ash, 68.57% starch digestibility, 63.32% protein digestibility, 52.21% antioxidants, 5.92% total dietary fiber, 0.56% soluble dietary fiber, and 5.63% insoluble fiber.

Sorghum tempeh could be produced through the following process. First, sorghum is soaked for 12 hours, boiled until it reaches a softer texture, and then left to cool, Furthermore, tempeh yeast which contains *Rizhopus oligosporus* is added to start the fermentation process **(Hartanti, Rahayu and Hidayat, 2015)**. The resultant mold produces various protease enzymes with proteolytic activity, which makes sorghum tempeh easier to digest **(Endrawati and Kusumaningtyas, 2017)**. Moreover, the fermentation process lasts for 60 hours and leads to the formation of β-glucosidase enzymes, which produce flavanone, flavonols, and aglycone isoflavones **(Salazar-López et al., 2018; Verni, Verardo and Rizzello, 2019)**. The final stage involves blanching for 5 minutes to stop the microbial activity **(Tian et al., 2018)**.

The advantages of products fermented by *Rhizopus oligosporus* were proven in other similar studies. Quinoa seed extract fermented by this fungi had increased antioxidant enzymes, such as *superoxide dismutase* (SOD) and *glutathione peroxidase* (GSHPx) which help to prevent peroxidative damage **(Matsuo, 2005; Hur et al., 2018)**. Furthermore, in line with the research on soybean and gembus tempeh, the results of yeast fermentation by *Rhizopus spp* was an increase in SOD enzyme which could reduce MDA levels **(Harun, Susanto and Rosidi, 2017; Kurniasari et al., 2017)**.

Sorghum tempeh has the potential as an antioxidant and anti-cholesterol agent, which could repair oxidative stress damage **(Pisoschi and Pop, 2015; Dykes, 2019)**. Moreover, fermented sorghum could reduce oxidative stress through the fermentation effect of its fiber. This fiber modulates the intestinal microbiota to improve immune function through the production of *short-chain fatty acid* (SCFA) **(Ohira, Tsutsui and Fujioka, 2017)**. SCFA reduces inflammation that triggers oxidative stress by suppressing T helper cells, neutrophils, macrophages, and cytotoxic activity in natural killer cells **(Tan, Norhaizan and Liew, 2018; Hu et al., 2020)**.

Sorghum tempeh also plays a role in controlling serum cholesterol. This is because the *cellulose synthase-like* (Csl) gene in the soluble fiber, (1,3;1,4)-β-glucan has the potential to increase fiber levels, which would reduce apoB-100 and LDL **(Ermawar et al., 2015; Ho et al., 2016)**. Additionally, fiber fermented by the intestinal microbiota produces SCFA, which influences lipid metabolism through transport and *de novo* synthesis to lower plasma cholesterol levels **(Chen et al., 2018)**.

From the above, the aim of the research, which is to prove the protective ability of sorghum tempeh to reduce MDA and LDL levels. Sorghum flour which was used as a comparison is sorghum seeds that were processed into flour without fermentation. These seeds were mashed with a grinder to achieve a powdery texture then sifted until they became soft. The previous study has been shown that sorghum flour improving lipid profile and MDA levels in rats **(Silva et al., 2020; Zakaria et al., 2011).** Besides, the flour (100 g) contains 315.11% energy, 69.75% carbohydrates, 8.12% protein, 1.31% fat, 10.55% water, 1.47% ash, 80.50% starch digestibility, 78.70% protein digestibility, 75.18% antioxidants, 8.79% total dietary fiber, 0.94% soluble dietary fiber, and 7.85% insoluble dietary fiber.

### Scientific Hypothesis

Sorghum tempeh decreases LDL and MDA levels in atherogenic diet-induced rats more effect than sorghum flour. Moreover, the levels of these variables were strong correlations before and after treatment.

## MATERIAL AND METHODOLOGY

### Samples

This study used white sorghum (Figure 1). The grain was soaked in water at a ratio of 1:3 for 12 hours and then boiled for 10 minutes. After that, the addition of the tempeh yeast at 0.25 g for every 100 g of white sorghum was carried out after the sorghum had cooled. The mixture was then incubated at room temperature (29 ±1 °C) for 60 hours. Subsequently, the sorghum tempeh was blanched at 90 °C for 5 minutes (Figure 2).

Sorghum flour was used as a comparison for sorghum tempeh and was produced by processing sorghum seeds without fermentation. The white sorghum was purred using a grinder to achieve a powdery texture and subsequently sieved with 70 mesh (Figure 3). Moreover, the dosage of sorghum tempeh and flour was based on human fiber needs, which is 20 $g.d^{-1}$. This amount was then converted to 200 g of body weight for the rats. Finally, the dosage for T1, T2, T3-groups at 4.095 g, 3.041 g, and 6.081 g, respectively.

### Chemicals

Two milliliters of blood were obtained from the *retro-orbital plexus* of the rats for total cholesterol ($mg.dL^{-1}$), LDL ($mg.dL^{-1}$), and MDA ($ng.mL^{-1}$) serum were assessed in PSPG UGM Yogyakarta Laboratory, Indonesia.

### Animal and Biological Material

This study used male *Sprague Dawley* rats supplied by PSPG UGM Yogyakarta Animal Laboratory, Indonesia. The white sorghum was produced by Sedana Panen Sejahtera Ltd., Jombang, Indonesia. The tempeh yeast was produced by Aneka Fermentasi Industri Ltd., Bandung, Indonesia.

### Instrument

The weights and diet consumed of the rats were measured by Digital Camry EK5055 Max.5 kg d = 1 g. Total cholesterol levels were tested using the Cholesterol FS (10`) 5 x 25 mL.1 x 3 $mL^{-1}$ STD. Meanwhile, LDL levels were tested using the LDL Precipitant 250 mL Kit. Finally, MDA levels were ascertained using the General Malondialdehyde ELISA Kit (MDA) 96 wells (IP). The kits of these tests were supplied by Khairos Jaya Sejahtera Ltd., Yogyakarta, Indonesia.

### Laboratory Methods

Total cholesterol and LDL levels were tested according to the CHOD-PAP method (WHO/IFCC). MDA levels were tested according to the ELISA method **(ISO 9001:2008; ISO 13485:2003)**.

### Description of the Experiment

This study used a true experimental design with a randomized pre-post test control group design. Furthermore, it was conducted at the PSPG UGM Yogyakarta Animal Laboratory from October-December 2020.

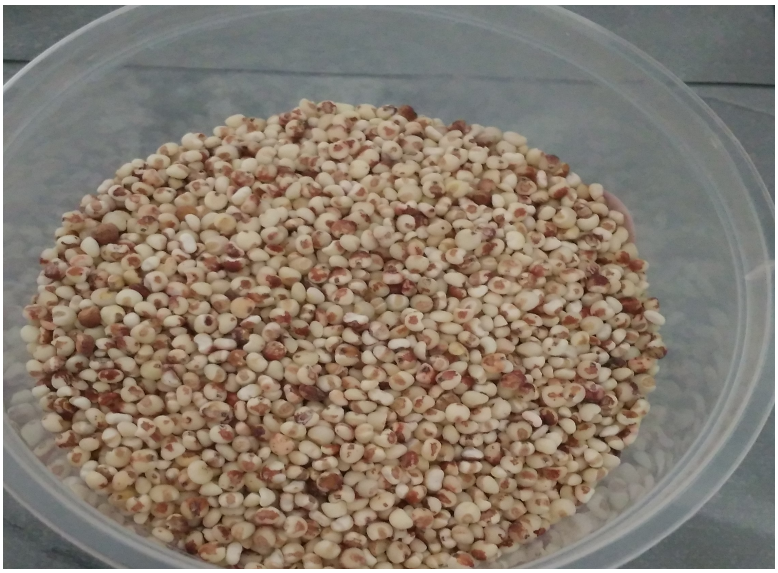

**Figure 1** Preparation of Sorghum Tempeh and Flour.

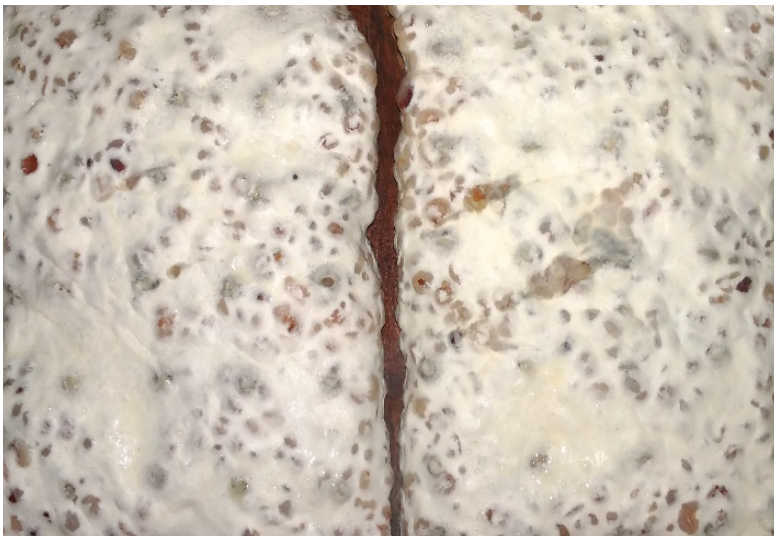

**Figure 2** Sorghum Tempeh.



**Figure 3** Sorghum Flour.

**Sample preparation:**

The inclusion criteria were male *Sprague Dawley* rats aged 2 months and weighing 150 – 200 g, that were in a healthy condition during the acclimatization period. Moreover, the acclimatization period was 1 week and aimed to get the animals acquainted with the type of feed and new way of life. The atherogenic criteria were based on the hypercholesterolemic condition of the rats (total cholesterol levels more than 100 ml.dL$^{-1}$). Meanwhile, the dropout criteria were rats that their body weight decreased to <150 g during the adaptation period and those that died during the study.

**Number of samples analyzed:**

There were 30 male *Sprague Dawley* rats. Additionally, they were divided by simple random sampling into five groups, namely C1, C2, T1, T2, and T3-groups, and were housed individually. The cage room had an ambient temperature of 28 – 32 ºC with a lighting cycle of 12 hours dark and 12 hours light. Furthermore, the rat feeds used were standard (Comfeed AD II) and atherogenic feeds (Table 1). Assessment of diet consumed was based on the weight of feed given minus the weight of the remaining feed.

The rats in C2, T1, T2, and T3-groups were induced using an atherogenic diet for 2 weeks. Moreover, this feed was given at 20 g.d$^{-1}$ and drinking water ad libitum. C1-group was in a normal condition as only the standard feed at 20 g.d$^{-1}$ and drinking water ad libitum was given therein. For the remaining groups, they received interventions for 4 weeks in a sonde, where the T1 group received sorghum flour at a dose of 4.095 g, while T2 and T3-groups received the tempeh at doses of 3.041 g and 6.081 g, respectively. The treatment groups were also given standard feed at 15 g.d$^{-1}$ and drinking water ad libitum. Finally, C1-group was maintained at normal conditions, while C2-group was continuously induced using an atherogenic diet until the end of the research.

**Number of repeated analyses:**

The blood was obtained from the *retro-orbital plexus* of the rats for total cholesterol, LDL, and MDA serum was carried out two assessments namely before and after the treatment period.

## Statistical Analysis

Data analysis was carried out using IBM SPSS Statistics 21 software. Furthermore, data normality was tested using the Shapiro-Wilk test. Bivariate analysis was used to analyze the levels of total cholesterol, LDL, and MDA through Paired t-test when they were normally distributed. Meanwhile, when the data was not normal, the Wilcoxon Signed Ranks test was used. The differences in the influence of the conditions of C1, C2, T1, T2, T3-groups on bodyweight, weights of rat feed consumption, and total cholesterol, LDL, and MDA levels were analyzed using the One Way Anova test when the data were normally distributed and homogeneous. However, in a condition where the data were not normally distributed or not homogeneous, used the Kruskal Wallis test. The correlation test used for the levels of LDL and MDA was the Pearson test when the data were normally distributed, however, when not normally distributed the Rank Spearman test was used. Finally, the value of significance in the study was $p < 0.05$.

## RESULTS AND DISCUSSION

The bodyweight of the rats at the beginning and end of the study did not differ significantly between the groups ($p = 0.383$ and $p = 0.136$). After the provision of the treatment products in T1, T2, and T3-groups, there was a significant difference between the groups ($p = 0.010$), that the treatment groups were the same as the normal control ($p = 0.793$, $p = 0.189$, and $p = 0.057$), and significant difference compared to the atherogenic diet induction control ($p = 0.006$ and $p = 0.001$) except for T1-group ($p = 0.091$). Additionally, the mean body weight in T1-group was lower compared to that of the atherogenic diet induction control. Table 2 shows the significant difference in delta (Δ) between the groups after treatment ($p = 0.001$), where the biggest change was in the T1-group at +18.83 ±13.00 g.

The mean weight percentage of the feed consumed in the groups during the induction period with an atherogenic diet was not significantly different ($p = 0.836$), while during the treatment period there was a significant difference ($p = 0.009$). Moreover, based on further tests, T1, T2, T3-groups had no significant difference in the weights percentage of feed consumed ($p > 0.05$). Table 3 shows that the T3-group from the induction to treatment period had a higher average percentage of feed consumed compared to the other two treatment groups, at 90.65% and 97.81%.

Table 4 shows that the levels of total cholesterol before treatment in T1, T2, and T3-groups were significantly different from the normal control (C1) ($p = 0.001$) and the same as the atherogenic diet induction control group (C2) ($p = 0.852$, $p = 0.729$, and $p = 0.498$). There was a significant increase in cholesterol total levels in the C2-group ($p = 0.001$), while in C1-group, there was the least change (Δ), at +2.01 ±1.36 mg.dL$^{-1}$ among all groups. Furthermore, the three treatments with their respective doses significantly reduced atherogenic parameters by lowering total cholesterol ($p = 0.001$) and were significantly different from the atherogenic diet induction control ($p = 0.001$), but still not the same as the normal control ($p = 0.001$). Meanwhile, the biggest change (Δ) occurred in T3-group at -80.55 ±5.79 mg.dL$^{-1}$.

LDL levels in T1, T2, and T3-groups after induction with the atherogenic diet was significantly different from normal control (C1) ($p = 0.001$), however, not significantly different from atherogenic diet induction control (C2) ($p = 0.947$, $p = 1.000$, $p = 0.833$). Table 5 shows that after the administration of the treatments, there was a significant decrease in the LDL levels ($p = 0.001$) across the groups, differ to the atherogenic diet induction control ($p = 0.001$) although, they were still significantly different from normal control ($p = 0.001$, $p = 0.001$, $p = 0.008$). When viewed from delta (Δ), it shows that the T3-group (sorghum tempeh a dose of 6.081 g) had the greatest influence, namely -44.19 ±2.58 mg.dL$^{-1}$ when compared to the other two treatments. The treatment in this group had the same effect as in the T1-group ($p = 0.265$), but a significantly different effect compared to the T2-group ($p = 0.020$). Meanwhile, the treatment in T1 and T2-groups had the same effect ($p = 0.225$).

**Table 1** Composition of Standard and Atherogenic Feed.

| Standard Feed | Atherogenic Feed |
|---|---|
| 12% water, 51% carbohydrates, 15% crude protein, 3-7% crude fat, 6% crude fiber, 7% ash, 0.55% phosphorus, 0.9 – 1.1 % calcium. | 12% water, 51% carbohydrates, 15% crude protein, 3-7% crude fat, 6% crude fiber, 7% ash, 0.55% phosphorus, 0.9 – 1.1 % calcium, cholic acid, pure cholesterol. |

**Table 2** Bodyweight of Rats.

| Groups | Mean ± SD (g) | | | | |
|---|---|---|---|---|---|
| | The Beginning of The Study | After Atherogenic Diet Induction | Δ1 | The End of The Study | Δ2 |
| C1 (normal control) | 189 ±10.81 | 215.83 ±9.37 | 26.83 ±7.94 | 252.33 ±19.56 | 36.50 ±17.23 |
| C2 (atherogenic diet induction control) | 192 ±13.67 | 229.83 ±18.57 | 37.83 ±10.63 | 288.17 ±38.65 | 58.33 ±24.79 |
| T1 (sorghum fluor a dose of 4.095 g) | 192.83 ±12.90 | 229.33 ±11.86 | 36.50 ±8.41 | 248.17 ±13.83[a,c] | 18.83 ±13.00 |
| T2 (sorghum tempeh a dose of 3.041 g) | 191.17 ±6.37 | 231 ±11.95 | 39.83 ±9.45 | 237.50 ±15,29 [a,b] | 6.50 ±8.46 |
| T3 (sorghum tempeh a dose of 6.081 g) | 181.50 ±8.34 | 219.17 ±6.74 | 37.67 ±3.45 | 230.50 ±8.74[a,b] | 11.33 ±8.73 |
| *p* | 0.383[1] | 0.136[1] | 0.091[1] | 0.010[2]* | 0.001[1]* |

Note: Δ1 = Bodyweight of after atherogenic diet induction – Bodyweight of the beginning of the study; Δ2 = Bodyweight of the end of the study – Bodyweight of after atherogenic diet induction; [1]One Way ANOVA Test; [2]Kruskal Wallis Test; [a]Not significantly different from normal control; [b]Significantly different from atherogenic diet induction control; [c]Not significantly different from atherogenic diet induction control. *Significant ($p$ <0.05).

**Table 3** Weights of Rat Feed Consumption.

| Groups | Mean ± SD (g) | |
|---|---|---|
| | Atherogenic Diet Induction Period | Treatment Period |
| C1 (normal control) | 17.94 ±1.43 (89.71%) | 17.82 ±0.71 (89.11%) |
| C2 (atherogenic diet induction control) | 17.49 ±1.87 (87.45%) | 16.66 ±2.07 (83.31%) |
| T1 (sorghum fluor a dose of 4.095 g) | 17.56 ±1.36 (87.79%) | 14.19 ±0.63 (94.61%)[a] |
| T2 (sorghum tempeh a dose of 3.041 g) | 17.18 ±1.47 (85.90%) | 14.24 ±0.39 (94.95%)[a] |
| T3 (sorghum tempeh a dose of 6.081 g) | 18.13 ±1.55 (90.65%) | 14.67 ±0.40 (97.81%)[a] |
| *p* | 0.836[1] | 0.009[2]* |

Note: [1]One Way ANOVA Test; [2]Kruskal Wallis Test; [a]The comparison between T1, T2, and T3-groups was not significantly different. *Significant ($p$ <0.05).

MDA levels in the T1, T2, T3-groups after induction were also significantly different compared to normal control (C1) ($p$ = 0.001, $p$ = 0.006, $p$ = 0.004) and not significantly different from atherogenic diet induction control (C2) ($p$ = 0.818, $p$ = 0.818, $p$ = 0.896). Additionally, after the administration of the treatment, there was a significant decrease in MDA levels ($p$ <0.05) across the groups and the levels were also significantly different from the atherogenic diet induction control ($p$ = 0.001, $p$ = 0.018) except for T3-group ($p$ = 0.238). The administration of sorghum tempeh at the dose of 6.081 g caused the smallest effect (Δ) among the three treatments at -3.97 ±0.33 ng.mL$^{-1}$, which was still the same as the atherogenic diet induction control ($p$ = 0.238), however, significantly different from normal control ($p$ = 0.001). Likewise, sorghum tempeh at a dose of 3.041 g did not have the same effect as normal control ($p$ = 0.018). The administration of sorghum flour at a dose of 4.095 g had the most effect on improving MDA levels to a similar level as normal control ($p$ = 0.238) with the largest delta (Δ), namely -7.67 ±0.37 ng.mL$^{-1}$. Statistically, the treatment in T1-group had a significantly different effect compared to the T3-group ($p$ = 0.018) and the same effect as in the T2-group ($p$ = 0.237). Meanwhile, the treatment in T2 and T3-groups had the same effect ($p$= 0.237) (Table 6).

Table 7 shows that LDL and MDA levels had a strong positive correlation before treatment, meaning that the higher the LDL levels, the higher the MDA levels ($p$ = 0.001, $r$ = 0.610). After the treatment period, LDL and MDA levels had a strong positive correlation, because the lower the LDL levels, the lower the MDA levels ($p$ = 0.001, $r$ = 0.805).

The rats induced with an atherogenic diet for 2 weeks had significantly increased total cholesterol levels, which could lead to atherosclerosis, with levels more than 100 mg.dL$^{-1}$ in rats **(Zárate et al., 2016)**.

**Table 4** Changes in Blood Cholesterol Total Levels Before and After Treatment.

| Groups | Mean ± SD (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Before Treatment | After Treatment | Δ | *p* |
| C1 (normal control) | 83.22 ±2.36 | 85.23 ±2.61 | +2.01 ±1.36 | 0.015[1]* |
| C2 (atherogenic diet induction control) | 192.01 ±4.78 | 222.75 ±3.91 | +30.74 ±6.28 | 0.001[1]* |
| T1 (sorghum fluor a dose of 4.095 g) | 189.61 ±4.19[a,c] | 122.88 ±3.50[a,b] | -66.74 ±5.50 | 0.001[1]* |
| T2 (sorghum tempeh a dose of 3.041 g) | 189.04 ±4.84[a,c] | 133.60 ±3.28[a,b] | -55.45 ±6.44 | 0.001[1]* |
| T3 (sorghum tempeh a dose of 6.081 g) | 188.13 ±4.96[a,c] | 107.58 ±2.69[a,b] | -80.55 ±5.79 | 0.001[1]* |
| *p* | 0.001[2]* | 0.001[2]* | 0.001[2] | |

Note: [1]Paired t Test; [2]One Way ANOVA Test; [a]Significantly different from normal control; [b]Significantly different from atherogenic diet induction control; [c]Not significantly different from atherogenic diet induction control. *Significant ($p$ <0.05).

**Table 5** Changes in Blood LDL Levels Before and After Treatment.

| Groups | Mean ± SD (mg.dL$^{-1}$) | | | |
|---|---|---|---|---|
| | Before Treatment | After Treatment | Δ | *p* |
| C1 (normal control) | 23.88 ±2.50 | 25.44 ±3.20 | +1.56 ±1.18 | 0.023[1]* |
| C2 (atherogenic diet induction control) | 76.12 ±1.38 | 85.37 ±1.30 | +9.24 ±1.69 | 0.001[1]* |
| T1 (sorghum fluor a dose of 4.095 g) | 76.93 ±1.27[a,c] | 39.95 ±2.48[a,b] | -36.98 ±3.16[d] | 0.001[1]* |
| T2 (sorghum tempeh a dose of 3.041 g) | 76.36 ±2.04[a,c] | 52.27 ±2.89[a,b] | -24.09 ±3.70[d] | 0.001[1]* |
| T3 (sorghum tempeh a dose of 6.081 g) | 74.97 ±2.08[a,c] | 30.78 ±2.04[a,b] | -44.19 ±2.58[d] | 0.001[1]* |
| *p* | 0.001[2]* | 0.001[2]* | 0.001[3]* | |

Note: [1]Paired t Test; [2]One Way ANOVA Test; [3]Kruskal Wallis Test; [a]Significantly different from normal control; [b]Significantly different from atherogenic diet induction control; [c]Not significantly different from atherogenic diet induction control. [d]The comparison was significant (T2 and T3-groups), was not significant (T1 and T2-groups, T1 and T3-groups). *Significant ($p$ <0.05).

**Table 6** Changes in Blood MDA Levels Before and After Treatment.

| Groups | Mean ± SD (ng.mL$^{-1}$) | | | |
|---|---|---|---|---|
| | Before Treatment | After Treatment | Δ | *p* |
| C1 (normal control) | 1.00 ±0.22 | 1.03 ±0.02 | +0.02 ±0.01 | 0.001[1]* |
| C2 (atherogenic diet induction control) | 9.36 ±0.51 | 9.65 ±0.40 | +0.29 ±0.14 | 0.004[2]* |
| T1 (sorghum fluor a dose of 4.095 g) | 9.36 ±0.28[a,d] | 1.69 ±0.18[b,c] | -7.67 ±0.37[e] | 0.001[1]* |
| T2 (sorghum tempeh a dose of 3.041 g) | 9.29 ±0.21[a,d] | 2.95 ±0.18[a,c] | -6.34 ±0.32[e] | 0.028[1]* |
| T3 (sorghum tempeh a dose of 6.081 g) | 9.30 ±0.29[a,d] | 5.33 ±0.47[a,d] | -3.97 ±0.33[e] | 0.001[1]* |
| *P* | 0.007[3]* | 0.001[3]* | 0.001[3]* | |

Note: [1]Paired t Test; [2]Wilcoxon Signed Ranks Test; [3]Kruskal Wallis Test; [a]Significantly different from normal control; [b]Not significantly different from normal control; [c]Significantly different from atherogenic diet induction control; [d]Not significantly different from atherogenic diet induction control; [e]The comparison was significant (T1 and T3-groups), was not significant (T1 and T2-groups, T2 and T3-groups).*Significant ($p$ <0.05).

**Table 7** Correlation between LDL and MDA Levels.

| LDL and MDA | *p* | *r* |
|---|---|---|
| Before Treatment[1] | 0.001* | 0.610[a] |
| After Treatment[2] | 0.001* | 0.805[a] |

Note: [1]Rank Spearman;[2]Pearson; [a]Strong Correlation.*Significant ($p$ <0.05).

Moreover, these characteristics describe atherosclerotic conditions and require intervention **(NCEP, 2001)**. Induction with the atherogenic diet contains high cholesterol and cholic acid continued for up to 6 weeks in C2-group and caused the increase of cholesterol total, LDL, and MDA levels **(Yamada et al., 2017)**.

Cholesterol abnormalities lead to changes in cholesterol content, including LDL **(Pirillo et al., 2018)**. Moreover, LDL levels have a strong positive correlation with MDA levels and this proves that LDL contributes to the oxidative stress process, which is marked by increased MDA **(Tsikas, 2017)**. Other studies stated that increased LDL and MDA have a positive correlation to vascular inflammation which is a clinical marker of atherosclerotic plaque, evaluated with $^{18}$F-FDG PET/CT **(Kaida et al., 2014)**. LDL accumulates in artery walls causing monocytes to differentiate into macrophages and secrete inflammatory mediators such as Interferon-γ (IFN- γ) and *tumor necrosis factor* (TNF). This inflammation triggers oxidative stress through the activity of myeloperoxidase, lipoxygenase, and ROS **(Gisterå and Hansson, 2017)**.

In this research, to tackle the impact of the development of atherosclerosis, sorghum tempeh and flour were used at different doses calculated based on the needs of human fiber and converted to 200 g of body weight of rats. The results showed a significant decrease in LDL levels followed by total cholesterol and MDA levels.

When traced from fiber content, the *cellulose synthase-like* (Csl) gene insoluble fiber (1,3; 1,4) -β-glucan in sorghum tempeh and flour has the potential to increase sorghum fiber content **(Ermawar et al., 2015; Ho et al., 2016)**. The digested fiber in the colon, fermented by the intestinal microbiota produces SCFA. SCFA is divided into three substrates regulating plasma cholesterol reduction, namely acetate, propionate, and butyrate. Cholesterol synthesis is reduced in the liver due to decreased acetyl CoA by the inhibition of *3-hydroxyl-3-methylglutaryl-CoA* (HMG-CoA) reductase. This increases the activity of the LDL receptor to reduce LDL levels **(Feingold, 2000; Chen et al., 2018)**.

The group treated with sorghum tempeh a dose of 6.081 g (T3) had the most reduced LDL levels, although it was not maximal until it was close to normal control. However, it was lower than the atherogenic diet induction control. This is because at this dose, the fiber needs were satisfied and the percentage of group feed consumption was greater than the two other treatments >90%. Other studies also suggested that food fermented by *Rhizopus oligosporus* has the potential as an anti-hyperlipidemia agent to inhibit cholesterol through SCFA in animals fed with high-fat diets **(Huang et al., 2018; Dimidi et al., 2019)**.

MDA is a parameter of oxidative stress in atherosclerosis **(Czerska et al., 2015)**. The results showed that its levels significantly decreased with treatment using sorghum tempeh and flour. When viewed from the antioxidant content in sorghum, 3-deoxyanthocyanidin is a phenolic that affects reducing oxidative stress **(Afify et al., 2012; Ramatoulaye et al,. 2016)**. Sorghum tempeh and flour both contain antioxidants at 52.21% and 75.18%. Meanwhile, high antioxidant activity reduces oxidative stress by increasing the antioxidant enzymes such as SOD and GSHPx **(Akyol et al., 2017; Hasanuzzaman et al., 2020)**.

Sorghum flour at a dose of 4.095 g (T1) was the most effecive at reducing MDA levels to the same as normal control. This is possible because the antioxidant content of this flour is greater than that of sorghum tempeh. Moreover, the flour does not go through immersion or heating processes such as in the process of producing sorghum tempeh, thus it is more likely to maintain greater amounts of antioxidants **(Liu et al., 2020)**. In line with similar studies tested on rats, it was stated that sorghum flour increased lymphocyte proliferation activity leading to higher SOD enzyme, CAT, and GSHPx activity. Therefore, the balance of antioxidants could be improved and MDA levels decreased **(Zakaria et al., 2011)**.

LDL and MDA levels had a strong positive correlation after treatment. This shows that efforts to reduce LDL levels also caused a reduction in MDA levels. Similar studies where sorghum was administered showed similar results. The administration of sorghum kafirin inhibited the increase in serum LDL in hyperlipidemic rats **(Cruz et al., 2015)**. Likewise, fermented sorghum reduced LDL levels in hypercholesterolemic rats **(Laleye et al., 2016)**. Another study where sorghum rice was administered along with black rice to rats with induced hypercholesterolemia in their liver for 12 weeks showed a significant reduction in hepatic LDL **(Liu, Huang and Pei., 2021)**. The same was true for interventions with fermented foods against MDA. There was a decrease in serum LDL and MDA in the liver with the intervention using fermented tea in rats induced by high fat **(Sun et al., 2019)**. Additionally, fermented tempeh also reduced MDA levels in rats induced by an atherogenic diet for 4 weeks **(Kurniasari et al., 2017)**.

The changes in body weight of the rats at the end of the study in T2 and T3-groups were not different from the normal control, although, they were lower than those of the atherogenic diet-induced control. Therefore, sorghum tempeh in the treatment of atherosclerosis following the method used in this study had the protective ability to repair molecules in blood circulation and the advantage of slowing weight gains.

## CONCLUSION

The rats induced with the atherogenic diet that received the intervention of sorghum tempeh and flour had decreased LDL and MDA levels. Furthermore, sorghum tempeh a dose of 6.081 g was the most effect at reducing LDL levels, although not the same effect as sorghum flour at reducing MDA levels. Sorghum tempeh and flour could be functional food for atherosclerosis treatment.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This study obtained ethical clearance from the Health Research Ethics Commission (KEPK), Faculty of Medicine, Diponegoro University Semarang No.94/EC/H/FK-UNDIP/IX/2020.

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# MONITORING OF RISK FACTORS OF CARDIOVASCULAR DISEASES IN ADULT MEN

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## ABSTRACT

The study aims to evaluate the monitoring of risk factors of cardiovascular diseases in the young population, which significantly contribute to the origin and development of cardiovascular diseases, such as peripheral artery diseases, atherosclerosis, stroke, and others. We focused on a group of young adult men (n = 110) in the age range of 30 to 50 years, which we selected from the database of 800 patients hospitalized in the Cardio Center in Nitra during 2010 – 2020. When evaluating the influence of meat products consumption frequency on biochemical parameters and BMI, we recorded a statistically significant effect at the level of $p$ <0.05 in the evaluation of meat products such as salami, brawn, and sausages. When consuming sausages, BMI values also increased with increasing frequency of consumption. The effect on BMI was also observed when eating salami, between consuming 1 – 2 times a week and not at all. We recorded a statistically significant effect ($p$ <0.05) in frequent consumption of brawn (1 – 2 weeks) on the level of HDL cholesterol. The most frequent fish consumption was 1 – 2 times a month for freshwater (51.8%) and marine fish (56.3%). Daily consumption of fruit was reported in 64.6% of men, while daily consumption of vegetables was recorded in only 44.6% of men. In the lifestyle assessment, we focused on probands' time spent on physical activity. Only 35.5% of men stated that they spend more than an hour a day on physical activity. 40% of men from the surveyed respondents were active smokers. Another risk factor for cardiovascular diseases is stress, which significantly affected up to 42.7% of respondents. Nutrition and lifestyle play an important role in the prevention of cardiovascular diseases, which significantly affect blood lipid parameters, vascular endothelial elasticity, and factors determining the etiopathogenesis of cardiovascular diseases.

**Keywords:** cardiovascular disease; risk factor; nutrition; lipid parameter; dietary habits

## INTRODUCTION

Cardiovascular disease (CVD) is a class of diseases constituting all conditions that affect the heart and the associated blood vessels, and it is currently the leading cause of death **(Villa et al., 2016; Lee and Chen, 2015)**. Cardiovascular events in young adults, particularly acute coronary syndrome, are considered to be critical health threats and a socio-economic burden, because such events may lead to devastating consequences including disability and mortality **(Tsai et al., 2018)**. While CVDs such as coronary heart disease and stroke manifest clinically in middle age or older adulthood, their origins begin much earlier **(Tarp et al., 2016; Ross, 1999)**. Risk factors for CVD are broadly classified as modifiable and non-modifiable risk factors. The latter include age, heredity, familial predisposition, gender, and ethnicity. Modifiable risk factors are divided into cardiometabolic factors such as hypertension, dyslipidemia, diabetes mellitus, and obesity (together constituting the metabolic syndrome) and lifestyle factors such as smoking, less activity, nutrition, and low socioeconomic status **(Kumar, 2014; Emery et al., 2018; Kataria et al., 2020)**. Since cardiometabolic risk factors for CVD have a significant genetic and familial basis, it might be relevant to especially target the young populations predisposed by family history of CVD risk **(Lin et al., 2020)**. Although women and men share most classic risk factors, the significance and the relative weighting of these factors are different. Some researchers have documented that age, hypertension, total cholesterol, and low-density lipoprotein (LDL-C) have a great influence on men. But smoking, diabetes, triglyceride, and high-density lipoprotein (HDL-C) levels mainly affect women **(Galiuto and Locorotondo, 2017; Gao et al., 2019)**.

Behaviors in young adulthood such as smoking, physical activity, and diet have a significant impact on lifespan and middle-aged cardiovascular health. Approximately 80% of premature CVD deaths could be prevented through regulation of these behaviors **(Alwan, 2011; Liu et al., 2012)**. Most young adults have a low short-term risk of CVD but many have a higher lifetime risk due to lifestyle risk factors **(Van der Pol-Harney et al., 2020)**. In Europe, CVD prevention has been a priority since the late 90s: guidelines focus on the promotion of a heart-healthy lifestyle and management of risk factors for patients with

established CVD as well as for the general population and include health-risk behaviors, such as dietary changes, exercise, and cessation of smoking and alcohol consumption **(Perk et al., 2012; Kotseva et al., 2016; Abbate et al., 2020)**.

Dyslipidemia is a major modifiable contributor to cardiovascular diseases **(Pan et al., 2016)**, elevated blood cholesterol accounts for nearly one-third of ischemic heart diseases **(Tripathy et al., 2017)**. Therefore, dyslipidemia constitutes a serious threat to population health and has become an important public health challenge **(Xing et al., 2020)**. Recommendations ESC/EAS 2019 for the management of dyslipidemias specified the content of cardiovascular risk categories, defined a new subcategory of CV risk, the so-called extremely high CV risk, introduced new, clearly lower target values for LDL-C, further strengthened the position of statins, removed several indication limitations for innovative pharmacotherapy. LDL-C target values range from 4.4 to 3.0 $mmol.L^{-1}$ **(Semková a Pella, 2020)**. Overweight and obesity in childhood and adolescence are the most prevalent modifiable risk factor for later CVD. Long-term weight loss following lifestyle interventions in youth has generally been disappointing, but if overweight or obese children regain optimal body mass index by adulthood, cardiometabolic risks attenuate to baseline **(Juonala et al., 2011; Bekkering et al., 2020)**. Among the many established risk factors for CVD, diet plays an important role **(Forouzanfar et al., 2015)**. In the past several decades, numerous studies have enhanced our understanding of the relationship between diet and cardiovascular health **(Pan et al, 2018)**. Individual dietary choices are believed to play an important role in CVD, as indicated by recent studies that have evaluated the effects of various habitual dietary patterns on the cardiovascular health of numerous populations **(Pase et al., 2011; Tisdel et al., 2021)**. Individual diet indices differ in their components and weighting, but most emphasize the high intake of fruits and vegetables, whole grains, nuts; moderate intake of low/nonfat dairy and alcohol; and low intake of sodium, processed meats, added sugar, and saturated fat **(Kourlaba and Panagiotakos, 2009; Yu et al., 2018)**.

### Scientific hypothesis

This study aims to evaluate the monitoring of risk factors for cardiovascular diseases in young adult men, which significantly contribute to the origin and development of cardiovascular diseases, such as peripheral artery diseases, atherosclerosis, stroke, and others.

## MATERIAL AND METHODOLOGY

We evaluated to influence of risk factors of cardiovascular diseases on lipid profile and BMI of adult men in productive age. We focused on a group of adult men (n = 110) in the age range of 30 to 50 years, which we selected from the database of 800 patients hospitalized in the Cardio Center Nitra during the years 2010 – 2020. This study was approved by the Ethics Committee of the Specialized Hospital St. Zoerardus Zobor (protocol number 10.6.2014). Selected respondents have either overcome the myocardial infarction or were diagnosed with angina pectoris and hospitalized after a procedure so-called catheterization. We used the questionnaire method for the detection of dietary habits and lifestyle of respondents. The questionnaire was applied individually by a single interviewer and was compiled by the Department of Human Nutrition. The questionnaire included questions concerning the socio-demographic situation of the subjects, anthropometric parameters, physical activity, smoking, and the impact of stress in their lives. The questions concerning the analysis of selected dietary habits were focused on the number of the consumed meals, their regularity, and the 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 Cardio Center 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. The anthropometric parameters - body weight (kg) and height (cm) were measured on outpatient electronic medical scales (Tanita WB-3000, Tanita Co., Tokyo, Japan). The body mass index (BMI) was calculated by dividing the body weight in kilograms by the square of the height in meters.

### Statistical Analysis

Statistical analysis was carried out using the program Statistica Cz version 10 (TIBCO Software Inc., Palo Alto, California, USA) and MS Excel 2010. 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 $p < 0.05$.

## RESULTS AND DISCUSSION

### *Basic characteristics of study participants*

From the group of adult men, 110 patients met the required criteria with a mean age of 43.94 ±5.9 years. From the obtained individual values, we calculated the basic characteristics of males (Table 1).

The body mass index BMI is one of the easiest indices obtained and is one of the most commonly used methods to determine adiposity associated with risk factors CVD **(Sheibani et al., 2020)**. We divided the BMI values into four groups according to **Zheng et al. (2021)**. Category from BMI <18.5 $kg.m^{-2}$ - malnutrition, BMI 18.5 – 24.9 $kg.m^{-2}$ - normal weight, BMI 25 – 29.9 $kg.m^{-2}$ - overweight, BMI ≥30 $kg.m^{-2}$ – obesity. Obese probands with a BMI ≥ of 30 $kg.m^{-2}$ (41.8%) had the largest proportion in the observed group. In the group BMI 25 – 29.9 $kg.m^{-2}$ with overweight we recorded 39.1% participants. The demographic characteristics of the study participants show that the majority of participants were married (61.9%) and completed their studies (59.1%).

A study to examine the relationship between lipid profile and incidence of CVD in young adults is associated with high, low LDL cholesterol levels HDL cholesterol levels, and high levels of triacylglycerols with an increased incidence of heart failure.

**Table 1** Basic characteristics of study participants.

| Characteristic | category | % | Characteristic | category | % |
|---|---|---|---|---|---|
| **Age (yrs)** | 30 – 34 | 10.9 | **Education** | basic | 5.4 |
| | 35 – 39 | 14.6 | | apprenticeship | 18.2 |
| | 40 – 44 | 12.7 | | graduation | 59.1 |
| | 45 – 50 | 61.8 | | **higher** | 17.3 |
| **BMI (kg.m$^{-2}$)** | <18.5 | 2.7 | **Family status** | **married** | 61.9 |
| | 18.5 – 24.9 | 16.4 | | **divorced** | 35.6 |
| | 25 – 29.9 | 39.1 | | single | 2.5 |
| | ≥ 30 | 41.8 | | | |

**Table 2** Basic characteristics of biochemical parameters of study participants.

| Parameters | Average ± SD | Min | Max |
|---|---|---|---|
| GLU (mmol.L$^{-1}$) | 6.34 ±1.7 | 4.26 | 12.31 |
| TC (mmol.L$^{-1}$) | 4.73 ±1.2 | 1.99 | 6.94 |
| HDL-C (mmol.L$^{-1}$) | 1.45 ±0.4 | 0.55 | 2.44 |
| LDL-C (mmol.L$^{-1}$) | 2.95 ±0.9 | 1.04 | 4.47 |
| TG (mmol.L$^{-1}$) | 1.71 ±0.9 | 0.43 | 5.78 |

Note: SD – standard deviation; Min – minimum value; Max – maximum value; TC – total cholesterol; (LDL-C) – LDL cholesterol; (HDL-C) – HDL cholesterol; TG – triglycerides; GLU – glucose.

**Table 3** Incidence of risk factors of study participants.

| Risks factors | % | Risks factors | % |
|---|---|---|---|
| **BMI** ≥25 **(kg.m$^{-2}$)** | 79.1 | **TC** ≥5.2 **(mmol.L$^{-1}$)** | 40.9 |
| **Smoker** | 40.9 | **HDL-C** <1.0 **(mmol.L$^{-1}$)** | 40.9 |
| Physical activity <30 min/day | 25.2 | **TG** ≥1,7 **(mmol.L$^{-1}$)** | 46.4 |
| Positive family history of CVD | 40.0 | **GLU** ≥5.6 **(mmol.L$^{-1}$)** | 62.7 |
| Stres | 42.7 | Blood pressure > 130/85 mmHg | 61.6 |

**Table 4** Effect of the frequency of consumption of selected processed meat on lipid profil and BMI of probands.

| Sausage | GLU mmol.L$^{-1}$ | TC mmol.L$^{-1}$ | HDL-C mmol.L$^{-1}$ | LDL-C mmol.L$^{-1}$ | TG mmol.l$^{-1}$ | BMI kg.m$^{-2}$ |
|---|---|---|---|---|---|---|
| 1-2 times per week | 6.3 ±1.5 | 4.6 ±0.4 | 1.2 ±0,4 | 2.8 ±1,0 | 1.5 ±0,8 | 20 ±8.3 [a] |
| 1-2 times per mounth | 6.2 ±1.4 | 4.8 ±1.2 | 1.1 ±0,3 | 3.0 ±0,9 | 1.9 ±1,0 | 29.3 ±4.5 [b] |
| no consume | 6.7 ±2.1 | 4.8 ±1.2 | 1.0 ±0,3 | 3.0 ±0,9 | 1.8 ±0,8 | 33.1 ±4.9 [c] |
| p-value | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* <0.05 |
| **Salami** | | | | | | |
| 1-2 times per week | 6.4 ±1.8 | 4.8 ±1.3 | 1.2 ±0.4 | 2.9 ±1.0 | 1.9 ±0.9 | 30.7 ±7.5 [a] |
| 1-2 times per mounth | 6.5 ±2.0 | 4.5 ±1.1 | 1.1 ±0.3 | 2.9 ±0.8 | 1.5 ±0.8 | 28.5 ±4.0 |
| no consume | 5.9 ±0.8 | 4.7 ±1.0 | 1.2 ±0.3 | 3.0 ±0.9 | 1.2 ±0.6 | 28.3 ±6.1[b] |
| p-value | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* <0.05 |
| **Brawn** | | | | | | |
| 1-2 times per week | 7.4 ±2.6 [a] | 4.5 ±1 | 0.9 ±1.3 [a] | 3.0 ±0.9 | 1.7 ±0.7 | 30.2 ±6.5 |
| 1-2 times per mounth | 6.0 ±1.4 [b] | 4.6 ±1.1 | 1.1 ±0.3 | 2.8 ±0.9 | 1.7 ±1.0 | 30.3 ±7.3 |
| no consume | 6.5 ±1.5 | 5.1 ±1.4 | 1.3 ±0.5 [b] | 3.2 ±1.0 | 1.8 ±0.8 | 29 ±6.1 |
| p-value | ***p* <0.05** | *p* >0.05 | ***p* <0.05** | *p* >0.05 | *p* >0.05 | *p* >0.05 |

Note: SD – standard deviation; TC – total cholesterol; (LDL-C) – LDL cholesterol; (HDL-C) – HDL cholesterol; TG – triglycerides; [a] Significant difference between 1-2 times/week and 1-2 times/mounth, [b] Significant difference between 1-2 times/mounth and no consume, [c] Significant difference between 1-2 times/ week and no consume.

The accumulation of triacylglycerols in cardiac muscle tissue may induce lipotoxic cardiomyopathy and cardiac steatosis. The latest recommendations emphasize the importance of early screening for lipid profile in young people because cumulative exposure to dyslipidemia in adolescence increases the future risk of CVD **(Kaneko et al., 2021)**. An overview of the average values of biochemical parameters can be found in Table 2. We monitored levels of glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triacylglycerols.

Cardiovascular disease (CVD) can be thought of as a continuum that begins with the presence of cardiovascular risk factors and proceeds via the progressive vascular disease to target organ damage, end-organ failure, and death **(Dzau et al., 2006)**.

Current epidemiologic predictions show that the world is heading for a vascular tsunami of pandemic proportions. The number of people at high risk from cardio-vascular disease is increasing **(Dalhöf, 2010)**.

**Table 5** Effect of the frequency of physical activity on lipid profil and BMI of probands.

| | **GLU** mmol.L$^{-1}$ | **TC** mmol.L$^{-1}$ | **HDL-C** mmol.L$^{-1}$ | **LDL-C** mmol.L$^{-1}$ | **TG** mmol.l$^{-1}$ | **BMI** kg.m$^{-2}$ |
|---|---|---|---|---|---|---|
| 15-30 minutes per day | 6.65 | 4.40 | 1.01 | 2.70 | 1.65 | 32.20 |
| 30-60 minutes per day | 6.74 | 4.26 | 1.06 | 2.60 | 1.57 | 30.31 |
| >60 minutes per day | 6.32 | 5.05 | 1.28 [a] | 3.17 | 1.80 | 27.80 [a] |
| no physical activity | 5.73 | 4.95 | 1.15 | 3.17 | 1.76 | 30.29 |
| p-value | *p* >0.05 | *p* >0.05 | ***p* <0.05** | *p* >0.05 | *p* >0.05 | ***p* <0.05** |

Note: SD – standard deviation; TC – total cholesterol; (LDL-C) – LDL cholesterol; (HDL-C) – HDL cholesterol; TG – triglycerides; [a]Significant difference between 15-30 minutes/day and >60 minutes/day.

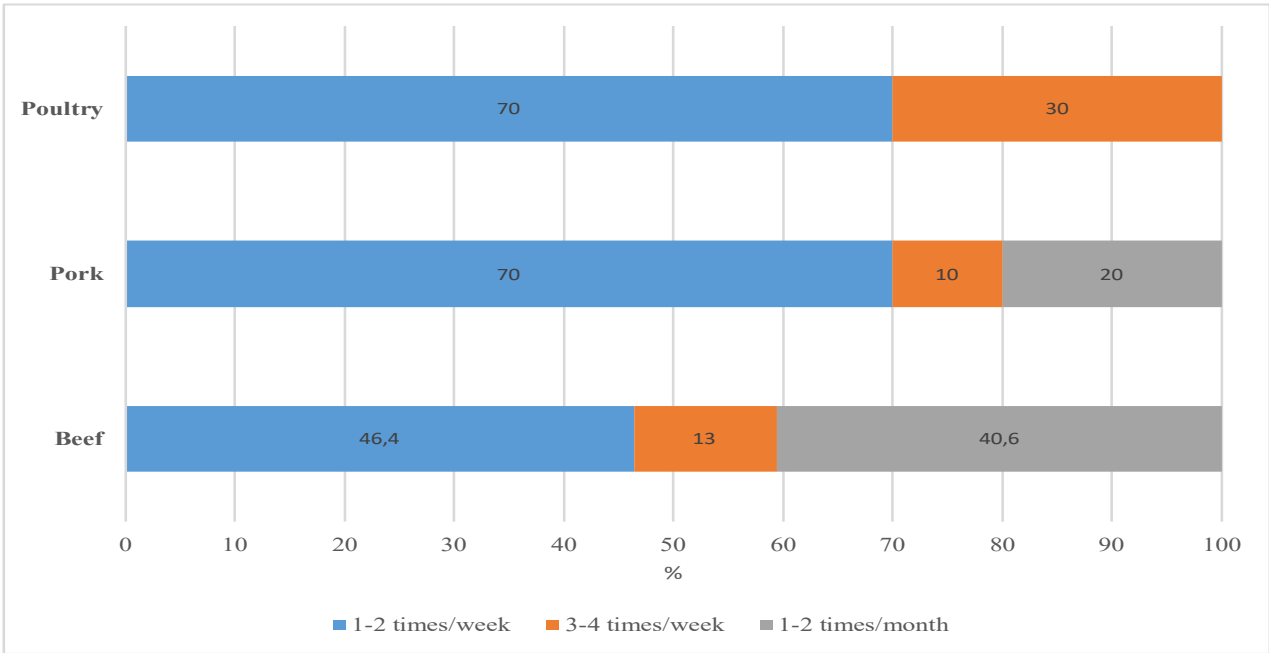


**Figure 1** Percentages of consumption frequencies meat of probands.

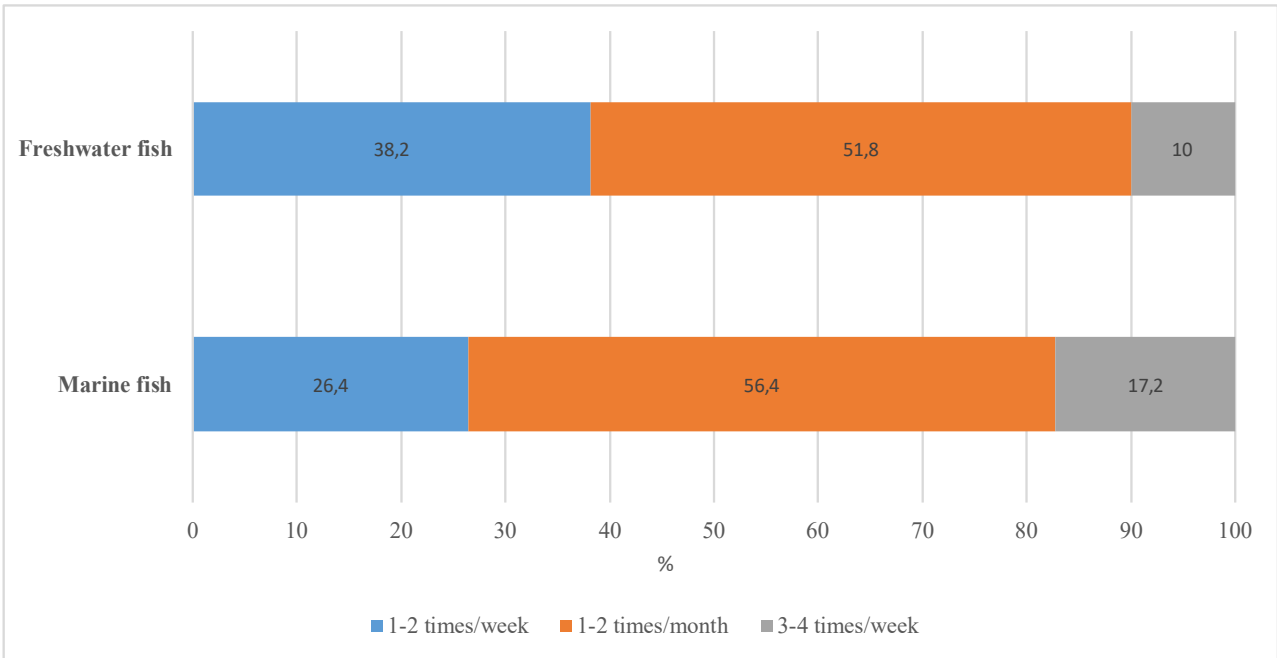


**Figure 2** Percentages of consumption frequencies fish of probands.

Table 3 shows the most serious risk factors for CVD according to the NCEP III. **(American Medical Association, 2001)** and the percentage of men in the study a set of probands. The most common risk factor in men was BMI ≥25 $kg.m^{-2}$, which we recorded 79.1% of probands. Elevated fasting glucose was observed in 62.7% of probands. Total cholesterol values ≥5.2 $mmol.L^{-1}$ we recorded 40.9% of men and HDL cholesterol <1.0 $mmol.L^{-1}$ was recorded in 40.9% of men. Increased concentration of triacylglycerols ≥1.7 $mmol.L^{-1}$had 46.4% of probands. Blood pressure > 130/85 mmHg had 61.6% of probands. **Wright et al. (2007)** observed the association of cardiovascular disorders with a positive family history of CVD risk. Positive family history of CVD risk affects the response to stress, which may, in turn, contribute to the risk of future CVD. 40% of men said it occurred in their family myocardial infarction or stroke. 40% of men from the surveyed probands were active smokers. Another risk factor for cardiovascular diseases is stress, which significantly affected up to 42.7% of probands (Table 3).

***The frequency of consumption of selected products of study participants***

Among the various foods, meat plays a key role in human diets, since it is the richest source of proteins, essential amino acids, minerals, vitamins, and other micronutrients **(Lafarga and Hayes, 2014)**. However, several studies have reported that diets high in red and processed meats are associated with metabolic syndrome and cardiovascular disease **(Simpson et al., 2019)**.

The most preferred meat in the monitored group of probands was poultry meat, which consumes 70% of participants 1 – 2 times a week and 30% of participants 3 – 4 times a week (Figure 1).

**Kopčeková et al. (2015)** have recorded of patients with cardiovascular diseases consumption of meat 3-4 times per week in 60.28% of men and **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.

**Hassannejad et al. (2021)** found that total red meat intake had a significant, direct association with lipid profile. **Cocate et al. (2015)** showed that those in the highest tertile of red meat consumption (≥81.5g/day) had a greater occurrence of hypertriglyceridemia. However, epidemiological studies reveal no authoritative connections between the intake of red and processed meat and the occurrence of CVD **(Delgado et al., 2021)**.

Our results show, that in the evaluation of the influence of the frequency of consumption of meat products on biochemical parameters and BMI, we recorded a statistically significant effect at the level of $p$ <0.05 of meat products such as salami and brawn. We also recorded a statistically significant effect ($p$ <0.05) of regular consumption of sausages (1 – 2 times a week compared to y 1 – 2 times a month and no consume) on BMI of probands (Table 4). In the consumption of salami, we found a statistically significant effect ($p$ <0.05) on the level of triacylglycerols in the group of probands who consumed salami 1-2 times a week and no consumption. HDL cholesterol levels were lower in probands who consumed brawn 1-2 times a week than in probands who did not consume. The highest TC, LDL-C, and TG values were seen in the male who consumes meat products 1 – 2 times per week. **Kontogianni et al. (2008)** found that a high intake of red meat (more than 8 servings/month) was associated with an increased risk of an acute coronary syndrome, but low income (less than four servings/month) showed no association.

Over the last few decades, the nutritional benefits of fish and polyunsaturated fatty acids on cardiovascular health have garnered great public health attention. Long-chain omega-3 polyunsaturated fatty acids (PUFAs) may prevent CVD by rendering antiarrhythmic effects and reduced blood viscosity, inhibiting platelet aggregation, lowering blood viscosity, suppressing inflammation, improving blood vessel function reducing plasma fibrinogen and insulin resistance **(Rhee et al., 2017)**.

Figure 2 shows the consumption of fish of study participants. They consumed freshwater fish more often, their consumption 1 – 2 times/week was reported by 38.2% of men, and 26.4% by sea fish. Most men consume fish 1-2 times/month in freshwater and marine fish. **Alhassan et al. (2017**) found that consuming fatty fish in the range of 20-150g/day leads to a significant reduction in plasma triacylglycerols and an increase in HDL cholesterol.

Many studies have shown a positive effect of fruit and vegetable consumption on some risk factors for CVD, such as lipid concentrations, inflammatory markers, and blood pressure **(Gan et al., 2015)**. As many as 64.6% of probands our study stated that they consume fresh fruit daily. 18.2% of probands consume 3-4 times/week and 17.3% consume fruit 1 – 2 times/week. 44.6% of men reported daily consumption of vegetables. 22.7% consumed vegetables 3 – 4 times/week and 23.6% consumed 1 – 2 times/week.

***Lifestyle and its effect on biochemical parameters of study participants***

In the lifestyle of the probands, we focused on their physical activity, smoking, stress factors, and sleep. Current physical activity guidelines for the secondary prevention of CVD prescribe at least 6500 – 8500 steps per day and 150 min of moderate-intensity aerobic exercise training per week, spread over at least five days **(Piepoli et al., 2016)**. **Williamson et al. (2021)** substantiate that as even modest levels of physical activity are associated with health benefits, the dose-response relationship observed between physical activity and CVD risk indicates that improving physical activity levels in small increments can also help mitigate CVD risk.

We found that only 35.5% of men engage in physical activity for more than an hour a day. 18.2% of men perform 30 – 60 min. physical activity a day, 20.5% only 15 – 30 min. a day, and 20.9% of men do not engage in any physical activity. The effect of the frequency of physical activity on the lipid profile and BMI of study participants is shown in Table 5. We recorded statistically significant results between frequency of physical activity 15-30 minutes/day and >60 minutes/day on the HDL cholesterol and BMI of probands ($p$ <0.05).

According to **Nayeri and Middlekauff (2020)** nicotine, although not a carcinogen, is sympathomimetic. The increased sympathetic tone is known to increase cardiac risk through many potential mechanisms, including increased heart rate and blood pressure, vasospasm, and arrhythmias,

and may contribute to inflammatory atherosclerosis. We found that 40% of men from the surveyed respondents were active smokers.

Another risk factor for cardiovascular diseases is stress, which significantly affected up to 42.7% of probands. Psychologically stressful experiences evoke changes in cardiovascular physiology that may influence risk for cardiovascular disease **(Ginty et al., 2017)**.

## CONCLUSION

Nutrition and lifestyle play an important role in the prevention of cardiovascular diseases, which significantly affect blood lipid parameters and factors determining the etiopathogenesis of cardiovascular diseases. Men aged 30-50 are often an underestimated group in cardiovascular disease because cardiovascular disease is most common in the elderly. From the point of view of the effect of risk factors on CVD, it is important to focus on early intervention in lifestyle changes of the young population to reduce the effect of these factors. The sooner the effect of risk factors can be reduced or eliminated, the lower the risk of CVD. In preventing CVD, we evaluated several risk factors, whose impact has been confirmed by many studies.

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# JUSTIFICATION OF THERMODYNAMIC EFFICIENCY OF THE NEW AIR HEAT PUMP IN THE SYSTEM OF REDISTRIBUTION OF ENERGY RESOURCES AT THE ENTERPRISE

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## ABSTRACT

The article evaluates the energy resources of the components of the environment and the prospects for their use on the redistribution with the creation of local zones of cooling and heating. The physical basis of the principle and systems of redistribution and transformations of energy resources of environments with coverage of the role of compensation processes is given. The use of closed energy circuits with intermediate energy sources, which are subject to phase transitions of evaporation and condensation, and data of energy potentials of ambient air, which are practically achievable for use on this basis, is proposed. The article shows the advantages of arranging systems for redistribution of thermal potentials based on the use of phase transitions of material media. Determination of energy balances of energy redistribution systems is carried out with the indication that in the end, such a method is the most energy-efficient. Upon completion of technological tasks, local areas with different energy potentials and temperatures degrade in dissipation processes and transform to the level of environmental indicators. This means interfering with the environment only at the level of energy costs in compensation processes. The article shows the transition to secondary recovery systems of energy resources based on the use of primary energy sources in environmental transformations at the levels of increasing their energy potentials and providing phase transitions with appropriate mathematical formalizations. A regression analysis of the feasibility of using primary energy potentials is given. It is proved that in the heat pump due to the generated mechanical energy the heat return at the level of the lost one. The estimation of the general condition of processes at power effects is given. The offered air pump and system of realization of a refrigerating cycle are considered. The redistribution of energy potentials of natural, forcibly created environments or systems and the synthesis on this basis of powerful heat fluxes in combination with advanced control methods, allows you to control their values of thermodynamic parameters.

**Keywords:** energy circuits; recuperation; phase transitions; internal resources; heat pump; condenser

## INTRODUCTION

The physical parameters of the globe, relating to the magnetosphere, atmosphere, lithosphere, and hydrosphere, correspond to the conditions of existence of the entire biological world and human society. In this definition, the energy component is no less important than the material, despite its differences from the coordinates, seasons, or time of day. After all, even under the harsh conditions of the Arctic and Antarctic, the air levels are quite far from absolute zero. Moreover, this corresponds to areas of human habitation with air temperatures above 0 °C.

The existence of the Earth's hydrosphere and lithosphere is accompanied by energy potentials created by interactions on a planetary scale. At the same time, the stabilization of the energy balance in this interaction ensures the existence of the biosphere under the accepted conditions. From this point of view, the authors **(Nochnichenko, and Yakhno, 2020)** believe that maintaining this balance or at least strict restrictions on its violation are crucial. Therefore, there is no alternative to the use of renewable resources and one of these areas is the use of technologies for redistribution of energy potentials of the environment based on heat pumps, air conditioners, refrigeration systems.

The properties of heat pumps provide **(Matsevity et all., 2014)** their significant use in connection with the possibility of transformation of energy potentials in a wide range of temperatures. However, the application of applied

problems often requires the expansion of these intervals. In the global dimension, one-third of energy consumption is associated with the industrial sector ultimately consumed as heat.

**Analysis of model approximations of medium types**

A significant amount of modern research concerns the topic of opportunities for transformations of energy potentials. Research **(Kosmadakis, 2019)** on the prospects of recovery of low-temperature thermal waste is devoted to assessing the potential of industrial (high-temperature) heat pumps for waste heat regeneration **(Xu et al., 2019)**. In the paper **(Lei Wang et al., 2018)** it is presented that the expansion of the theoretical base is supplemented by experimental studies of the characteristics of the heat pump of the triple loop for heat recovery of exhaust air in winter. The authors **(Hyunjeong et al., 2017)** made a clear technical addition in search of energy-saving potentials. They pointed out that the use of heat pipes on geothermal sources is relevant.

It should be noted that in the work of the authors **(Wang et al., 2018)** the traditional ways in the technical representation of heat transfer processes logically supplement by options for heat recovery in drying plants of the food industry.

The authors **(Lim et al., 2019)** focused on absorption heat pumps in heat flow recovery studies simultaneously with the water-saving modification for a cogeneration system with a steam-gas combined steam cycle. Geothermal heat pumps are recognized as one of the promising technologies. According to the authors **(Cui et al., 2019)**, attention to this needs a feasibility study due to high capital investment and the cost of installation and technological support. In experimental researches, the problem of the creation of the system of dehumidification of fresh air with the use of the heat pumps completed with the heat exchangers in which the working surfaces covered with a dehumidifier is solved. The research **(Bin Hu et al., 2019)** is investigated the centrifugal heat pumps with the utilization of the fulfilled heat with considerable thermal power and high temperature of water supply.

Therefore, the rational use of heat pumps with simultaneous heating and cooling of local areas is carried out based on exergy analysis. Here, the prototype heat pump operates in three main modes. The authors **(Paul Byrne, and Redouane Ghoubali, 2019)** consider these regimes quite clearly, who noted that:

- the first – the heating mode which provides receiving of hot water with the use of heat available in ambient air;
- the second – the mode of cooling of cold water and heat removal to ambient air;
- in the third mode, hot water is supplied due to the heat taken away from the cold water.

Thus, the authors **(Woolley et al., 2018)** noted that the recovery of industrial thermal waste in a systematic approach is assessed at the level of limiting the negative effects on the environment.

Recent advances in heat pump systems **(Guo-Hua Shi et al., 2019)** involve the direct use of solar radiation, which is conducive to the environment through low-temperature environmental energy and solar radiation. It is shown that the integration of solar evaporators with photoelectric energy technologies in combination with thermal storage and the use of heat pipes is promising for use in different climatic conditions. Thus, thermal systems in which phase transitions are carried out with the relative ease of regeneration, remain relevant for theoretical and practical research.

**Scientific Hypothesis**

Defined in the form of phenomenological analysis of thermodynamic efficiency of air heat pump using the energy potential of the environment in the heat supply system and redistribution of energy resources and prospects for use in the food industry.

## MATERIAL AND METHODOLOGY

**Samples**

The system of redistribution and transformations of energy resources of environments is considered. Theoretical and practical experience of thermodynamics specialists has allowed, among other things, to generalize the relationship between air heat capacity and temperature. Molar heat capacity at p = const, kJ / (kmol • K) is represented by the dependencies in the actual value in the interval 0-1000 °C:

$$c_{\mu p} = 28{,}7558 + 0{,}0057208t \quad (1)$$

and on average

$$\bar{c}_{\mu p}\Big|_0^t = 28{,}827 + 0{,}002708t\,. \quad (2)$$

Numerical values of heat capacities are given in Table 1, from which follows the possibility of certain comparisons.

Estimation of energy potential in 1 m$^3$ of air at a temperature of 303 K at a pressure of 1 bar, is:

$$Q_{pot} = c_p\, vT = 1.2971\ 1.0\ 303 = 393.0213\ kJ \quad (3)$$

**Table 1** Heat capacity of air.

| Temperature, °C | Heat capacity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Molar, kJ / (kmol • K) | | | | Mass, kJ / (kg • K) | | Volume, kJ / (m$^3$ • K) | |
| | $c_{\mu p}$ | $c_{\mu v}$ | $\bar{c}_{\mu p}\Big\|_0^t$ | $\bar{c}_{\mu v}\Big\|_0^t$ | $\bar{c}_p\Big\|_0^t$ | $\bar{c}_v\Big\|_0^t$ | $c_p\Big\|_0^t$ | $c_v\Big\|_0^t$ |
| 0 | 29.073 | 20.758 | 29.073 | 20.758 | 1.0036 | 0.7164 | 1.2971 | 0.9261 |
| 100 | 29.266 | 20.951 | 29.152 | 20.833 | 1.0061 | 0.7193 | 1.3004 | 0.9295 |
| 200 | 29.676 | 21.361 | 29.299 | 20.984 | 1.0115 | 0.7243 | 1.3071 | 0.9362 |
| 300 | 30266 | 21.951 | 29.521 | 21.206 | 1.0191 | 0.7319 | 1.3172 | 0.9462 |

Cooling this volume of air to 272 K means heat transfer Q to the receiver. At the same time, we note that the same volume of air at -30 °C or 243 K has a potential of 315.1953 kJ. Thus, technological opportunities for the redistribution of energy potentials open up prospects for their transformation and use, due to the second law of thermodynamics.

**Chemicals**

Air is a mixture of gases. The composition of the air is not constant and varies depending on the area, region, and even the number of people around you. Air consists of about 78% nitrogen and 21% oxygen, the rest are impurities of various compounds. Its chemical formula is O2. Under normal conditions (temperature 0 °C, pressure 101.3 kPa) oxygen is in a gaseous state, with no mass of taste, odor, slightly heavier than air.

**Instruments**

The flow temperature at the pump inlet and outlet was determined, after which the temperature value was averaged. Several thermocouples were used to measure the flow temperature in the middle of the pipeline. The data of the primary temperature transducers were fed to the ADC, converted into a digital signal, and entered into the PC. Data registration interval - 10 s. Measurements were performed with chromel-kopel thermocouples, measuring device brand Expert (manufacturer Ukraine).

At regular intervals, the surface temperature t was measured. The surface temperature of the module was determined by a thermocouple embossed into the surface. At regular intervals, the air pressure was measured. Measurements were performed with a manovacuummeter OBMV1-1006f (manufacturer Ukraine). The value of pressure in the tables of properties of water vapor was determined by its temperature.

The determination of ambient air parameters was done by using a psychrometer brand VIT-2 (manufacturer Ukraine). There was a registration of air parameters, namely its temperature, humidity, moisture content, enthalpy. The measurement of these parameters was performed using the ADC module "Arduino" and a computer.

A digital humidity sensor SHT 10 was used to determine the parameters of the air coming from the pump. This allowed determining with high accuracy the characteristics of the air directly in the flow during the experiments. The program interface of the ADC converter is made in such a way that the change of air parameters from time to time is reflected in the form of graphical dependencies in the online mode. The use of such a scheme with the use of frequency control of the pump drive allowed to quickly intervening in the course of the experiment that simplifies its planning.

**Laboratory Methods**

The fluid flow simulation was performed using ANSYS CFD general-purpose computer software from ANSYS, Inc.

Software systems allow modeling and calculation of liquids and gases, heat and mass transfer processes, reacting flows. ANSYS CFD is fully integrated into the ANSYS Workbench environment, which is the basis of engineering modeling, it integrates all ANSYS tools and software.

The ANSYS Workbench environment provides general access to such tools as: communication with CAD-complexes, construction, and modification of geometry and calculation grid. The software package is widely used for modeling processes that take place in pumps, fans, compressors, gas, and hydro turbines, etc. The ANSYS CFD-Post postprocessor, which is part of the software package, can be used to create high-quality animations, illustrations, and graphics **(Bezbakh et al., 2009,; Basok, and Bazeev, 2017**; **Nochnichenko, and Yakhno, 2018)**.

Theoretical modeling of conditions of creation and reproduction of processes of redistribution of energy potentials of environment and recovery of secondary resources based on provisions and laws of technical thermodynamics is used.

The research of the process was carried out according to the Box-Benken plan, which allows obtaining the maximum amount of objective information about the influence of factors with the help of the smallest number of experiments.

Processing of the obtained experimental data set was performed according to well-known methods and statistical processing methods to obtain an empirical mathematical model:

$$Q_{ke} = f_Q(x_1; x_2; x_3)$$

using methods of correlation and regression analysis of the approximating function, which characterizes the influence of factors and their interaction on the optimization parameter, ie productivity.

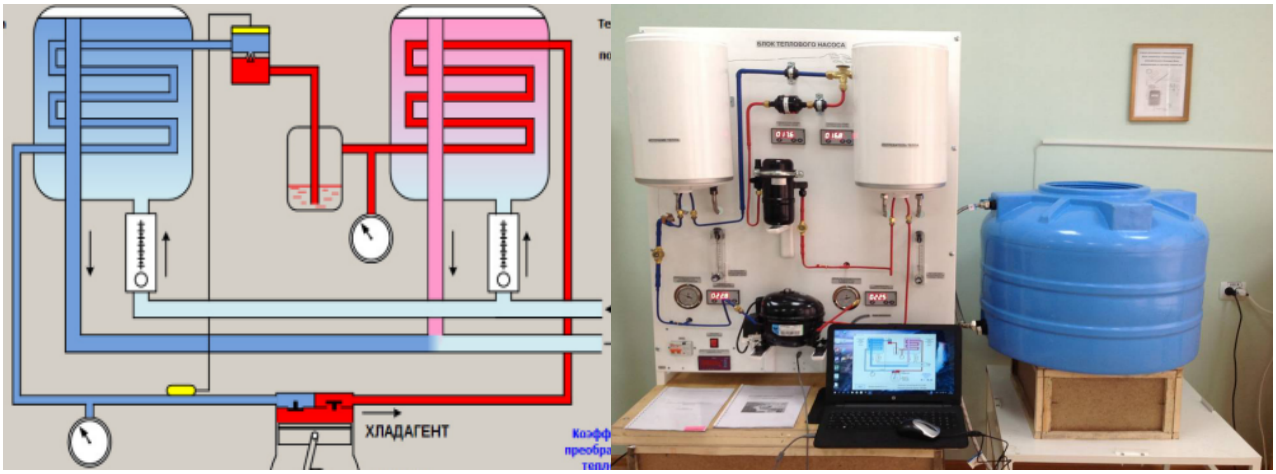
Based on the constructed regression equation, the contribution of each independent variable to the variation of the studied dependent variable is determined, ie the influence of factors on the performance indicator.

The experimental data set was processed using the Statistica-12 software package. The coefficients of the regression or approximating function equation, under the condition of orthogonality and symmetry of the plan-matrix of the planned factorial experiment, were determined according to the standard method according to known dependences.

The obtained results were statistically processed using the standard Microsoft Office software package.

**Description of the Experiment**

Theoretical and experimental research was performed fbased on the Problem Research Laboratory of the National University of Food Technologies. The study consisted of three parts. The first was to assess the energy potential of ambient air as the most accessible environment.




*a* *b*

**Figure 1** software module (a) and General view of laboratory experimental setup (b).

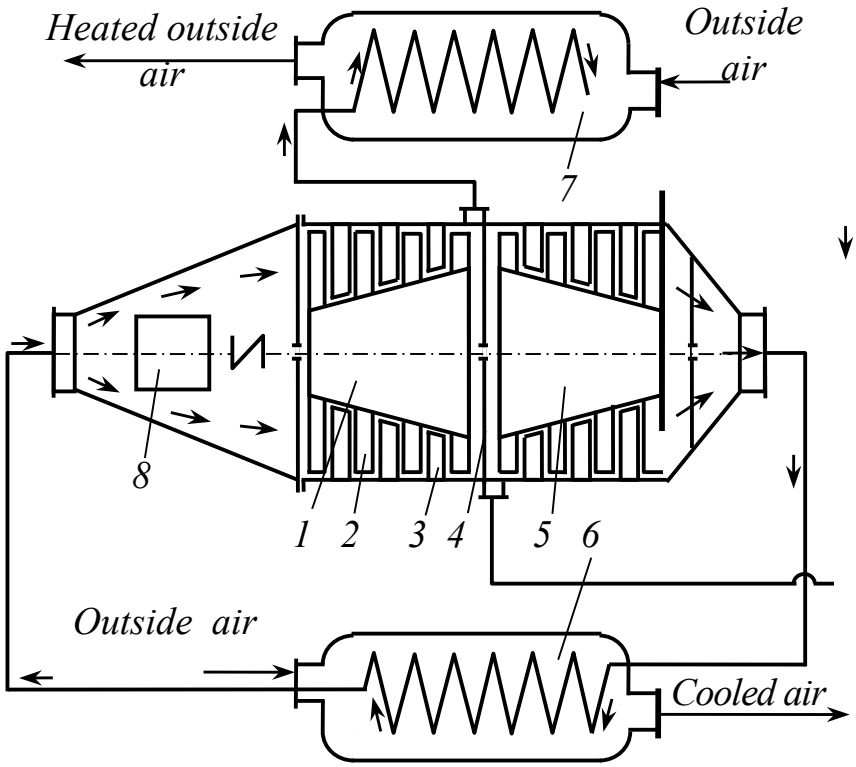



**Figure 2** Air heat pump.

This part of the research is the basis for the creation of a modern air heat pump ( a patent of Ukraine 17167) with the implementation of phenomenological studies relating to the redistribution of heat fluxes based on the second law of thermodynamics using a closed circulating air circuit.

The second part of the study concerns the thermodynamic features of achieving phase transitions of water as the main filler of food industry environments with the development of mathematical formalizations and estimation of energy consumption in the processes of heating the liquid phase and entropy transformations.

The third part is devoted to the implementation of phase transitions with the assessment of the energy potentials of the secondary pair. This creates a basis for the implementation of regenerative processes by increasing the pressures and temperatures in the compensation processes and subsequent condensation to obtain a liquid phase of $H_2O$ in closed energy circuits.

In this article, we examine the first and second parts of the research, which substantiates our further direction of theoretically directed mathematical analysis.

Historically, humanity has received such opportunities after the creation of heat engines. In 1852, Lord Kelvin proposed new use of the heating machine for space heating. Kelvin called such a machine a heat pump, the task of which was to cool the cold outside air and transfer the received thermal energy at a higher temperature in the room. This unnatural process of heat transfer from a cold

to a heated environment was carried out through the consumption of mechanical work. Each unit of mechanical work, brought to the ideal heat pump, before getting into the heated room "captured" 5 – 8 units of the heat of the outside air. Therefore, 427 k Gm of work at the inlet to the heat pump was converted into 6 – 9 kcal of heat at the outlet. The same kilocalorie formed by burning a certain amount of fuel is not supplemented by anything and remains one kilocalorie.

Burning some amount of fuel directly, bring in a heated room 1 kcal of heat. If the same amount of fuel will be burned in a heat engine, then only about 20%, equivalent to 85 kGm, will convert into mechanical work. If these 85 kGm are brought to the heat pump, it will provide 6 times more heat to the room, ie 6 • 85 = 510 kGm or 1,2 kcal. These ratios indicate the feasibility of using the primary energy potentials of the fuel in the circuit "heat engine - heat pump".

Thus, it is expedient to pay attention to equivalence in these thermodynamic transformations. Thus, the value of the coefficient of performance of heat engines is difficult or impossible to increase by 20%. In the heat pump due to the generated mechanical energy, heat returns to the level of lost.

**The general structure and principle of operation of the model of the laboratory installation and the "Air Heat Pump"** are shown in Figure 1 and Figure 2. The laboratory complex allows obtaining the dependence of the parameters of the heat pump installation on the temperature regimes of the heat supply and air conditioning system (Figure. 1 a, Figure 1b). In Figure 1a – the software module is given, and in Figure 1b – a general view of the laboratory experimental setup. The research aimed to establish the optimal parameters of the developed heat pump (Figure 2), which consists of a compressor 1 with blades 2, guides 3, partition 4, expander 5, heat exchangers 6 and 7, and a drive motor 8. It works as follows. The drive motor 8 provides rotational movement of the rotor of the compressor 1 and due to the interaction of the airflow with the blades 2 and the guide

Numerical data of energy costs and torque on the shaft of the electric drive depending on the load at a particular

**Statistical analysis**

It is worth recalling that a heat pump is an installation that converts low-potential natural thermal energy or heat from secondary low-temperature energy resources into the energy of higher temperature potential, which is already suitable for practical use. The transformations take place in the reverse thermodynamic cycle, and the transfer of energy from the lower temperature level to a higher one is performed due to a certain amount of mechanical (electrical) energy, which is externally supplied to the heat pump compressor and its design.

The algorithm for conducting experimental studies of the air heat pump, formalized in the form of a

the structural model scheme is shown in Figure 3. It involves determining the functional patterns of influence of individual input variables and their impact on the output

devices, 3 is its compression with increasing temperature. This compressed air enters the zone of separation of the internal volume of the pump and enters the heat exchanger 7, through which the external air flow. The latter absorbs thermal energy by cooling the compressed air. The cooled air is supplied to the expander 5, in which, expanding to a given final value of pressure, gives its energy. In this process, its temperature drops sharply to a temperature well below ambient temperature. Due to this, when in contact in the heat exchanger 6 with the flow of external air is heat transfer from the latter to the air circulating in a closed circuit. In the future, the cycle is repeated, and the cooled outside air is sent to further technological needs.

Pre-rough control of the compressor shaft speed of the compressor of the appropriate diameter was set using commands from the motor control panel of the control multisystem control and reading device Altivar 71 using Power Suite software version 2.3.0. The technical

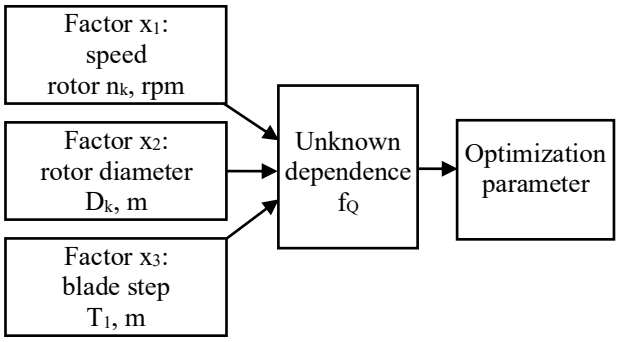



**Figure 3** Scheme of the model of the planned experiment of the PFE $3^3$ type.

capabilities of this device and software allow to smoothly change the speed of the motor shaft of the prototype laboratory installation in the range from 0 to 1300 rpm. The numerical value of the motor shaft speed (error within ± 1.5%) was recorded using a sensor type E40S6-10Z4-6L-5, which is connected simultaneously to the rotor of the motor and the multisystem device.

time of the experiment is displayed in the form of tabular data and graphical dependences on the PC monitor. value, or optimization parameter. To verify the adequacy of theoretical research (theoretical model) of productivity $Q_k$ , experimental studies of the model of the laboratory installation, which is shown in Figure 1 and Figure 2. To obtain an empirical regression equation characterizing the change in productivity $Q_k$ depending on the parameters of the compressor rotor, implemented a planned three-factor experiment such as PFE $3^3$.

The total number of experiments e N one repetition was determined by the formula:

$$Ne = P^k$$

Where:

$P$ – the number of levels of variation of the variable input factor;

$k$ – the number of active variables of input factors in the experiment.

**Table 2** The results of coding factors and levels of their variation.

| Factors | Marking | | Interval of variation | Levels of variation natural / coded | | |
|---|---|---|---|---|---|---|
| | natural | coded | | | | |
| Speed of rotation, $n_k$¢, rpm | $X_1$ | $x_1$ | 100 | 100/-1 | 200/0 | 300/+1 |
| Diameter $D_k$, m | $X_2$ | $x_2$ | 0.04 | 0.12/-1 | 0.16/0 | 0.2/+1 |
| Step $T_1$, m | $X_3$ | $x_3$ | 0.03 | 0.05/-1 | 0.08/0 | 0.11/+1 |

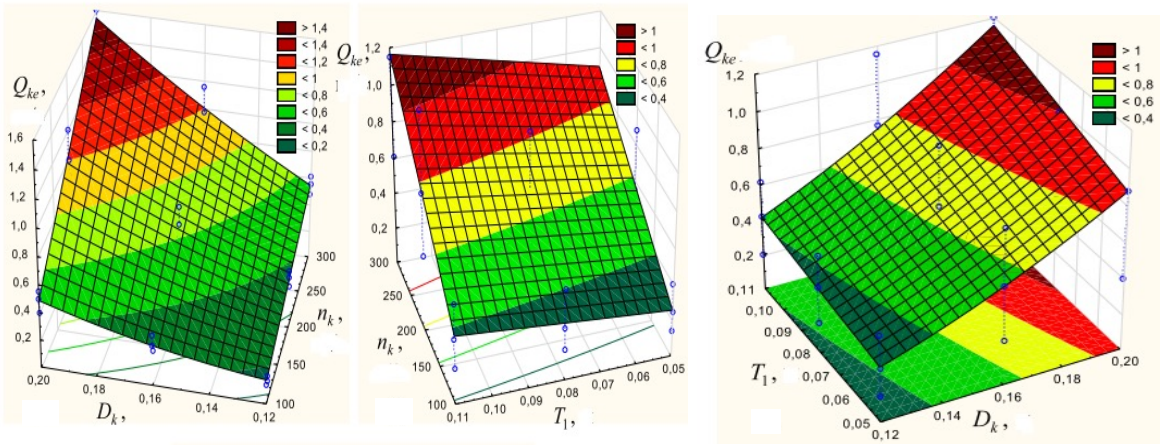



**Figure 4** Surface response changes in productivity in the form of functionality.

The experiments were performed in triplicate. The asymmetric plan-matrix of the planned three-factor PFE $3^3$-type Box-Benkin experiment for three factors and three levels of factor variation had a total number of experiments equal to 27.

The independent variables were: the speed of the rotor of the compressor $n_k$, which was encoded by the index $x_1$, ie $n_k$; the diameter of the rotor of the compressor $D_k$, which is encoded by the index $x_2$, ie $D_k$; blade pitch $T_1$, encoded by the index $x_3$, ie $T_1$.

Structural model of a planned three-factor **(Krutov, 1989)** PFE-type experiment $3^3$ shown in Figure 3.

Thus, to study the performance of $Q_{ke}$, an approximate mathematical model in the form of a functional dependence was chosen.

$$Q_{ke} = f_Q (x_1; x_2; x_3).$$

When compiling the plan-matrix of experiments, coded notations of upper (+1), lower (-1), and zero (0) levels of variation by factors were introduced **(Krutov, 1989)**, ie a three-factor experiment was performed at three levels of variation by input factors or a planned PFE $3^3$ type experiment was implemented.

The results of coding of variable input factors, the upper and lower level of variation of each factor, and the interval of its variation are given in Table. 2.

Because during the experiments independent variable input factors, ie $n_k$ are inhomogeneous, ie they all have different physical units and different orders of arithmetic numerical values of units, they were led to a single system of calculations by switching from the entered notation coded values to real (natural) values. Made a randomized plan matrix of the planned three-factor experiment type PFE $3^3$.

After estimating the statistical significance of the coefficients of the regression equation and checking the adequacy of the mathematical model of the logarithmic function, we obtained a regression equation that characterizes the functional change in productivity in natural quantities **(Krutov, 1989)**.

$$Q_{ke}=0.81+0.61ln(n_k)+1.33ln(D_k)+0.31ln(T_1) \quad (4)$$

With the probability level p = 0.95 and the value of the t-alpha criterion equal to 2.053, the following statistics were obtained: coefficient of multiple determination D = 0.893; multiple correlation coefficient R = 0.945; standard deviation of the estimate s = 0,150; Fisher's F-test is 64,212. The coefficient D is significant with the probability level P = 1.00000. The regression equation (4) characterizes the change in the performance of the air heat pump depending on the design and kinematic parameters within the following limits of change of input factors: speed nk (from 100 to 300 rpm); diameter Dk (from 0.12 to 0.2 m); blade pitch T1 (from 0.05 to 0.11 m). The functional change in productivity depending on the change in Qke factors is directly proportional - with increasing speed, diameter and pitch, the value of productivity also increases.

According to the regression equation (1), the response surface of the functional change in the productivity of Qke in the form of a functional is constructed:

$Q_{ke}= f_Q(n_k; D_k)$ *(Figure 4, a);*
$Q_{ke}= f_Q (n_k;T_1)$ *(Figure 4, b);*

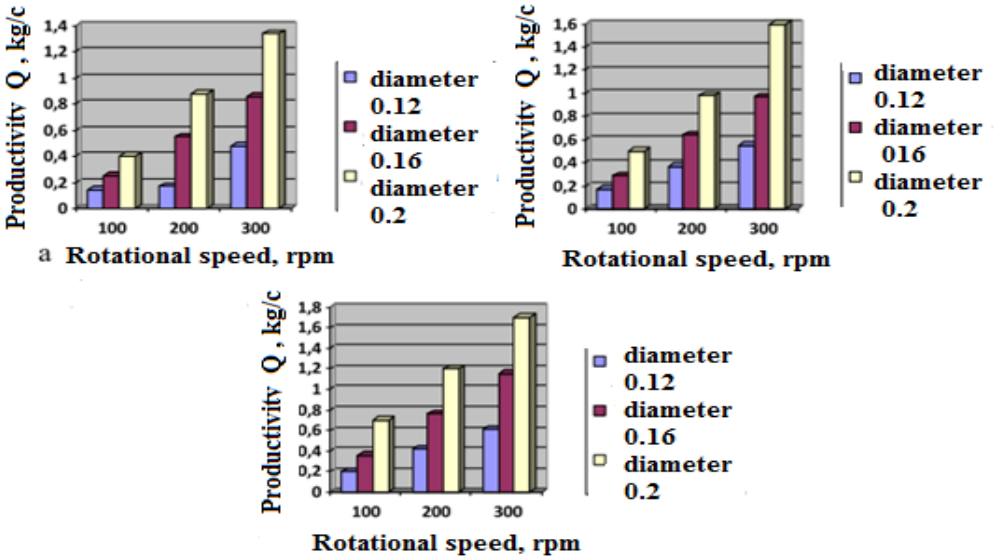



**Figure 5** The diagram of change of productivity Qke of the heat pump. Note: a, b, c – T1 = 0.05; 0.08 and 0.11 m.

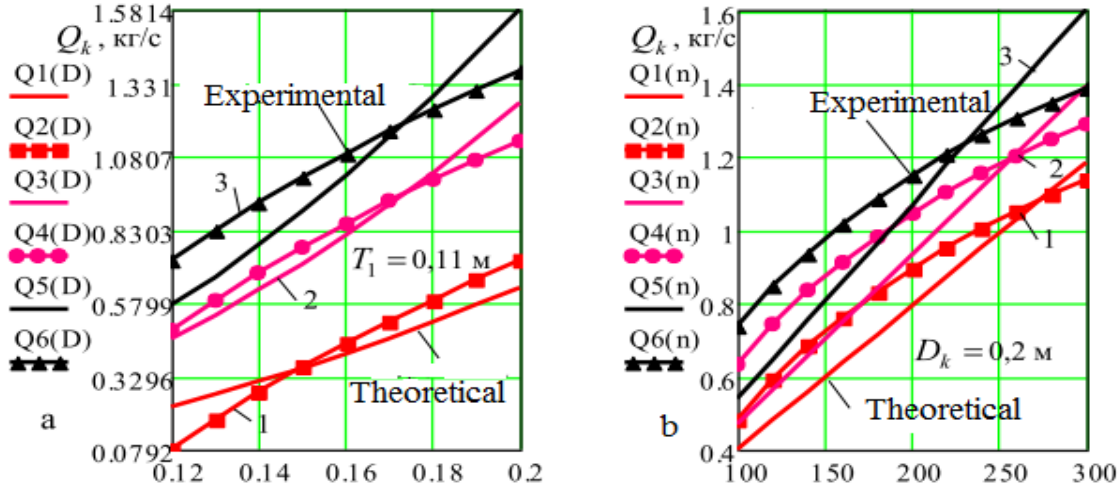



**Figure 6** Dependence of change in productivity Qe as a functional:a – $Q_k = f_{Qk}$ (D) , 1, 2, 3 – in accordance, $n_k$= 100; 200; 300 rpm; b – $Q_k = f_{Qk}$ ($n_k$), 1, 2, 3 – in accordance, $T_1$ =0,05; 0,08; 0,11 m.

*$Q_{ke} = f_Q$ ($D_k$;$T_1$) Figure 4, c).*

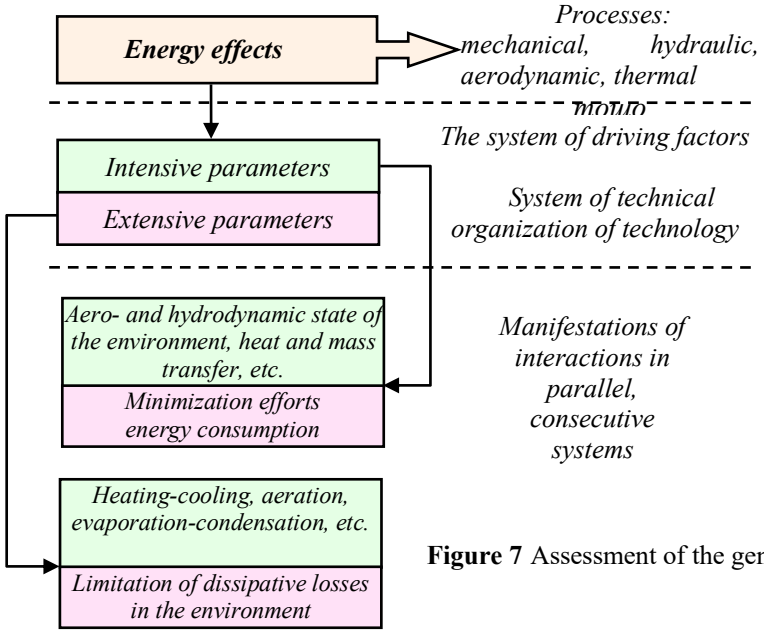



**Figure 7** Assessment of the general state of processes in energy effects.

The dominant factors that have a significant functional impact on the increase in productivity $Q_{ke}$ are the speed nk and diameter $D_k$, which is characteristic of the graphical interpretation of the response surface, and this is the regulation of energy potential, ie temperature.

Figure 5 represents a diagram of the change in the productivity of the $Q_{ke}$ heat pump, based on the obtained average results of experimental studies with three repetitions of each numbered factor field experiment according to a randomized plan matrix of the planned experiment type PFE $3^3$.

Based on the graph-analytical analysis (Figure 5) it can be stated that the nature of the functional change in the productivity $Q_{ke}$ of the heat pump. It is obtained for the limit values of the corresponding points of the a three-factor experiment type PFE $3^3$ compositional plan is quite adequate model $Q_{ke} = f_Q (x_1; x_2; x_3) = Q_{ke} = f_Q (n_k; D_k; T_l)$, Figure 4, Figure5, which is also characteristic of the dependence, which is shown in Figure 6.

The discrepancy between the experimental values of the performance $Q_{ke}$ of the pump obtained according to the regression equation (4) and the experimental values of the performance $Q_k$ (graphical dependences of Figure 7) is in the range of 5 – 10%.

## RESULTS AND DISCUSSION

This feature of energy transformations is based on the second law of thermodynamics with an indication of the need to use compensation systems by increasing the temperatures and pressures of energy sources in closed circuits.

An important advantage of the heat pump is that it implements "reverse" processes in the modes of heating and cooling of the premises as an ideal air conditioner.

The technical implementation of heat pumps and refrigeration machines based on the Carnot reverse cycle, which is the only achievement of humanity in the implementation of the principle of energy redistribution in existing parallel systems.

Returning to condition (3) we obtain an estimate of the heat flux dissipated from the cooling medium:

$$Q' = c_p v' \left(T_{(п)} - T_{(к)}\right) = c_p v' \left(t_{(п)} - t_{(к)}\right), \text{ kW},$$

Where:

$v'$ – volumetric flow of the gas phase supplied to the evaporator as part of the heat pump, $m^3$ / s;

$T_{(n)}$, $T_{(к)}$, $t_{(п)}$ and $t_{(к)}$ – initial and final absolute temperatures and temperatures in degrees Celsius.

Similarly, it is possible to determine the energy potentials of the liquid phases of lakes, rivers, seas and oceans, which are largely used.

The study **(Nikitin and Krylov, 2012)** estimates the ratio of redistribution of energy flows, according to which from 60 to 70% of energy costs related to the circulating circuits.

Understanding this situation in a significant number of cases **(Mandryka, 2017)** prompted attempts to use this energy component in favor of energy resources. It should be noted that the kinematic parameters of the gas-liquid medium are estimated to be approximately stable. Their transfer to the regimes characteristic of transitional processes should be assessed as a promising direction of intensification of energy resources.

Another direction **(Sniegkin et al., 2008)** concerns the use of secondary energy resources that accompany most industrial technologies. Their energy potentials elate to the solid and liquid phases of the input raw materials, to which the potentials of the vapor phases and gases are added in the transformation processes. The latter applies to a significant number of technological processes and individual complexes. Solving the problems of recovery of secondary energy resources is most appropriate to solve in parallel-synchronized flows. This largely applies to thermal systems in which phase transitions are carried out due to the relative ease of regeneration in them.

In cases of asynchronous situations, there is a need to use energy-saving storage devices. However, the positive results for parallel system designs are quite achievable even in mechanical systems in which transients are generated. In this case, in addition to energy effects, it is possible to regulate the movement of machines with restrictions on total dynamic loads.

In the general list of processes that take place in food, chemical, microbiological and other technologies, there are mechanical, hydraulic, aerodynamic, thermal interactions or various combinations of them (Figure 7). Manifestations of such interactions are in series and parallel systems with corresponding intensive and extensive parameters, based on driving factors, aero- and hydrodynamic states of environments, heat, and mass transfer surfaces, means of increasing energy potentials, energy loss compensators, and so on. The technical organization of technologies in general and at the level of individual processes require the interaction of material, energy, and information flows, the task of which is to achieve appropriate technological effects related to heating-cooling, evaporation-condensation, formation of concentrated media, gas-saturated systems, aeration, etc. At the same time, efforts to minimize energy costs and limit dissipative losses to the environment at a certain level of opportunity in the implementation of these provisions remain fundamental. Assessing the general condition, we turn to the example of the features of only one component of convective heat transfer.

During convection, heat is transferred during the mixing of cold and warm layers of liquids or gases, and therefore this process is inextricably linked with the mechanical motion of liquid and gas flows. Their theoretical basis relates to the relevant sections of hydro- and aerodynamics, but the level of complexity, even for simple cases in mathematical formalizations, is so significant in combination with thermal processes that it has led to limitations in the relevant scientific interests. However, it is convection in heat transfer mechanisms in heating systems, technological devices, electric drives, brakes, compressors, refrigeration units, etc. in terms of significance that led to the development and solution of applied problems.

Most of them concern the determination of heat transfer coefficients, which may depend on the thermal conductivity of the media, viscosity, density, heat capacity, kinematic parameters, and geometry of the media volumes. The effects of all these parameters are combined by the phenomenon of the boundary layer. The imaginary shirt creates the main barrier to heat transfer, which barriers that most effectively overcome in the modes of phase transitions

of boiling and condensation due to the activation of heat transfer coefficients. An additional positive effect of the phase transition concerns the production of a coolant with a thermodynamic parameter supplemented by the heat of vaporization.

Phase transitions open additional possibilities of transformation of parameters of pressure and temperatures that allow overcome natural prohibitions which features are formulated by the second law of thermodynamics. In the classical definition, this is achieved by supplementing the closed or partially closed circuits with a *compensatory process* of mechanical compression or the introduction of additional thermal potential with increasing pressure and temperature of the coolant circuit. As a result, such transformations in the reversed Carnot cycle, it is possible to transfer heat from less heated media and bodies to more heated ones, and the purpose of such transformations may relate to the problem of cooling (heating) the local zone. In the first case there is a use of the refrigerating machine, and in the second - the heat pump.

However, in addition to information on the creation of Lord Kelvin air heat pump with the transformation of energy potentials of air flows due to the relationship

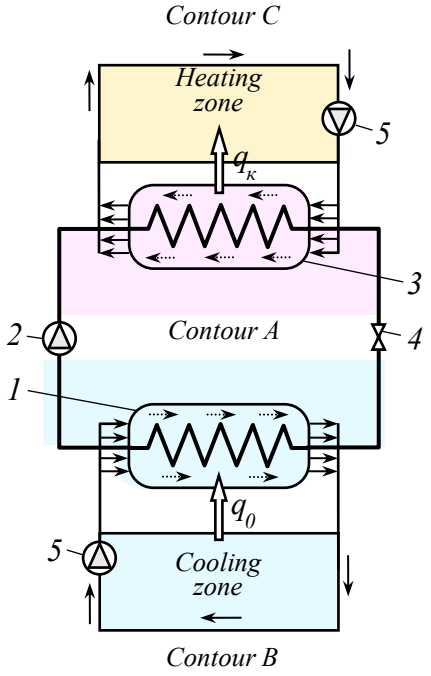



**Figure 8** System of realization of a refrigerating cycle: 1 – the evaporator; 2 – compressor; 3 – capacitor; 4 – throttle; 5 – fan.

between pressure and temperature, we note the consequences of continuing attempts to create their modern structures, which we consider in this paper.

This applies to the development of the patent of Ukraine 17167 "Air heat pump" (Figure 2).

The creation of initial energy potentials in such systems is carried out using primary energy sources. The latter in most cases relate to the resources of the generated vapor phase, electricity, energy of hydraulic systems, compressed air systems, or chemical energy of incoming raw materials. The presence of the latter is a constant factor in any technology, according to which the energy potential of recycled raw materials should be preserved as much as possible. However, the appropriate set of energy-material transformations provide by the influences of external energy flows due to which the set temperatures of technological processing of environments are reached. It can be carried out without achieving the modes of phase transitions or with their implementation. Consider the transformation of the energy potentials of airflows due to the relationships between the design parameters of the pump and the process.

***System of realization of energy potentials***

The potential of devices for increase of energy efficiency and intensification of processes of a mode of phase transitions and generation of steam, gas phase, or steam-gas mixes is high enough. Development of new designs of heat exchangers, evaporators, the definition of rational modes of their operation is possible only based on the data received at comprehensive researches of the processes proceeding in devices.

It is expedient to refer to the peculiarities of the cycles of refrigeration units or heat pumps from the point of view of creating analogies for systems of industrial devices in which there are modes of phase transitions and generation of steam, gas phase, or steam-gas mixtures. The existence of a closed circuit in the refrigeration cycle involves the combination of an evaporator as a steam phase generator (Figure 8), a compressor, a condenser, and a choke operating in synchronized parallel modes with the appropriate thermodynamic parameters.

In the closed circulation circuit A of the refrigerant, phase transitions occur due to the supply of heat flow $q_0$ from the cooling zone with circuit B and the removal of heat flow $q_k$ from the condenser in circuit C. Depending on the technological tasks, circuits B and C can be closed or open.

This leads to the conclusion that the total energy balance of circuit A is supplemented by the energy consumption $\ell_к$ of compressor 2, which meets the condition:

$$q_к = q_o + \ell_к, \qquad (6)$$

and the whole system in balance calculations must take into account power consumption in the circuits B and C. The decision of technical problems is achieved in one of the circuits B or C, or both simultaneously. It is important that the arrangement of energy-material connections of circuits B and C following the evaporator and condenser can be realized due to convective air flows of the medium, which are formed in response to the existence of a gravitational field. This solution is present in the installation of most domestic and industrial refrigeration systems and systems.

Cooling and heating zones can exist as local, but in cases where they are open, it means that they are interconnected through the environment, and technical systems of refrigeration systems, heat pumps, and air conditioners *act as programmable energy redistributors*. Important is the ratio of the potentials of the synthesized energy flows in the direction from zone B to zone C and the potential of the compensation processes, which can be up to 5 – 10 units. This means the possibility of creating powerful energy-intensive systems using compensatory processes, the limited structure of which is the ultimate negative result of the impact on the ecosystem. The last statement is because

the synthesized heat fluxes at the end of technological processes are dissipated with the equalization of temperatures following the law of the most probable state. To prevent further negative effects on the environment the power supply of the electric drive of the compressor-compensator deserves attention. The use of modern systems for the transformation of world energy into electricity in such cases would practically solve the
problems of energy security in a maximum way.

***Thermodynamic analysis of energy resources.***

A scientifically based analysis of energy processes is essential for an active energy-saving policy. Modern laws of thermodynamics **(Annex 49 summary report IEA ECBCS. –Fraunhofer IBR. -2011)** include the study of the properties of energy in its transformations in two approaches to efficient use: energy and exergy. Such approaches involve the use of two thermodynamic characteristics of energy - quantity and quality: quantity - in energy, both - in exergy.

Thus, the authors **(Kudelya, and Dubovskyi, 2020)** consider the possibility of obtaining work when the characteristics of the system (pressure, temperature, velocity, chemical composition, and potential energy of the system) differ from the characteristics of the state (parameters) of the environment. This possibility is completely lost when the system and the environment are in balance and calm concerning each other.

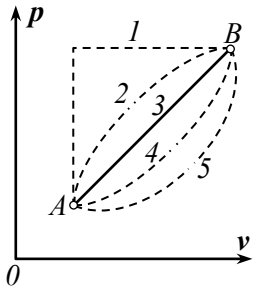



**Figure 9**. Dependence of p = p (v) in thermodynamic transformations parameters.

The magnitude of the work, as a quantitative measure of energy quality, is included in the equation of energy balance (First law of thermodynamics), and the condition of convertibility S gene ≥0 - in the equation of entropy balance (Second law of thermodynamics). Therefore, the authors **(Ebrahimi, 2012)** propose the use of low-potential heat in optimizing the generation of electricity taking into account the depth temperatures and heat exchange of wells with side rocks. In this case, the Rankine cycle was chosen as the theoretical basis for the calculation of a heat engine with distributed parameters.

The calculation of authors **(Domschkea, 2017)** based on the total energy consumption for main gas transportation for a multi-threaded network, which includes compressor stations, heat exchange areas with the environment, bridges, and branches

Modeling and optimization of gas flow through the pipeline network by the authors **(Domschkea, 2017)** used a hierarchical model based on one-dimensional isothermal Euler equations of fluid dynamics. Therefore, in the works **(Liu, et al., 2017; Orga, et al., 2017; Pouladi, 2017)** based on the analysis of the topological structure of the heating network, its hydraulic and thermodynamic parameters, a method of counting the heat flow of the network was developed. Taking into account the external heat inflow in the sections of pipelines minimizes the total heat consumption and improves the management of technological processes.

Based on the above, the physical state of the system is determined by the values of two variables out of three, namely pressure, volume, and temperature.

It is known that the physical state of the system is determined by the values of two variables out of three, namely pressure, volume, and temperature. There is a functional connection between these three parameters. In what follows, we will consider the pressure p and the volume v as independent variables, and then we will display this relationship in the form:

$$\mathrm{T} = \mathrm{f}(\mathrm{p},\ \mathrm{v}). \tag{7}$$

The set of values of p and v determines the position of a point on the plane p-v. Each such point corresponds to a certain value of temperature T (Figure 9).

Differential

$$\mathrm{dT} = \frac{\partial \mathrm{f}}{\partial \mathrm{p}}\mathrm{dp} + \frac{\partial \mathrm{f}}{\partial \mathrm{v}}\mathrm{dv} \tag{8}$$

is a complete differential. By changing the state of the system from the parameters at point A to the parameters of point B, the temperature at point B can be determined in the form:

$$\mathrm{T_B} = \mathrm{f}(\mathrm{p_B},\ \mathrm{v_B}). \tag{9}$$

Determining the values of the work performed by the system as a result of changing its state during the transition of parameters from point A to point B and, considering the process inverse, we reflect the dependence:

$$\mathrm{W_{A-B}} = \int_{\mathrm{v_A}}^{\mathrm{v_B}} \mathrm{p}\,\mathrm{dv}. \tag{10}$$

The graphical interpretation of the given integral is the plane under the transition curve on the p-v diagram. Since the transition from point A to point B can be done with different trajectories, it means that these areas will be different. Their area is to some extent determined by the design parameters and speed of the drive. It follows that the value of W depends on not only the coordinates of points A and B, but also on the selected transition trajectories. It is logical to assume that the amount of heat perceived in this transition of the system also depends on that, but the difference between the amount of perceived heat Q and energy W does not depend on the shape of the transition trajectory. The conclusion about the constancy of the difference Q – W, which corresponds only to the state of the system at points A and B, indicates a change in the internal energy u:

$$\Delta \mathrm{u_{A\to B}} = (\mathrm{Q} - \mathrm{W})_{\mathrm{A}\to\mathrm{B}} = \mathrm{F}(\mathrm{p_B},\ \mathrm{v_B}) - \mathrm{F}(\mathrm{p_A},\ \mathrm{v_A}). \tag{11}$$

In another form, expression (11) has the form:

$$\mathrm{du} = \mathrm{dQ} - \mathrm{dW} = \frac{\partial \mathrm{u}}{\partial \mathrm{p}}\mathrm{dp} + \frac{\partial \mathrm{u}}{\partial \mathrm{v}}\mathrm{dv}. \tag{12}$$

For the case of a closed trajectory from point A we obtain a curvilinear integral from du, then we have:

$$\int_{\mathrm{v_A}}^{\mathrm{v_A}} \mathrm{du} = \mathrm{u_A} - \mathrm{u_A} = 0. \tag{13}$$

The written curvilinear integral is called the circulation and is denoted by a symbol $\oint_{(\ell)}$,

Where:

$\ell$ – closed curve.

Bringing the heat flux Q to the medium means corresponding changes in the value of entropy. The latter is determined only by the variables that characterize the physical state of the system, and in the transition from point A to point B changes in entropy do not depend on its trajectory.

Herewith

$$\Delta s_{A\to B} = \int_A^B \frac{dQ_{зв}}{T}, \quad (14)$$

Where:

$dQ_{зв}$ – the amount of heat passing through the system boundaries during the reverse process.

In the elementary process we have:

$$ds = \frac{dQ_{зв}}{T}; \quad dQ_{зв} = T\,ds. \quad (15)$$

Replacement of values dQ і dW provided (12) leads to the form:

$$du = T\,ds - p\,dv, \quad (16)$$

in which there are point functions and complete differentials.

Integration (16) leads to the value of u as a function of the variables s and v in the form:

$$u = f_u(s, v), \quad (17)$$

or, disclosing condition (17), write:

$$du = \left(\frac{\partial u}{\partial s}\right)_v ds + \left(\frac{\partial u}{\partial v}\right)_s dv, \quad (18)$$

and by comparison with condition (16) we obtain:

$$\left(\frac{\partial u}{\partial s}\right)_v = T \text{ і } \left(\frac{\partial u}{\partial v}\right)_s = -p. \quad (19)$$

If condition (19) is known for a mass of any homogeneous liquid, then the parameters T, p and u can be calculated for any physical state of the medium, which is determined by the independent variables s and v. Therefore, the performance of the heat pump sets the value of the internal energy **u** of the process.

***Methods for determining the temperature field of a low potential energy source***

The paper **(Oosterkamp, Ytrehus, and Galtunget, 2016)** is aimed at analyzing the influence of extreme temperature conditions on heat transfer between a low-potential energy source of soil massifs and an underground pipeline. One- and two-dimensional models were used to calculate thermal conductivity. It is shown that the accuracy of soil temperature forecasting deteriorates when using air temperatures to assess the marginal condition of the soil surface. It is proposed to take into account the effect of heat accumulation, as well as the actual temperature of the gas in the pipeline for more accurate efficiency of heat transfer prediction.

Therefore, the calculation of the temperature field of a low-potential energy source in the zone of influence of the compressor rotor diameter and the blade pitch reduced to solving the equation of nonstationary thermal conductivity.

In a cylindrical coordinate system characteristic of a heat pump, the equation has the form **(Lykov, 1967)**:

$$\frac{\partial t}{\partial \tau} = a\left(\frac{\partial^2 t}{\partial r^2} + \frac{1}{r}\frac{\partial t}{\partial r} + \frac{1}{r^2}\frac{\partial^2 t}{\partial \theta^2} + \frac{\partial^2 t}{\partial z^2}\right)$$

Where:

t – the ambient temperature, °C;

τ – hour, s;

a – thermal conductivity, $m^2/s$;

r is the radial coordinate, m;

θ is the polar angle (the angle between the radius vector r and the x axis).

This is a three-dimensional problem, but given the shape and length of the rotor relative to the radius of impact, as well as the heterogeneity of the blades, it can be reduced to two-dimensional with a sufficient degree of accuracy. For this problem statement, taking into account the symmetry of the temperature field, it is proposed to make a simplified solution, provided that τ>; 0 до rp< r< rк **(Rudenko, 2012)**:

$$\frac{\partial t}{\partial \tau} = a\left(\frac{\partial^2 t}{\partial r^2} + \frac{1}{r}\frac{\partial t}{\partial r}\right)$$

Where:

rp – average rotor radius, m;

$r_к$ – radius of the contour of influence, m.

Simplifying the task by switching from a three-dimensional to a two-dimensional model eliminates heat flow along the rotor axis. However, the heat flow in the vertical direction, despite its small orders, must be taken into account due to its continuity in time, even when the heat pump is stopped. To solve the problem, we introduce an amendment that compensates for bulk sources and heat fluxes (environment). Then equation (2.2) will take the following form **(Rudenko, 2012; Sniegkin et al., 2008)**:

$$\frac{\partial t}{\partial \tau} = a\left(\frac{\partial^2 t}{\partial x^2} + \frac{\partial^2 t}{\partial r^2} + \frac{1}{r}\frac{\partial t}{\partial r}\right) + \frac{g_v}{c}$$

Where:

t – ambient temperature, °C;

r – time, s;

a – thermal conductivity, $m^2$ /s;

r – is the radial coordinate, m;

qv-sources and heat runoff due to heat fluxes of the environment and heat release through the surface, W / $m^3$;

c – heat capacity, J / ($m^3$ • °C).

The paper **(Biletsky, 2013)** presents the concept of calculating the mode parameters of gas transportation through the network of gas wells, and the authors **(Romaniuk, et al., 2019)** reveal the features of calculating electricity losses in systems. The heat exchange between the transported gas and the external environment at each of the sections of the pipeline network is not taken into account. The problem of cork and hydrate formation in the pipeline is not considered. The calculation of electricity losses does not fully reflect the effects of the external environment, heat fluxes.

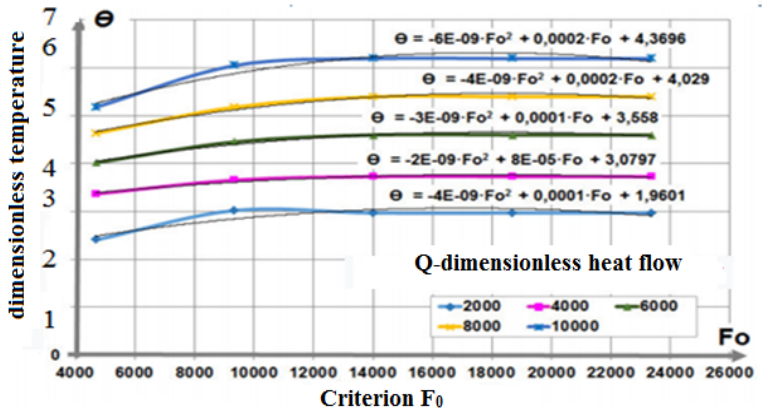



**Figure 10** The results of the calculation of the function from the defining parameters.

Therefore, the set of factors: background temperature of low potential energy source ($t_{fon}$, °C), ambient temperature, heat runoff (qv, W), thermophysical characteristics, the intensity of incident solar radiation, are the basic data in the calculations. The set of factors: background temperature of low potential energy source ($t_{fon}$, °C), ambient temperature, heat runoff (qv, W), thermophysical characteristics, the intensity of incident solar radiation, are the basic data in the calculations.

Execution of the technical and economic analysis is reduced to the definition of the temperature of the heat carrier selected from the heat pump that in turn, is defined by the created temperature. The second feature is the need to assess the operating conditions of the heat pump in the worst, in terms of the coefficient of thermal transformation, conditions. This corresponds to the time period of completion of the heating (cooling) cycle.

The known similarity criteria do not fully reflect the studied phenomena, in connection with which the following dimensionless complexes are proposed in the research work: relative heat flux (Q), modified dimensionless temperature (Θ), and criterion Fo.

The temperature field of a low-potential energy source is described by a dimensionless function **(Rudenko, 2012)** with three dimensionless parameters:

$$f = (Fo, \Theta, Q), \quad (1)$$

Where:
Fo –Fourier criterion,
Θ – dimensionless temperature,
Q – relative heat flux.

Considering the active load on the rotor with blades, which perturbs the temperature field factor, we propose to introduce the relative heat flux (Q), which is accepted in the research work to calculate the formula:

$$Q = g_{na}/g_{fon} \quad (2)$$

Where:
$q_{na}$ – specific heat flux per unit area of the rotating rotor with blades, heat load on the pump in a certain period of operation, W / m$^2$;
$q_{fon}$ – background heat flux (specific, per unit area of the environment), W / m$^2$

The operating parameters of the heat pump depend on the difference between the generated temperature and the ambient temperature.

The Fo criterion was formed by a known formula (**Lykov, 1967**). The determining size is proposed to take the radius of the rotor:

$$F_0 = \frac{a\,\tau}{r_p^2},$$

Where:
a - thermal conductivity coefficient equal to a =λ/cρ m$^2$/s;
λ - thermal conductivity, W / m ˚C;
ρ – air density, kg / m$^3$;
$c_p$ - heat capacity of air, J / (kg × °C);
τ - characteristic time of change of external conditions, s; $r_p$ - characteristic body size (rotor radius), m.

The complex nature of the mutual influence of the defining parameters does not allow to formalize an unambiguous solution, in connection with which the traditional approach to the type of criterion equation as a static dependence is used. The generalizing equation for single-stream operation can be described as a regression:

$$\theta = \kappa_1 F_0^2 Q + k_2 Q F_0 \ldots + k_n F_0 Q$$

Where:
$k_{1,2\ \ldots\ n}$ – determining factors.

The results of the calculations are well approximated by second-order polynomials. Applying the methods of statistical processing, the criterion equation is obtained:

$$\theta = -5\ 10^{-9} Q F_0^2 + 2\ 10^{-8} F_0 Q + 0.0003\ Q + 5.1$$

The results of the calculation of the temperature at the outlet of the partition as a function of the defining parameters in the dimensionless form are presented in Figure 10 **(Saprykina, 2016)**. This allows you to predict the temperature change of a low-potential energy source operating in the cyclic (seasonal) mode without reversing the heat flow.

## CONCLUSION

The process of supplying and discharging heat to a low-potential energy source is a function of time and space. The temperature field is formed by the design parameters of the pump. Analysis of performance data and these formed temperature fields showed the predominance of heat flux in the radial direction and a small amount of heat flux in the axial direction. Nevertheless, due to the design, the formation of heat flow and heat dissipation from the circuit are stabilizing factors that provide a quasi-stationary state.

The phenomenological analysis of the materials given in researches allows noting perspective directions of use of the closed power circuits in the following list:

- Technologies of redistribution of energy potentials of the environment based on heat pumps, refrigeration units, and air conditioners;
- Recuperative transformations of heat fluxes of industrial environments based on thermodynamic connections between pressures and temperatures of gas and steam phases;
- Technologies of creation and transformations of steam, gas, and steam-gas streams with the application of modes of adiabatic phase transitions;
- Creation of technologies based on use of dynamic systems in which continuous processes of heating, endurance, and cooling of streams of environments for use of primary potentials in the closed system without initial streams with the subsequent compensation of losses in the environment with the subsequent transition to dynamic modes are realized;
- In cooling systems of environments in large volumes of technological devices based on the use of cooling jackets or external heat exchangers, an important disadvantage is the gradual reduction of temperature differences on heat exchange surfaces and restrictions on heat recovery. Avoidance of such shortcomings is associated with the transition to a dynamic system with constant temperature differences.

According to the results of theoretical and experimental studies, taking into account thermodynamic analysis, the temperature field of a potential energy source, the main rational parameters of the heat pump rotor are set. So, diameter - 0.2 m; step of the first turn of the blade - 0.11 m; step increment - 0.03 m; the number of blades that are installed between one a pair of adjacent turns - 4 pcs; rotor speed - 300 rpm.

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# COMPETITIVENESS OF AGRI-FOOD FOREIGN TRADE IN CONDITIONS OF THE SLOVAK REPUBLIC

*Anna Látečková, Michaela Trnková, Jozef Palkovič, Ivan Holúbek*

## ABSTRACT

The presented paper aims to evaluate the development and competitiveness of agri-food foreign trade of the Slovak Republic for the period 2016 – 2020. In the article, we present the priorities of Slovakia within the commodity structure. We use the RCA indicator to identify comparative advantages. In this paper, we also compare the actual and estimated development of agri-food foreign trade of the Slovak Republic in the years 2018 – 2020. Based on the reached results, we can state that we achieve an ever-increasing trade balance deficit in agri-food foreign trade. Produced results confirmed all the research assumptions presented in the paper. Despite the increase in export and import of agri-food products, the negative trade balance is still deeper. A significant deviation of real development from the predicted values can be explained by the influence of the worldwide pandemic of the coronavirus COVID-19, which slowed down the possible further development of agricultural trade.

**Keywords:** agri-food foreign trade; commodity structure; competitiveness of agri-food foreign trade; time series analysis

## INTRODUCTION

Competitiveness is a concept that has attracted a great deal of attention from economic theorists for decades. This concept is still not clearly defined due to its complexity and different perceptions **(Šegota, Tomljanović and Huđek, 2017)**.

The issue of competitiveness is constantly the subject of various researches and there are several definitions in various professional publications. The individual definitions of competitiveness are based on:

- the relationship between competition and competitiveness,
- the links between performance and competitiveness,
- understanding of competitiveness at the level of country, industry, enterprise, product, and the like **(Kadárová and Janeková, 2019)**.

Under the term competitiveness, we mean the ability of a certain economic entity to participate in the competition with similar economic entities **(Fabová, 2015)**.

In foreign countries, competitiveness is one of the most popular topics in the economic analysis of various business activities carried out by market players. This is mainly due to the general intensification of international and internal competition over the last few decades **(Borodin, 2006)**.

For the present, global changes are characteristic, the scope and pace of which are constantly increasing. Interdependence and interconnectedness between countries are increasing, and the volume of international transactions is growing. Significant international trade promotion organizations are being set up to remove obstacles and facilitate international exchanges between countries. International trade is constantly evolving and is becoming increasingly important for countries.

The main reasons for the emergence of foreign trade include climatic conditions, production conditions, consumer preferences, economies of scale, and absolute and comparative advantages **(Jurečka, 2011)**.

The movement of goods between countries is the oldest form of international economic relations. The growing exchange between countries leads from isolated national markets to the creation of a world market - to the emergence of world trade **(Tuleja, Nezval and Majerová, 2012)**.

Information on the commodity structure of foreign trade provides a basic overview of the nature of foreign trade, while it is important to observe changes in the structure of imports and exports **(Spěváček et al., 2016)**.

The trade balance expresses the movement of goods and services across national borders. It is given in monetary units and most often, it is monitored over one year **(Michník et al., 1998)**.

A deficit between exports and imports of goods and services, otherwise known as a deficit of trade balance, where imports predominate over exports, could mean that a country imports more to increase productivity, which ultimately leads to more exports. This could ultimately finance the deficit reduction. The deficit may also stem from

an increase in foreign investment and an increased obligation on the local economy to pay the return on investment. However, foreign investment can also have a positive impact on the local economy, and if used wisely, it can increase market value and production for the country's economy in the future. This may allow the local economy to eventually increase exports and reverse its deficit again. A deficit is not necessarily a bad thing for a country's economy, especially for an economy in development or reform. The economy sometimes has to spend money to make money. If a country wants to run a deficit on purpose, the economy must be prepared to finance this deficit **(Heakal, 2021)**.

## Scientific hypothesis

The main goal of the proposed paper is to evaluate the development and competitiveness of agri-food foreign trade in the Slovak Republic for the period 2016–2020. To meet it, we have set the following partial goals:

- analysis of the development of exports and imports of agri-food foreign trade,
- analysis of the commodity structure of agri-food foreign trade,
- forecast of further development of exports and imports of agri-food foreign trade,
- impact of the change in export and import conditions in 2020 through a comparison of actual and forecasted values,
- formulation of recommendations for foreign trade activities of the Slovak Republic in the next period.

Following the set partial goals, the following research hypotheses were formulated:

H1: Based on the forecast of the trend of the further development, we assume an increase in exports and imports in the foreign agri-food trade of the Slovak Republic in the next period.

H2: Despite the expected increase in exports and imports in the next period, we expect further deepening of the passive balance of foreign agri-food trade in the Slovak Republic.

H3: We assume that the actual development of exports and imports of foreign agri-food trade in 2020 will be lower than the values forecasted based on its trend.

## MATERIAL AND METHODOLOGY

In the presented paper, we evaluate the agri-food foreign trade of the Slovak Republic for the period 2016–2020 based on numerical data obtained from the website of the Statistical Office of the Slovak Republic. We will use mathematical-statistical methods, graphical method, comparison, analysis, and synthesis to solve the problem. Percentage results will be rounded to two decimal places for more accurate interpretations.

We will also calculate and evaluate the well-known indicators of foreign trade according to **Kuzmišin P. et al. (2010)**:

$$Turnover = export + import$$

$$Trade\ balance = export - import$$

$$Active\ trade\ balance = export > import$$

$$Passive\ trade\ balance = export < import$$

The comparative advantage indicator

$$RCA = ln\left[\frac{(EC/IC)}{(CAEC/\ CAIC)}\right]$$

where:
EC – commodity export
IC – commodity import
OAEC – overall agro export of the country
OAIC – overall agro import of the country

RCA > 0 – comparative advantage
RCA < 0 – comparative disadvantage

Based on the obtained and processed data, we will create tables and graphical presentations, which will provide us with better insight into the analyzed topic.

## Statistical analysis

In this paper, we will compare the actual and estimated development of agri-food foreign trade in the Slovak Republic in the years 2018 – 2020. We followed up on the results of the research, which was part of the scientific work of the author Michaela Trnková. In this paper, a decomposition approach was applied to a time series analysis. For a more accurate estimate, data from 2000 to 2017 were collected in a time series. In 2017, we worked with preliminary data obtained from the Statistical Office of the Slovak Republic. **Matejková, Pietriková and Poláková (2015)** stated that in a time series, the values were arranged chronologically over time.

The principle is to understand the mechanism through which the observed data are generated and based on which the future values are predicted. A decomposition approach was used to predict the time series. The time series were decomposed into individual components that were predicted. Given that the analysis was based on annual data, the forecast was based on an extrapolation of the further development of the trend function. The most suitable function for the trend forecasting was evaluated based on the R-Squared indicator (a coefficient of determination). The trend captures the changes that have occurred in the average behavior of the time series in the long run. The forecast, therefore, expresses the expected further development of the time series values in case that the indicator continues in the current trend. From all the possible functions that were estimated for forecasting, the most accurate trend function expressing the development of exports was the power function, which has the form:

$$y_j = b_0 * {t_j}^{b_1}$$

The development of imports was most accurately expressed by a linear function in the form of:

$$y_j = b_0 + b_1 * t_j$$

where:
$b_0$ – locating constant,
$b_1$ – regression coefficient,
$y_j$ – j-th value of the dependent variable,
$t_j$ – j-th value of the independent variable.

## RESULTS AND DISCUSSION

Agricultural development depends increasingly on integration into the global economy. International trade, including food trade, is a link for the economies of different countries, a point of their political and economic convergence. The turnover of food and agricultural raw materials has a special place in international trade because domestic food adequacy and the ability to sell surplus food on foreign markets characterize not only the country's level of food independence but also the qualitative structure of its foreign trade **(Maslova, Chekalin and Avdeev, 2019)**.

The Slovak Republic is a small country, both in Europe and globally. Slovakia's agri-food foreign trade does not have an important position for these countries. It is therefore important for the Slovak Republic to engage in international trade **(Podolák et al., 2007)**.

According to **Tong Soo (2013)**, the benefit of exports for smaller countries, which is also the case of Slovakia, is always higher compared to larger countries.

The share of agri-food foreign trade in total foreign trade fluctuated in the period under review. We recorded both growth and decline, but there were no major deviations from the average, which was 4.98%. In 2016, the share of agri-food foreign trade in total foreign trade was 5.11%. The lowest share was in 2018 – 4.71%. In 2020, the highest share was recorded – 5.35%. We can state that the position of agri-food commodities within the total foreign trade was strengthened especially in 2020 when the territory of our country was also hit by the global pandemic of the coronavirus COVID-19.

In the observed period of 2016 – 2020, the agri-food sector of the Slovak Republic exported goods with an average value of 2 931 million eur per year. The total agri-food export in 2016 was at the level of 2 827 million eur. In the observed period, it also represented the lowest value of exports. In 2020, it was 3 136 million eur, which represented an increase compared to 2016 by 309 million eur, in relative terms by 10.93%. In the observed period, it also represented the highest value of exports.

Import in the agri-food foreign trade of the Slovak Republic in the observed period reached an average annual value of 4 485 million eur. The lowest value of imported agri-food products was in 2016, amounting to 4 104 million eur. In 2020, we recorded the highest value of imports, which amounted to 4 831 million eur. Compared to 2016, it increased in absolute terms by 727 million eur, which in relative terms represents an increase of 17.71%.

The value of imports is almost more than double that of exports. This is a long-term trend, which is confirmed by **Smutka, Rovný and Hambálková (2016)** in their research.

The turnover of agri-food foreign trade developed similarly to exports. In 2016, it was at the lowest level, amounting to 6 931 million eur. In 2020, it reached its highest value of 7 966 million eur. The increase compared to 2016 was 1 035 million eur, i.e. 114.93%.

In the period 2016 – 2020, the average annual trade balance deficit of agri-food foreign trade amounted to 1 554 million eur. From year to year, this average deficit increases. Imports are growing faster than exports. In 2016, the balance of agri-food foreign trade was at the lowest level of 1 277 million eur. We recorded the highest value in 2020 when it reached the amount of 1 695 million eur. Compared to 2016, this trade balance deficit increased by 418 million eur, which represents an increase of 32.73%.

The commodity structure of the agri-food foreign trade of the Slovak Republic is constantly changing. These changes are caused by fluctuating development of the domestic market, but also of the foreign markets.

The negative balance is increasing and poses a major threat that affects the competitiveness of domestic products in domestic and foreign markets **(Récky and Hambálková, 2014)**.

We divide agri-food foreign trade into trade in agricultural products and trade in food products. Food products have the

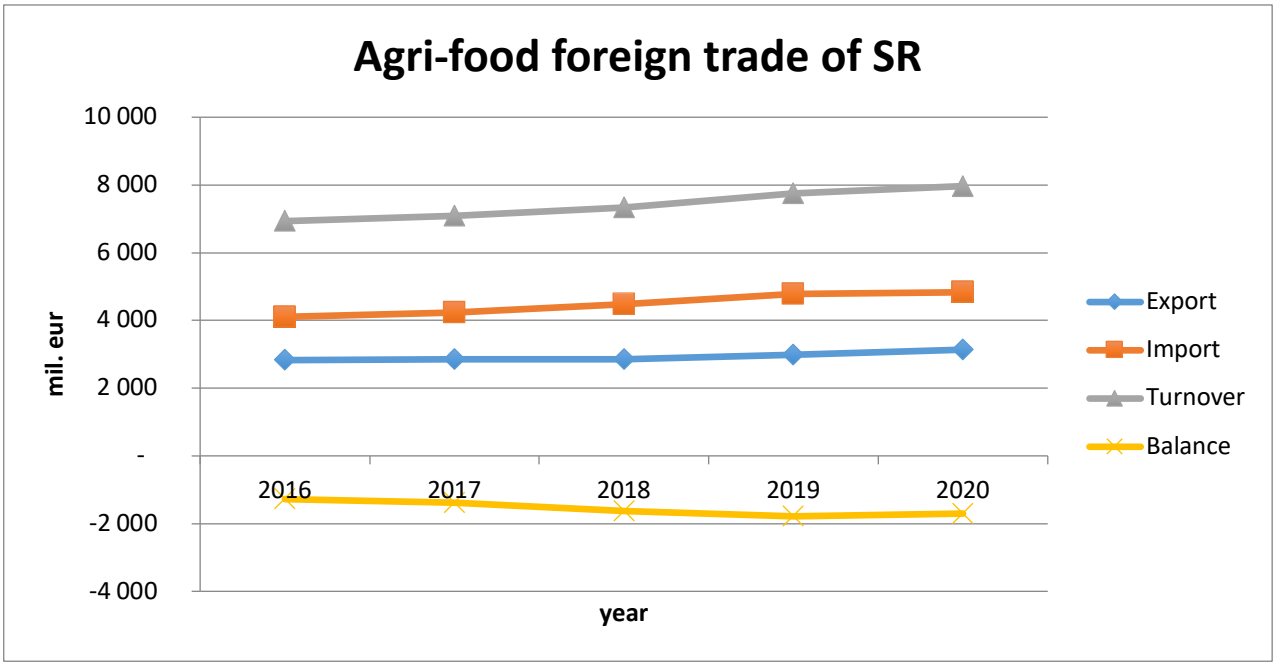


**Figure 1** Agri-food foreign trade of SR in the years 2016 - 2020 in mil. eur.
Source: own calculation according to Statistical office of the Slovak Republic.

**Table 2** Agri-food foreign trade of SR by classes of the harmonized system in 2016 and 2020.

| | | 2016 | | 2020 | |
|---|---|---|---|---|---|
| | | mil.eur | % | mil. eur | % |
| Export | SUM | 2826 | 100.00 | 3 148 | 100.00 |
| | I. Live animals and animal products | 575 | 20.35 | 549 | 17.43 |
| | II. Plant products | 968 | 34.25 | 1 051 | 33.38 |
| | III. Animal and crop fats, oils, waxes | 122 | 4,32 | 119 | 3.78 |
| | IV. Products of the food industry, beverages and tobacco | 1161 | 41.08 | 1 430 | 45.41 |
| Import | SUM | 4104 | 100.00 | 3 821 | 100.00 |
| | I. Live animals and animal products | 890 | 21.69 | 1 153 | 30.17 |
| | II. Plant products | 1051 | 25.61 | 1 146 | 29.99 |
| | III. Animal and crop fats, oils, waxes | 192 | 4.68 | 164 | 4.29 |
| | IV. Products of the food industry, beverages and tobacco | 1971 | 48.03 | 1 358 | 35.54 |

Source: own calculation according to Statistical office of the Slovak Republic.

largest share in the agri-food foreign trade of the Slovak Republic. When broken down into substitute and irreplaceable products, the substitute products account for the largest share of foreign agribusiness. This is confirmed by the statements of **Zoborský (2006)** as well as **Podolák et al. (2007)** on the predominance of the competing products in agri-food foreign trade.

The growing negative balance poses a threat to the competitive commodities we can produce in the domestic market **(Récky and Hambálková, 2014)**.

The role and importance of agricultural and food products in world trade have changed over time **(Jovovic, Jovanovič and Dasic, 2014)**.

Their production, unlike industrial production, has many peculiarities. Foreign trade in agricultural and food products is influenced not only by several predictable factors but also by unpredictable factors **(Gálik, 2011)**.

Production and trade in agricultural and food products are becoming increasingly globalized **(Vinciuniene and Dadurkaite, 2011)**.

We include classes I. – IV. of a harmonized system to agri-food foreign trade, namely:

I. Live animals and animal products,
II. Plant products,
III. Animal and crop fats, oils, waxes,
IV. Products of the food industry, beverages and tobacco.

In Table 2, we can see the structure of agri-food foreign trade of the Slovak Republic according to the classes of the harmonized system. In 2016 and also in 2020, class IV. Products of the food industry, beverages and tobacco had the largest share in Slovakia's foreign agribusiness. In 2016, this class accounted for 41.08% of agro export and in 2020, for 45.41%. In agroimport, in 2016, it was 48.03%, and in 2020, 35.54%. Class II. Plant products follow, followed by class I. Live animals and animal products and in the last place with the smallest share in agri-food foreign trade, there is class III. Animal and crop fats, oils, waxes. For more detailed information, we work with the division according to the chapters of the harmonized system, where agri-food foreign trade consists of chapters 01 – 24. These are made up of individual items, each of which is assigned with an appropriate four-code.

The most important item of agri-food exports in 2016, and also in 2020, occurred to be wheat and meslin. The value of its exports increased by 12 million eur, i.e. by 5.08%. In 2016, it participated in agro export by 8.35% and in 2020, by 7.89%.

For many items of agri-food exports, we have noticed that their share is declining from year to year. The Slovak

**Table 3** The most important items of agri-food export of SR in 2016 and 2020.

| | 2016 | | |
|---|---|---|---|
| Code | Item name | Value of export in mil. eur | Share in agroexport in % |
| 1001 | Wheat and meslin | 236 | 8.34 |
| 1806 | Chocolate | 232 | 8.21 |
| 0901 | Coffee | 181 | 6.40 |
| 2106 | Food preparations | 108 | 3.82 |
| 0406 | Cheese and cottage cheese | 99 | 3.50 |
| | 2020 | | |
| Code | Item name | Value of export in mil. eur | Share in agroexport in % |
| 1001 | Wheat and meslin | 248 | 7.88 |
| 1806 | Chocolate | 231 | 7.34 |
| 2106 | Food preparations | 224 | 7.12 |
| 0406 | Cheese and cottage cheese | 142 | 4.51 |
| 1005 | Maize | 129 | 4.10 |

Source: own calculation according to Statistical office of the Slovak Republic.

**Table 4** The most important items of agri-food import of SR in 2016 and 2020.

| 2016 | | | |
|---|---|---|---|
| Code | Item name | Value of import in mil. eur | Share in agroimport in % |
| 1806 | Chocolate | 252 | 6.14 |
| 0203 | Pork | 248 | 6.04 |
| 0901 | Coffee | 196 | 4.78 |
| 1905 | Bakery goods | 173 | 4.22 |
| 2106 | Food preparations | 162 | 3.95 |
| 2020 | | | |
| Code | Item name | Value of import in mil. eur | Share in agroimport in % |
| 0203 | Pork | 308 | 6.38 |
| 2106 | Food preparations | 247 | 5.12 |
| 1806 | Chocolate | 236 | 4.89 |
| 1905 | Bakery goods | 225 | 4.66 |
| 0406 | Cheese and cottage cheese | 196 | 4.06 |

Source: own calculation according to Statistical office of the Slovak Republic.

Republic is less and less focused on the export of agri-food commodities and on the support of Slovak farmers and food producers. An increasing agro import, and thus an increasing trade balance deficit, are then the results.

The most significant imported item in 2016 was chocolate, whose share in agro import was 6.15%. In 2020, its share decreased to 4.90% and first place was occupied by pork with a 6.37% share in agro import of the Slovak Republic.

For most commodities, we are seeing an increase in their share of total agri-food imports. This is the opposite of the situation when exporting agri-food commodities. Furthermore, we can state that exports with a lower added value prevailed in contrast to imports, where products with a higher added value predominated.

Chocolate is one of the most important long-term commodities of agri-food exports as well as imports from the Slovak Republic. It reached first place in the monitored period. This is also confirmed by the research of **Hambálková and Kádeková (2017)** for the years 2013 – 2015 when chocolate also took the first place in exports and imports.

The diversity of foreign trade decreased between 2000 and 2015, which corresponded to development in other post-communist countries, too **(Benešová I. et al. 2020)**.

Price development is closely related to the commodity structure of agri-food foreign trade in the Slovak Republic **(Gálik, 2011)**.

The Slovak agrarian sector imports goods that are more expensive than our exported agricultural products. This is mainly because the Slovak exports are focused mainly on agricultural raw materials, and imports are dominated by processed food products with a higher added value **(Gálik, 2013)**.

In the long run, it is more appropriate to focus on the export of products with a higher added value, i.e. food products, and direct agricultural production to provide basic raw materials for the food industry **(Hambálková and Vakoš, 2017)**.

Based on the calculated values of the RCA indicator, we claim that in 2016, and also in 2020, the agri-food foreign trade of the Slovak Republic developed unfavorably. This was also confirmed by **Rumankova et. al. (2020),** when comparing the development of exports within the V4 countries, but also by the results of the research of **Nagyova et al. (2018)**.

Within the framework of agri-food foreign trade in 2016, out of the total number of 197 items, Slovakia had a comparative advantage for 58 items of the harmonized system and a comparative disadvantage for up to 128 items of the harmonized system. Commodities with a good comparative advantage in 2016 included cereal straw and husks; tapioca and starch substitutes; other live animals; live sheep and goats; wheat and meslin; malt; cocoa mass; glycerol; and residues resulting from the manufacture of starch and oats. On the contrary, commodities with the

**Table 5** Competitiveness of agri-food foreign trade of SR – the RCA indicator.

| Code | Item name | 2016 | Code | Item name | 2020 |
|---|---|---|---|---|---|
| 1213 | Cereal straw and husks | 5.55 | 1107 | Malt | 4.70 |
| 1903 | Tapioca and starch substitutes | 5.37 | 1213 | Cereal straw and husks | 4.42 |
| 0106 | Other live animals | 5.07 | 0106 | Other live animals | 4.30 |
| 0104 | Live sheep and goats | 4.68 | 1001 | Wheat and meslin | 4.16 |
| 1001 | Wheat and meslin | 4.49 | 1507 | Soybean oil | 3.25 |
| 1511 | Palm oil | -13.44 | 1804 | Cocoa butter, fat and oil | -10.90 |
| 1521 | Crop waxes | -8.23 | 1801 | Cocoa beans | -10.49 |
| 1404 | Crop products | -6.41 | 0205 | Meat of horses, asses and mules | -9.39 |
| 0601 | Tubers, bulbs and roots | -5.74 | 1521 | Crop waxes | -7.34 |
| 1603 | Extracts and juices of meat, fish or crustaceans, mollusks and other aquatic invertebrates | -5.48 | 1510 | Other oils | -7.01 |

Source: own calculation according to Statistical office of the Slovak Republic.

greatest comparative disadvantage included palm oil; crop waxes; crop products; tubers, bulbs and roots; extracts and juices of meat, fish or crustaceans, mollusks, and other aquatic invertebrates; peanut oil; cocoa butter, fat and oil; the fish; bones; and leaves, branches and other parts of plants.

The Slovak Republic was not a net exporter even for 1 item. However, it was a net importer for 9 commodities. Among them, we include meat from horses, asses, and mules; aquatic invertebrates other than crustaceans and mollusks; human hair; swine and boar bristles and hair, badger and other hair; mate; wheat gluten; dill; cocoa beans; cocoa shells, husks, skins, and other cocoa waste; oilcake and other solid residues and wine lees, raw tartar. For 2 commodities, foreign trade was not realized at all - dill; oilcake, and other solid wastes.

In 2020, the Slovak Republic had a comparative advantage in 59 items and a comparative disadvantage in 126 items of the harmonized system within agri-food foreign trade. Among the commodities with a good comparative advantage in 2020, there were included malt; cereal straw and husks; other live animals; wheat and meslin; soybean oil; cocoa mass; residues resulting from the manufacture of starch; eggs; live sheep and goats; and rye. The items with the greatest comparative disadvantage included cocoa butter, fat, and oil; cocoa beans; meat of horses, asses, and mules; crop waxes; other oils; vanilla; the fish; other processed tobacco products; sheep wool and fat; plant materials and plant waste.

The Slovak Republic was not a net exporter even for one item. On the contrary, it was a net importer for 5 items of the harmonized system - human hair; peanut oil; degras; wine lees, raw tartar, and cocoa shells. For 3 commodities, foreign trade was not realized at all – dill; oilcake and other solid wastes; swine and boar bristles and hair, badger and other hair.

Compared to 2016, we can say that the situation in agri-food foreign trade has not changed for the better in the past 5 years. Although Slovakia is a small country, it would be able to be largely self-sufficient in agriculture and food production. Imports of many agri-food commodities would not be necessary. However, the situation is different. The competitiveness of the Slovak agri-food sector is decreasing, exports are declining, imports are increasing, and domestic products are being pushed out by the foreign.

No country in the world can be completely self-sufficient and competitive. However, each country should maintain Ricardo's theory of comparative advantage, where the country should focus on the production and export of those products at which it achieves the highest productivity **(Rhee, 2012)**.

In the last part of the paper, we compared the actual and estimated values of the development of agri-food foreign trade of the Slovak Republic in 2018 – 2020.

Based on the analysis of the time series of the development of agri-food foreign trade of the Slovak Republic in the years 2018 – 2020 with unchanged internal and external factors, we assumed that exports in 2018 would reach the value of 3 230 million eur, in 2019, would be in the amount of 3 414 million eur and in 2020, would increase to 3 527 million eur, provided that any unforeseeable events do not occur.

Comparing the forecasted values with the real development of exports, we can observe that the expected development of the export trend was significantly affected by its maximum values in 2010 and 2014. Export growth in the following years slowed down significantly, compared to the estimated trend, resulting in a large difference between actual and forecasted values according to the power trend function. With three forecasted values for the years 2018 to 2020, the forecasted values were on average by 421 million eur higher, although the increase in exports in this period was slightly higher than its stagnation in the period 2014 to 2017. It can be stated that the export forecast was affected by its maximum values in 2010 – 2014, and its further development will follow a trend that would forecast without

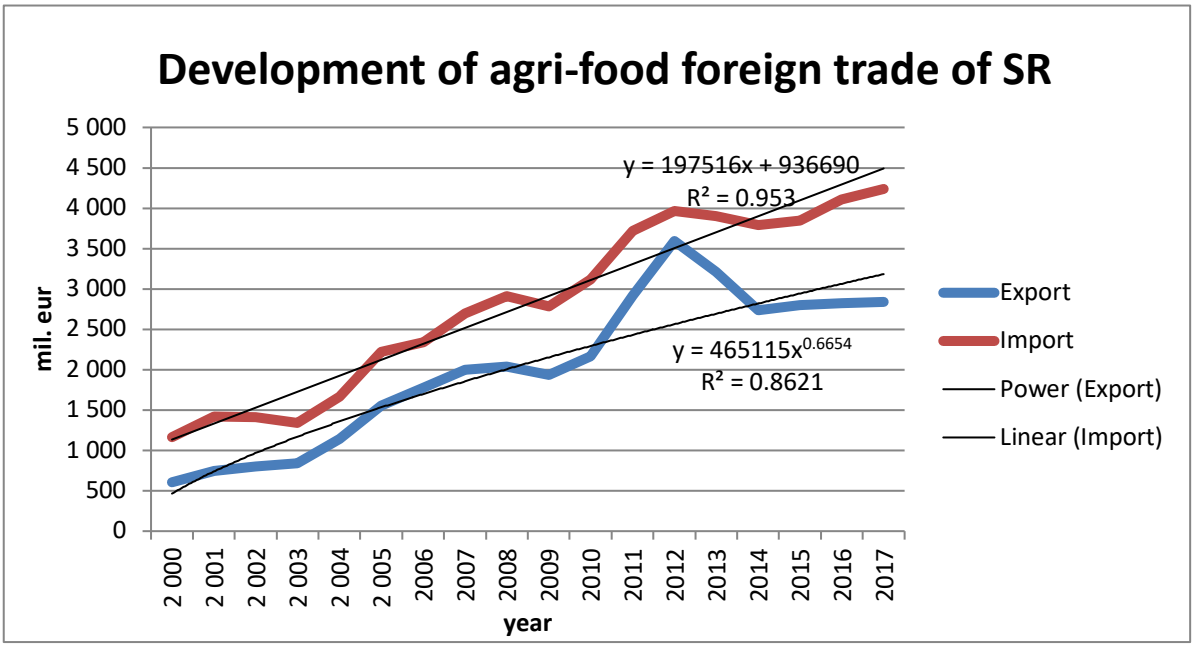


**Figure 1** Export and import of agri-food foreign trade of SR in the years 2000-2017 in mil. eur.
Source: own calculation according to to Statistical office of the Slovak Republic.

**Table 7** Estimated values of the basic indicators of agri-food foreign trade of SR in the years 2018-2020 in mil. eur.

| | Export | Import | Turnover | Balance |
|---|---|---|---|---|
| **2018** | 3 300 | 4 689 | 7 989 | - 1 389 |
| **2019** | 3 414 | 4 887 | 8 301 | - 1 473 |
| **2020** | 3 527 | 5 085 | 8 612 | - 1 558 |

Source: own calculation according to Statistical office of the Slovak Republic.

**Table 8** Actual values of the basic indicators of agri-food foreign trade of SR in the years 2018-2020 in mil. eur.

| | Export | Import | Turnover | Balance |
|---|---|---|---|---|
| **2018** | 2 856 | 4 486 | 7 342 | -1 630 |
| **2019** | 2 986 | 4 772 | 7 758 | -1 786 |
| **2020** | 3 136 | 4 831 | 7 967 | -1 695 |

Source: own calculation according to Statistical office of the Slovak Republic.

taking the high values from that period into account. A linear trend function was used to forecast imports. The forecast assumes an increase in the values of imports similarly to the previous one, at a faster pace than for exports. In 2018, it should amount to 4 689 million eur, in 2019, it will increase to 4 887 million eur and in 2020, it should reach 5 085 million eur. Compared to the actual values, the increase in imports was slower and lagged behind the extrapolated values of the linear growth trend by an average of 191 million eur at the three forecasted values.

On the contrary, in contrast to the expected forecasted growth with the same average growth rate, import growth slowed down, especially between 2019 and 2020.

At the moment, imports are in a phase of slowing down, after which we can expect their recovery and return to the values that would be closer to the originally forecasted trend. Based on the forecast, with the growing value of exports and imports, we assumed that the turnover of foreign agri-food trade in the Slovak Republic would also increase every year. Despite the significantly overestimated values originally forecasted for exports and imports, the actual trade balance deficit of the Slovak Republic was significantly higher than the forecasted. Compared to the originally forecasted values of the trade balance deficit for the years 2018 – 2020, which were 1 389 million eur; 1 473 million eur; and 1 558 million eur, its actual values in this period were 1 630 million eur; 1 786 million eur; and 1 695 million eur. The originally forecasted values of the development of exports and imports of agri-food foreign trade in the Slovak Republic can help to form an idea of their development in the situation if the previous conditions were maintained without the influences that affected their development mainly in 2020. This year, the actual exports have been lower than the forecasted by 391 million eur and imports by 254 million eur.

The possibilities of transformation not only for expansion but also for maintaining the achieved export values in the current conditions are undermined by the negative dynamics of demand, which is exacerbated by the economic downturn due to the COVID-19 pandemic **(Kiselev, Romashkin, 2021)**.

The competitiveness of the Slovak agri-food sector is declining, exports are declining, imports are increasing, and domestic products are being pushed out by foreign ones. There are many problems and causes of the ever-deepening deficit of agri-food foreign trade.

The annual increase in the deficit balance of the agri-food foreign trade of the Slovak Republic is also related to the high share of foreign agri-food products in retail chains in the territory of the SR. On the contrary, the share of agri-food products produced in Slovakia is decreasing from year to year. It is necessary to support the production and sale of products from domestic producers. Possible solutions include in particular the expansion of advertising for the purchase of Slovak products. Recently, some well-known retail chains have focused their attention on the promotion of Slovak products. It is necessary to continue in this direction and draw people's attention to the purchase of domestic products. In this way, we can greatly increase the share of domestic agri-products and gradually reduce the share of foreign agri-food products, which would subsequently lead to a reduction in the deficit balance of foreign agribusiness and growth in prosperity in Slovakia.

Support of exports in the agri-food foreign trade of Slovakia is another significant problem. The Slovak Republic should pay more attention to supporting the export of those commodities in which we achieve a comparative advantage. We achieve this in many agri-food commodities. Even though we have enough agricultural production to produce many food products, we still import them from abroad. It is necessary to reduce the import of the substitute commodities, which can also be produced in our conditions. Farmers should reconsider growing crops to create some added value. Cultivation of crops for seeds and not for fodder, which consequently has a lower price, is a suitable example. Also, growing fruit and vegetables for further processing can increase employment and reduce imports. We export raw materials in large quantities and import processed products, which are much more expensive. For the Slovak Republic, it is necessary to focus on the processing of raw materials and materials at its disposal, and thus to create products with a higher added value and not to import these more expensive products from abroad.

## CONCLUSION

In this paper, we evaluated the development and competitiveness of agri-food foreign trade in the Slovak Republic for the period 2016–2020. Based on the achieved results, we can state that we have reached an ever-increasing trade balance deficit in agri-food foreign trade. Finally, we compared the actual and estimated values of the development of agri-food foreign trade in the years 2018 – 2020. With the unchanged internal and external conditions, exports and imports will continue to grow, but imports will grow faster than exports. As a result, the trade balance deficit will become more and more intensified.

The area of agri-food foreign trade of the Slovak Republic is connected with several problems that need to be solved. The main problems include export of the unprocessed commodities and import of the finished products with a high added value, which are consequently also more expensive for domestic consumers. This is also related to insufficient support for the export of those commodities and products for which we achieve a comparative advantage. We import easily replaceable products on a large scale, which can also be produced on the territory of our country. The change in this situation will provide new job opportunities, increase employment, and produce products with a high added value and lower prices for domestic consumers.

At present, it is very difficult to anticipate the future development and competitiveness of the agri-food foreign trade of the Slovak Republic, as the pandemic of the coronavirus COVID-19 has significantly affected the entire business environment. Despite the above, we consider our contribution to be beneficial, as it analyzes in detail the development of the competitiveness of agri-food foreign trade in the Slovak Republic and points out the problems in this area.

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# THE IMPROVEMENT OF INSULIN RESISTANCE AND THE ANTIOXIDANT CAPACITY IN TYPE 2 DIABETES MELLITUS RATS WITH WHITELEG SHRIMP SHELL POWDER (*LITOPENAEUS VANNAMEI*)

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## ABSTRACT
As estimated having an increased incidence of about 50% until 2040, the diabetic condition could be augmented primarily from astaxanthin contained in carotenoids. This research examines and compares the influence of WSSP and AST complement on Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) level and Total Antioxidant Capacity (TAC) induced high-fat diet streptozotocin (HFD-STZ) in T2DM rats. WSSP 0.89gr/200gr/d (X1) and 1.77gr/200gr/d (X2) groups; and AST supplement 0.09mg/200gr/d (X3) groups were compared with both of positive (K1) and negative (K2) groups. The treatments were administered orally for 21 days to 25 Wistar rats which each group consisted of 5 rats. HOMA-IR and TAC levels were measured by ELISA and ABTs method respectively. TAC levels significantly increased in treatment groups than K1 group ($p$ = 0.008). The increase in the TAC level of the X2 group was greater than the X1 group ($p$ = 0.017). HOMA IR levels were significantly lower in treatment groups than K1 group ($p$ = 0.009). X2 group had a greater decrease in HOMA IR levels compared to X1 ($p$ = 0.016). In consequence, the research findings show a utilitarian effect of WSSP in increasing TAC and decreasing the HOMA-IR index.

**Keywords:** astaxanthin; HOMA-IR; antioxidant; T2DM; whiteleg shrimp shell powder

## INTRODUCTION
Type 2 Diabetes Mellitus (T2DM) is a non-communicable disease that remains to be a global issue in which the incidence continues to increase every year. In 2015, the International Diabetic Federation (IDF) estimated that the number of people with diabetes will reach 415 million people with an estimation that will increase to 642 million people in 2040 **(IDF, 2015)**. The occurrence of diabetes in Indonesia reached 10 million in 2015, and it continuously increases each year for 4-6% of the world population until 2020, including Gestational Diabetes Mellitus (GDM) **(IDF, 2017; Primal, Putri and Meiriza, 2021)**. Type 2 Diabetes Mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia due to impaired metabolism of nutrients, as a result of decreased secretion and sensitivity toward insulin known as insulin resistance. Abnormality of metabolic nutrients induces increasing oxidative stress due to an imbalance between the levels of Reactive Oxygen Species (ROS) and Total Antioxidant Capacity (TAC) in the body **(Nolan, Damm and Prentki, 2011)**.

A decrease in TAC in the body results in damage of insulin receptor cell insulin resistance by activating several enzymes. The increase in circulating fatty acids in the liver and muscle results in increased activity of Protein Kinase C (PKC) followed by a disruption of phosphorylation in Insulin Receptor Substrate (IRS-1 and IRS-2) as an indication of liver and muscle insulin resistance. Circulating fatty acids can also activate the Hypoxia-Inducible Factor-1 (HIF-1) gene which will increase Jun N-terminal Kinase (JNK) and IkB Kinase (IKK) activity which will lead to insulin resistance to adipose tissue. The condition of chronic insulin resistance causes glucolipotoxicity to pancreatic β cells which results in a decrease in insulin secretion and an increase in blood glucose levels which is known as T2DM **(Sears and Perry, 2014; Samuel and Shulman, 2012; Kupsal et al, 2015)**.

The decrease of TAC in T2DM exacerbates insulin resistance which can be measured by the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR). This method illustrates the feedback response between insulin secretion by pancreatic β cells with glucose uptake from the circulation by insulin receptor cells into glucose target cells **(Antunes et al., 2016)**. Traditional medicine derived from nature is currently one of the most popular medical techniques. Whiteleg shrimp shells are part of unused shrimps in the shrimp processing industry, especially in Indonesia. The benefits of shrimp shells for health have not been widely known, especially for T2DM.

Whiteleg shrimp shells contain several bioactive components such as AST of 101.7 ±11.2 µg / gr which is the main antioxidant in Whiteleg shrimp shells. The other bioactive components are chitosan, Mono Unsaturated Fatty Acid (MUFA), Poly Unsaturated Fatty Acid (PUFA), vitamin E, vitamin A, and calcium **(Hue et al, 2008; Nair et al, 2017; Cahú et al, 2012; Suptijah, Jacoeb and Deviyanti, 2012).**

Each bioactive component that is also found in WSSP content has been investigated for its protective role in T2DM. The protective mechanisms used are suppressing oxidative stress, suppressing the process of gluconeogenesis in the liver, increasing uptake of glucose into the tissue, and stimulating insulin secretion in pancreatic β cells **(Ambati et al, 2014; Liu, Chang and Chiang, 2010; Hodgkin, Hills and Squires, 2008).** Astaxanthin is an important component of WSSP and pure AST from many sources that have been studied for its protective role in T2DM and obese animal model **(Sila et al, 2015; Li et al, 2016; Chan, Pen and Yin, 2012)**. The treatment of shrimp extract oil is proven to be protective as indicated by improvement of HOMA-IR in HFD-STZ induced. Our study used WSSP to avoid decreasing levels of an active component during extraction. This study has never been done before; therefore, it is expected that this study can provide knowledge to the community that the whiteleg shrimp shell is the potential to improve T2DM conditions and it is expected to produce a product that can prevent T2DM using Whiteleg shrimp shell-based ingredients.

### Scientific hypothesis

The research hypothesis hinged on the assumptions that the whiteleg shrimp shell powder (*Litopenaeus Vannamei*) could be positively improving the insulin resistance and antioxidant level of T2DM rats. As an object of the *in vivo* study in Wistar strain rats, WSSP in different doses and AST supplement has been approved as an improvement of total antioxidant capacity and insulin resistance.

## MATERIAL AND METHODOLOGY

**Samples**

The drying process of shrimp shells using the freeze-drying method was aimed at maintaining the quality and quantity of bioactive substances found in shrimp shells **(Shofian et al., 2011**). The WSSP processing referred to the previous manufacturing technique which is suitable for maintaining the bioactive component in WSSP. Drying was carried out at -40°C for 3 – 4 days after separating each part of the shrimp shell (abdomen, head, and thorax). Dry shrimp shells were then mashed with a food processor for 2 – 3 minutes then sifted using a 60 mesh sieve. WSSP was stored in dark glass bottles coated with aluminum foil at 4°C. 19.20 The WSSP was added by suspending WSSP doses of 0.89gr/200gr/day and 1.77gr/200gr/day into Natrium Carboxymethylcellulose 0.5%. DPPH free radical scavenging and ABTs radical scavenging activity assays are used subjected to determine the antioxidant activity. WSSP and AST supplement in this study had IC50 mentioned in Table 1.

**Chemicals**

STZ (C8H15N3O7) and nicotinamide were obtained from Nacalai Tesque, Kyoto-Japan. Natrium Carboxymethyl Cellulose was obtained from Sigma-Aldrich, Japan. TAC examination used the ABTs method with TAC kit obtained from Randox Laboratory, United Kingdom. Insulin examination using ELISA reagents was obtained from Wuhan-China, Fine test. Blood glucose examination reagents were obtained from Diasys-German and measurement of insulin resistance used the HOMA-IR method.

**Animals and Biological Material:**

This study used 25 male white rats Wistar (Rattus novergicus) strain which was obtained from Central Food and Nutrition Laboratory, Gadjah Mada University-Yogyakarta, Indonesia with inclusion criteria of male, age of eight weeks, the weight of 150 – 200 grams, healthy and active.

**Instruments**

STZ-NA, ELISA test for fasting insulin measurements, ABTs test for TAC examination. A microtube and centrifuge for 4000 rpm spinning.

**Laboratory Methods**

The examination of the HOMA-IR and TAC levels was done twice which were three days after STZ-NA induction and at the end of the research. Before taking rats' blood, they have fasted for 8 – 10 hours. Two ml of blood from *plexus retroorbitalis* was taken and placed in a microtube then centrifuge and spin at 4000 rpm for 15 minutes. HOMA-IR index examination was obtained from fasting blood glucose levels and fasting blood insulin. Fasting glucose examination was performed by spectrophotometry and fasting insulin was measured by ELISA method in which TAC examination was carried out by ABTs method.

**Description of the Experiment**

**Sample preparation:** Rats were acclimatized for seven days in individual stainless-steel cages at 21°C. It also had adequate air circulation and was exposed to light for 12 hours. Feed type of Comfeed II with the composition of 15% of crude protein, 3 – 7% of crude fat, 12% of moisture content, 6% of crude fiber, 7% of ash, 0.5% of phosphorus, and calcium 0.9 – 1.1% were given during the study. Rats in the K2 group received standard feed until the end of the study, while the remaining rats were induced to be T2DM by High-Fat Diet (HFD) followed by STZ-NA. HFD was given from the second week to the third week. The feed composition of HFD used was a mixture of 90% of Comfeed II, 10% of lard, and 1.25% of cholesterol of the total HFD feed weight. Comfeed II feed and HFD were given as much as 15 g/d. STZ-NA induction was carried out after rats received HFD for 14 days, STZ-NA dose was 45 mg/kg and 110 mg/kg (i.p). Rats induced by STZ-NA were declared to have T2DM if blood glucose levels were >250 mg/dl **(Ghasemi, Khalifi and Jedi, 2014)**.

**Number of samples analyzed:** 25 male white rats Wistar (Rattus novergicus) strain with the weight of 150-200 grams, healthy and active. This research is a true experiment containing a randomized pre-posttest with a

control group design. Rats were split up into five groups of which each group consisted of five. K1 group was a group of T2DM rats, while K2 group was a group of healthy rats which did not receive any treatment. X1 and X2 groups were groups of T2DM rats treated with WSSP in doses of 0.89 gr/200gr/d and 1.77gr/200gr/d respectively. Finally, X3 group was a group of T2DM rats treated with AST supplement in a dose of 0.09mg/200gr/d. WSSP and AST supplements were carried out for 21 days through the oral gavage.

**Number of repeated analyses:** 0

**Number of experiment replication:** 0

**Statistical Analysis**

Statistical analysis was used to see the effect of giving WSSP and AST supplements on HOMA-IR and TAC levels. Paired t-test analysis was utilized to see dissimilarity of the HOMA-IR and TAC level before and after WSSP and AST supplement treatment in the data with normal distribution, whereas the Wilcoxon test was used to see differences of the HOMA-IR and TAC level before and after WSSP and AST supplement treatment in the data with the abnormal distribution. Analysis of HOMA-IR and TAC level between groups used the ANOVA test followed by the Poshoc Bonferoni test for the normally distributed data, otherwise, the Kruskal Wallis test was followed by Mann-Whitney test for the abnormally distributed data. Statistical analysis was done by using SPSS 21 software, the difference was significant when the p-value is less than 0.05 and CI of 95%.

## RESULTS AND DISCUSSION

The weighing of the rats was carried out every week upon which the average weight of the rat at the beginning of the study was 192.23 gr. The average weight and blood glucose of rats after the induction of HFD-STZ was 226.23 gram and 420.03 mg/dl; hence, this indicates that the induction of HFD-STZ can increase the blood glucose levels and weight of the rats.

The effect of WSSP and AST supplement treatments on the TAC of rats induced by HFD-STZ can be seen in Table 2. Both K2 and K1 groups had a reduced TAC level in the post-experiment and these differences were not significant ($p = 0.180$ and $p = 0.063$). All treatment groups (X1, X2, and X3) show increase TAC levels in the post-treatment, these were demonstrated by higher TAC levels in the post-treatment than those of pre-treatment, and these were significantly different ($p = 0.039$, $p = 0.001$, and $p = 0.041$). These findings indicated that WSSP in doses used in this study were affiliated with the rise of TAC levels in T2DM rats, this association was also observed in AST treatment.

Because of Kruskal-Wallis test showed a significant difference of TAC level change ($p < 0.001$, Table 2), the Mann-Whitney test was then performed and can be seen in Table 3. TAC level change of K2 compared with K1 groups was not different ($p = 0.408$, Table 3). Both K2 and K1 groups were shown significant differences in TAC level change than each treatment group, i.e. X1, X2, and X3. The negative TAC level change in both control groups was significantly different than those of positive TAC level change in each treatment group (Table 2 and Table 3). The TAC level change of X2 group was higher than X1 group ($\Delta$TACX2 = 0.86mmol/l $\pm$0.19, $p = 0.017$) and this was notably contrast ($p = 0.017$ Table 3). This indicated that the dose of 1.77gr/200gr/d WSSP showed a better effect on TAC level change than the dose of 0.89gr/200gr/d WSSP. The TAC level change of both X1 and X2 groups was lower than X3 groups (Table 2). This indicated that the effect of WSSP on TAC level change was lower than AST in T2DM rats. There is a slight decrease in TAC in the group of K2 (-0.15 mmol / l) which is followed by an increase in blood glucose levels ($p = 0.007$) and a decrease in insulin levels ($p = 0.012$) in Table 4; therefore, this indicated that there is an association between the decrease of TAC level with T2DM.

There is a declining number in blood glucose levels and an inclining in insulin levels after the WSSP and AST supplement treatment in T2DM rats ($p < 0.05$, Table 4). Both K2 and K1 groups had increase blood glucose levels in the post-experiment and this difference was significant ($p = 0.007$, $p = 0.013$, Table 4). K2 group has decreased blood insulin level in the post-experiment, and these differences were significant ($p = 0.012$, Table 4).

Because the Kruskall-Wallis test showed a significant difference in blood glucose and insulin change ($p = 0.000$, Table 4), the Mann-Whitney test was then performed (Table 5 and Table 6). Blood glucose level changes of K2 and K1 groups were different ($p = 0.009$, Table 5). Both K2 and K1 groups were shown significantly different blood glucose level changes than each treatment group i.e. X1, X2, and X3. The blood glucose level change of X2 group was higher than X1 group (Table 4), upon which this was significantly different ($p = 0.009$, Table 5). This indicates that the dose of 1.77gr/200gr/d WSSP showed a better effect on blood glucose level change than the dose of 0.89gr/200gr/d WSSP. The blood glucose level change of X2 group was higher than X3 group (Table 4), upon indicated that the effect of WSSP on blood glucose level change was better than AST supplement in T2DM rats.

The blood insulin level changes of K2 and K1 groups were different ($p = 0.008$, Table 6). Both K2 and K1 groups were shown significantly different blood insulin level changes than each treatment group. The blood insulin level change of X2 group was higher than X1 group (Table 4), this indicated that the dose of 1.77gr/200gr/d WSSP showed a better effect on blood insulin level change than the dose of 0.89gr/200gr/d WSSP. The blood insulin level change of X2 group was lower than X3 group (Table 4), this indicated that the effect of WSSP on blood insulin level change was lower than AST supplement.

The effect of WSSP and AST supplement treatment on the HOMA-IR of the T2DM rat is shown in Table 7. HOMA-IR index of K1 group was higher at the end of the experiment than those on the starting point and this was significantly different ($p = 0.001$, Table 7). Healthy rats in K-group showed no difference of HOMA-IR index in those to the final point ($p = 0.235$, Table 7). HOMA-IR index in each treatment group (X1, X2, and X3) was significantly lower in the post-treatment than in the pre-treatment.

**Table 1.** IC50 Value of WSSP and AST Supplement using DPPH and ABTs Radical Scavenging.

| Method | Sample | IC50(ppm) |
|---|---|---|
| **DPPH** | WSSP | 47,639.9 |
| | AST Supplement | 26,309.05 |
| **ABTs** | WSSP | 242,783 |
| | AST Supplement | 154,347.7 |

**Table 2.** Effect of Whiteleg Shrimp Shell Powder and Astaxanthin Supplement on TAC (mmol/l) Level.

| Groups | Pre-Treatment Mean ± SD | Post-Treatment Mean ± SD | Difference (Δ) pre-post treatment | $p^a$ | $p^d$ |
|---|---|---|---|---|---|
| **K2** | 2.06 (1.91 – 2.06) | 1.91 ±0.18 | -0.15 (0.15 – 0.15) | 0.180[b] | 0.000* |
| **K1** | 0.32 ±0.12 | 0.15 (0.15 – 0.29) | -0.14 (0.01 – 0.15) | 0.063[b] | |
| **X1** | 0.29 ±0.15 | 0.74 (0.74-0.88) | 0.59 (0.30 – 0.59) | 0.039[b]* | |
| **X2** | 0.44 (0.29 – 0.44) | 1.24 ±0.17 | 0.86 ±0.19 | 0.001[c]* | |
| **X3** | 0.26 ±0.12 | 1.91 (1.76 – 1.91) | 1.61 (1.47 – 1.62) | 0.041[b]* | |

Note: K2 = healthy rat + standard feed; K1 = T2DM + standard feed; X1 = T2DM + WSSP 0.89 gr/200 gr/d; X2 = T2DM + WSSP 1.77 gr/200 gr/d; X3 = T2DM + AST supplement 0.09 mg/200 gr/d; data with normal distribution represented as mean ± SD, otherwise it was written as median (min-max); a =*p* value of the differences between pre- and post-treatment; b = Wilcoxon; c = paired t-test; d = *p* value for the differences between pre- and post-treatment among five groups (Kruskal Wallis); * = *p* value significant.

**Table 3.** Mann Whitney Test of TAC Level Change (mmol/L) Pre-Post Treatment.

| Groups | *p* value | | | | |
|---|---|---|---|---|---|
| | **K2** | **K1** | **X1** | **X2** | **X3** |
| **K2** | - | 0.408 | 0.006* | 0.007* | 0.007* |
| **K1** | | - | 0.008* | 0.008* | 0.008* |
| **X1** | | | - | 0.017* | 0.008* |
| **X2** | | | | - | 0.008* |
| **X3** | | | | | - |

*$p$<0.05 = significant level

**Table 4.** Effect of Whiteleg Shrimp Shell Powder and AST Supplement on Blood Glucose (mg/dl) and Insulin (µIU/ml) Level.

| Groups | | Pre Mean ± SD | Post Mean ± SD | Difference (Δ) pre-post treatment | *p* value | $p^c$ |
|---|---|---|---|---|---|---|
| **K2** | Blood Glucose (mg/dl) | 76.41 ±6.01 | 77.49 ±5.79 | 1.08 ±0.48 | 0.007[a]* | 0.000 |
| | Blood Insulin (µIU/ml) | 17.32 ±0.20 | 16.49 ±0.30 | -0.38 ±0.20 | 0.012[a]* | 0.000 |
| **K1** | Blood Glucose (mg/dl) | 433.62 ±22.37 | 440.17 | 6.55 ±3.41 | 0013[a]* | |
| | Blood Insulin (µIU/ml) | 12.21 ±0.13 | ±22.42 | 0.35 (0.09-0.35) | 0.042[b]* | |
| | | | 12.49 ±0.20 | | | |
| **X1** | Blood Glucose (mg/dl) | 391.74 (383.76-423.93) | 138.08 ±3.39 | -258.11 ±15.16 | 0.000[a]* | |
| | Blood Insulin (µIU/ml) | 12.46 ±0.24 | 13.88 ±0.32 | 1.41 ±0.14 | 0.000* | |
| **X2** | Blood Glucose (mg/dl) | 477.78 (393.45-484.90) | 107.70 ±1.91 | -370.25 (-287.51 – -376.95) | 0.043[b]* | |
| | Blood Insulin (µIU/ml) | 12.28 ±0.15 | 14.68 ±0.20 | 2.40 ±0.32 | 0.000[a]* | |
| **X3** | Blood Glucose (mg/dl) | 405.87 ±27.78 | 97.24 ±1.24 | -308.63 ±28.09 | 0.000[a]* | |
| | Blood Insulin (µIU/ml) | 12.18 ±0.12 | 15.49 ±0.18 | 3.31 ±0.07 | 0.000[a]* | |

Note: K2 = healthy rat + standard feed; K1 = T2DM + standard feed; X1 = T2DM + WSSP 0.89 gr/200 gr/d; X2 = T2DM + WSSP 1.77 gr/200gr/d; X3 = T2DM + AST supplement 0.09 mg/200 gr/d; data with normal distribution were represented as mean ± SD, otherwise it was written as median (min-max); a = paired t-test; b = Wilcoxon; c = p value for the difference between pre-post treatment among five groups (Kruskal Wallis); * = *p* value significant.

**Table 5.** Mann Whitney Test of Blood Glucose Change Pre-Post Treatment.

| Groups | *p* value | | | | |
|---|---|---|---|---|---|
| | K2 | K1 | X1 | X2 | X3 |
| K2 | - | 0.009* | 0.009* | 0.009* | 0.009* |
| K1 | | - | 0.009* | 0.009* | 0.009* |
| X1 | | | - | 0.009* | 0.028* |
| X2 | | | | - | 0.175 |
| X3 | | | | | - |

Note: *$p$<0.05 = significant level.

**Table 6.** Mann Whitney Test of Blood Insulin Change Pre-Post Treatment.

| Groups | *p* value | | | | |
|---|---|---|---|---|---|
| | K2 | K1 | X1 | X2 | X3 |
| K2 | - | 0.008* | 0.009* | 0.009* | 0.009* |
| K1 | | - | 0.008* | 0.008* | 0.008* |
| X1 | | | - | 0.009* | 0.009* |
| X2 | | | | - | 0.009* |
| X3 | | | | | - |

Note: *$p$<0.05 = significant level.

**Table 7.** Effect of Whiteleg Shrimp Shell Powder and AST Supplement on HOMA-IR Index.

| Groups | Pre-Treatment Mean ± SD | Post-Treatment Mean ± SD | Difference (Δ) pre-post treatment | $p^a$ | $p^d$ |
|---|---|---|---|---|---|
| K2 | 2.94 ±0.26 | 2.92 ±0.26 | -0.03 ±0.04 | 0.235[c] | 0.000* |
| K1 | 11.77 ±0.66 | 12.21 ±0.63 | 0.45 ±0.12 | 0.001[c]* | |
| X1 | 10.97 ±0.36 | 4.26 ±0.08 | -6.71 ±0.42 | 0.000[c]* | |
| X2 | 12.20 ±1.26 | 3.51 ±0.06 | -9.41 (7.22,9.63) | 0.043[b]* | |
| X3 | 10.98 ±0.73 | 3.35 ±0.08 | -7.64 ±0.74 | 0.000[c]* | |

Note: K2 = healthy rat + standard feed; K1 = T2DM + standard feed; X1 = T2DM + WSSP 0.89 gr/200 gr/d; X2 = T2DM + WSSP 1.77 gr/200 gr/d; X3 = T2DM + AST supplement 0.09 mg/200 gr/d; data with normal distribution were represented as mean ± SD, otherwise it was written as median (min-max); a = $p$ value of the difference between pre and post treatment; b = Wilcoxon; c = paired t-test; d = p value for the difference between pre and post treatment among five groups (Kruskal Wallis); * = $p$ value significant.

**Table 8.** Mann Whitney Test of HOMA-IR Index Change Pre-Post Treatment .

| Groups | *p* value | | | | |
|---|---|---|---|---|---|
| | K2 | K1 | X1 | X2 | X3 |
| K2 | - | 0.009* | 0.009* | 0.009* | 0.009* |
| K1 | | - | 0.009* | 0.009* | 0.009* |
| X1 | | | - | 0.016* | 0.028* |
| X2 | | | | - | 0.175 |
| X3 | | | | | - |

Note: *$p$ <0.05 = significant level.

This indicated that WSSP and AST supplement treatments were reduced insulin resistance in T2DM rats. Additionally, T2DM rats without treatment showed an increase in insulin resistance at the end of treatment. There are differences in HOMA-IR among the groups after WSSP and AST supplements treatment ($p$ = 0.000, Table 7). The statistical analysis of the HOMA-IR index change was shown in Table 7.

The increase in HOMA-IR index change found in K1 group was significantly different than those of a decrease in the HOMA-IR index change in the treatment group (X1, X2, and X3) (Table 7 and Table 8). This indicated that WSSP and AST supplement treatment was associated with reduced insulin resistance in T2DM rats. Decreasing the index in the HOMA-IR change of the X2 group was more obvious than those in X1 group, and this difference was significant ($p$ = 0.016, Table 8). This suggested that WSSP

in a dose of 1.77gr/200gr/d has a better effect on insulin resistance than the dose of 0.89gr/200gr/d on T2DM rats. Interestingly, decreasing HOMA-IR index change of X2 group was not different than those of X3 group ($p = 0.175$, Table 8). This suggested that the dose of 1.77gr/200gr/d WSSP has no different than AST supplements in their ability to reduce insulin resistance.

The most interesting finding in this study was WSSP and AST had no different abilities in reducing insulin resistance in T2DM rats. This was shown by HOMA-IR index change analysis (Table 7 and Table 8). It was previously hypothesized that WSSP treatment has a higher ability than AST supplement in reducing insulin resistance. To our knowledge, this study was the first that shows this interesting finding. Previous studies open the possibility of many mechanisms involved in reducing insulin resistance.

Although the results of the activity test of antioxidant WSSP was a low category, the bioactive component in WSSP will be digested by digestive enzymes including the pepsin and lipase enzyme in the body. This digestive process causes WSSP to be more representative when digested in the body. Pepsin is a hydrolase enzyme that catalyzes the hydrolysis reaction of a substrate using water molecules as a mediator. Pepsin can break down protein molecules to be smaller; therefore, the AST can separate itself from its bonds, in this case, are amino acids. In addition to the enzyme pepsin, the lipase enzyme, which is one of the enzymes that play a role in breaking down fat molecules into fatty acids, also plays a pivotal role in the release of AST from fatty acid bonds. The digestive process of AST follows the process of fat metabolism in the body. The absorption of AST greatly depends on the steps of food digestion to ease the absorption of AST into the intestinal lumen starting from the mechanical and chemical digestion of the digestive tract, emulsification assisted by bile salts, absorption by microvilli, and binding of AST with some lipoproteins. The emulsion of the fat types can increase the bioavailability of AST by expanding the surface for the release of AST. This emulsion's works resemble the mechanism of release of bile salts during lipolysis and help the absorption of lipophilic compounds that will increase the solubility of AST. By formulating AST in the form of fat-based emulsions, AST absorption can get into the lymphatic circulation without having to follow fat metabolism from the beginning. Polar carotenoids such as zeaxanthin, lutein, AST are transported by High-Density Lipoprotein (HDL). The fat composition in WSSP and digestive processes in animal bodies can predictably increase AST bioavailability in WSSP, hence it can improve TAC and insulin resistance **(Ayudiarti, 2011; Affandi, Julianto and Majeed, 2012; Ambati et al., 2014; Benarroch et al., 2016)**.

An increase in the production of ROS was accompanied by a decrease in TAC levels results in the release of fatty acids into the blood circulation. This mechanism impacts the activation of HIF-1 gene. The gene will have an impact on an increase in the activity of several enzymes such as JNK and IKK which are known to cause damage to insulin receptor cells in adipose tissue **(Ye, 2013)**. Oxidative stress also results in insulin resistance in the liver and muscles by increasing Diasyl Glycerol (DAG) which increases Protein Kinase C (PKC), this is followed by a disruption of phosphorylation in Insulin Receptor Substrate (IRS-1 and IRS-2) as an indication of liver and muscle insulin resistance. An increase in fatty acids in the circulation due to oxidative stress also causes an increase in the formation of ceramide which will produce large amounts of Nitric Oxide (NO). This substance is toxic to pancreatic β cells and results in the death of pancreatic β cells which is known as T2DM **(Al-Goblan, Al-Alfi and Khan, 2014; Sears and Perry, 2015)**.

Whiteleg Shrimp Shell Powder contains several bioactive components that can improve T2DM. One of the main antioxidants in the carotenoid category found in WSSP is AST, AST is known as a powerful antioxidant that works from inside and outside the cell **(Ambati et al., 2014)**. Astaxanthin acts as a powerful antioxidant by the mechanism of supplying electrons and reacting with free radicals. One of which is ROS which will form more stable free radicals and stop the systemic reaction of free radicals. Astaxanthin can inhibit the production of ROS that occurs in mitochondria in antioxidant activity from AST. Therefore, it will increase antioxidant status in the bodies of rats induced by T2DM **(Yang et al., 2015)**. The previous study shows that giving AST extract from shrimp waste increases the levels of Catalase (CAT), Superoxide Dismutase (SOD), and Glutathione Peroxide (GPx), particularly GPx in diabetic induced rats (**Sila et al., 2015)**. Astaxanthin supplements resulted in an increase in TAC in overweight and obese humans **(Choi et al., 2011)**. Giving HFD-STZ aims to form oxidative stress in the body especially pancreatic β cells which are known as one of the cells of the body that contains a little antioxidant **(Savu et al., 2012)**. Giving HFD-STZ results in a decrease in TAC levels which is caused by several things, namely an increase in the production of ROS and inadequate endogenous antioxidants due to competition between endogenous antioxidants and pro-oxidants in the body **(Asmat, Abad and Ismail, 2016)**. There was a significant decrease in the levels GPx, Glutathione Redoxin (GR), and SOD when they were compared to the non DMT2 group **(Aouacheri et al., 2015)**. A decrease in oxidative stress caused by the activity of AST antioxidant results in the improvement to insulin receptors which will lead to the improvement of the T2DM condition. There is a slight decrease in TAC in the group of K2 (-0.15 mmol / l) which is followed by an increase in blood glucose levels ($p = 0.007$) and a decrease in insulin levels ($p = 0.012$). This condition was predictably caused by psychological stress experienced in K2 group. The lack of antioxidant composition in the standard feed also predictably leads to a decrease in TAC in the group of K2. It illustrates the importance of exogenous antioxidant intake to maintain body health **(Shiloah et al., 2003)**.

The other study shows that there was a decrease in blood glucose levels in diabetic rats after the intake of AST with a dose of 50 mg/kg Body Weight and 100 mg/kg Body Weight for 14 days **(Li et al., 2016)**. Shrimp waste oil can lower blood glucose levels in rats that were given HFD after glucose tolerance testing **(Nair et al, 2017)**. The previous study shows that AST increases the production and secretion of insulin in T2DM rats. Regeneration of pancreatic β cells is caused by suppression of oxidative stress biomarkers such as $H_2O_2$ and MDA as well as an increase in total antioxidant capacity which results in a decrease in blood glucose levels in the circulation.

Therefore, it gives them time for β cells to repair to increase insulin secretion **(Al-Bulish et al., 2017)**.

An increase in TAC resulted in an improvement in the HOMA-IR index. This research finds that there was an increase in TAC and a decrease in the HOMA-IR index after the treatment of WSSP and AST supplements. Astaxanthin is not the only bioactive substance contained in WSSP, chitosan is a chitin deacetylation product which is a polymer of glucosamine long-chain (β-1, 4-2 amino-2-deoxy-D-glucose) which has an impact on the HOMA-IR index. Chitin and chitosan are known to have several activities such as antioxidants and antidiabetic. The mechanism of the improvement in T2DM done by chitosan through suppressing the process of gluconeogenesis in the liver is by suppressing the expression of Phosphoenolpiruvate Carboxykinase (PEPCK), p38 phosphorylation, and an increase in the activities of AMP-kinase (AMPK) in the liver so that the store of glucose in the liver can be maintained. Chitosan also increases kinase Protein Akt / PKB which is the main mediator in the translocation of GLUT4. This can increase the uptake of glucose by expediting the translocation of GLUT4 from the circulation into glucose target tissues such as the liver and muscles **(Liu, Chang and Chiang, 2010)**. The mechanism of T2DM repair done by chitosan provides an opportunity for Pancreatic β cells and insulin receptors to carry out cell regeneration due to a decrease in glucolipotoxicity and low calories from the standard feed. An increase in insulin secretion results in a decrease in blood glucose levels which leads to an improvement in the HOMA-IR index **(Sathananthan et al., 2015)**.

Another component that contains WSSP is calcium which has a role in insulin secretion by pancreatic β cells. The condition of T2DM leads to a decrease in calcium levels both in extracellular and intracellular. Hypocalcemia was caused by the accumulation of Advanced Glycation End Products (AGEs) in bone collagen fibers. Accumulation of AGEs increases the process of calcium resorption that results in Osteoporosis under the condition of T2DM. As many as 85.49% of the calcium content in shrimp shells is known to be able to meet calcium needs to stimulate insulin secretion by pancreatic β cells when the condition of pancreatic β cells has improved and insulin secretion by β cells is still normal **(Miyata et al., 1997)**. There was an increase in insulin secretion by pancreatic β after calcium supplementation for diabetic patients and those at risk of diabetes **(Mitri et al., 2011)**. The other study shows that there was a decrease in the HOMA-IR index in diabetic patients that were given fortified yogurt of vitamin D and Calcium **(Nikooyeh et al., 2011)**.

The results of this study showed that a higher increase of TAC was found in the treatment group of AST supplement, otherwise, the HOMA-IR index change showed that X2 group had better improvement compared to X1 and X3 treatment group. It indicated that WSSP has an advantage in improving the condition of T2DM. Bioactive substances contained in WSSP, such as AST, chitosan, and calcium, as well as various other substances, that get together with different mechanisms can reduce the HOMA-IR index as a form of improvement in T2DM conditions and insulin resistance in peripheral tissues. The results of this study provide a new insight into which WSSP can increase TAC and decrease the HOMA-IR index in T2DM conditions. Further researches need to be carried out to determine the in-depth mechanism for T2DM improvement by WSSP so it can make WSSP one of the products for prevention of T2DM disease and progressive prevention of T2DM itself.

## CONCLUSION

In a conclusion, the results of this study show a beneficial effect of WSSP in increasing TAC and decreasing the HOMA-IR index in Wistar rats induced by HFD-STZ. For further investigation of the proper use of pathohistological preview of the pancreatic tissue amelioration, it would be advisable to investigate the tissue remodeling and hematological profile in different stages of diabetic tissue damage.

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# THE EFFECT OF EXTRACTION TIME ON THE PHYSICOCHEMICAL CHARACTERISTICS OF NANOCALCIUM POWDER FROM CHICKEN AND DUCK EGGSHELLS

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## ABSTRACT
Calcium deficiency is associated with the risks of bone fracture and osteoporosis. This type of malnutrition has been a concern of governments and the World Health Organization for decades, and extensive efforts have been made to address it. There are several solutions to increase calcium intake. One is to take calcium in the form of nanocalcium. The objective of the present research was to determine the effect of extraction time on the physicochemical characteristics of nanocalcium powder extracted from chicken and duck eggshells through precipitation. This research was conducted by using a completely randomized factorial design with two factors, and each treatment analysis was repeated three times. The first factor was the type of eggshell (chicken and duck), and the second was the extraction time (1, 1.5, and 2 hours). The observed parameters were physical (yield and color) and chemical characteristics (moisture, ash, calcium content, and crystalline structure). The results showed that the type of eggshell had a significant effect ($p$ <0.05) on yield, color (lightness), and moisture content and that extraction time had a significant effect ($p$ <0.05) on yield, color (chroma and hue), moisture, and ash content. The highest content of the crystalline structure of nanocalcium formation (100%) was nanocalcium powder from chicken and duck eggshells, with 1 hour of extraction time. The particle size of the crystalline structure of nanocalcium from chicken and duck eggshells were 41.54 nm and 24.90 nm, respectively.

**Keywords:** nanocalcium; chicken; duck; eggshell; extraction

## INTRODUCTION
Calcium is the main mineral in the human body, with as much as 1.5% – 2% of adult body weight or about 1 kg **(Ariyanti, 2012)**. Calcium plays an important role in stiffness, bone strength, and most metabolic processes, including blood clots, muscle contractions, hormonal systems, glycogen metabolism, cell proliferation, and differentiation **(WHO, 2006)**. People's calcium consumption varies according to age and sex.

Indonesian people's dietary intakes of calcium, 254 mg/day, are far below the requirement to build proper bone mass. It is widely accepted in the literature that a low calcium intake over the years contributes to the development of bone and osteoporosis. Factors that lead to calcium deficiency among Indonesians are poor dietary habits, low intake of natural sources of calcium (e.g., dairy products), low intake of other calcium sources that must be consumed in large amounts to meet the recommended dietary intake for calcium, and low bioavailability (e.g., cereals, nuts, and green leafy vegetables). Another factor is consumer behavior, such as purchasing power, food preferences, and special conditions (e.g., teen years, young adult years, childbearing years, pregnancy, breastfeeding, later adult years, lactose intolerance, and a vegetarian diet).

There are several solutions to increase calcium intake. One of these is consuming calcium-fortified foods. However, calcium in these food products is more available in the form of microcalcium, so the absorption of calcium in the body (only about 50%) is not optimal **(Lekahena et al., 2014)**.

Current technological advances, particularly nanotechnology, can overcome the problem of eggshell waste by extracting nanocalcium powder with particle sizes of 10 – 100 nm in diameter. This process increases the economic value of abundant eggshell waste in line with the concept of zero waste products. It also reduces calcium deficiency by converting calcium from the eggshells into absorbable forms **(Suptijah, Jacoeb and Deviyanti, 2012)**.

Using nanotechnology in the fractionation process of the precipitation method converts calcium carbonate into calcium oxide so that it produces nanocalcium powder with good physical and chemical characteristics. This method controls the solubility of the material in the solution through pH and temperature changes by adding

certain chemicals to convert soluble compounds into insoluble solids or by mixing acidic bases, producing solids and water **(Purwasasmita and Gultom, 2008)**. This method is very effective because it produces nanoparticles through a simple and low-cost process **(Gulsun, Gursoy and Oner, 2009)**.

Eggs are the main protein source in Indonesia. They are popular with the community because they are highly nutritious, relatively cheap compared to other animal protein sources, delicious, and easily digested in the body. In Indonesia, the production of chicken eggs in 2018 reached 1,644,460 tons, with an average consumption per capita of as much as 302.71 grams per day **(CSA, 2016)**.

Increasing the value of egg production is in response to the increasing demand for eggs. Thus, the potential for eggshell waste in Indonesia is quite large but has not been used optimally. People generally dispose of eggshells without using them first even though 96% of their calcium content (94% calcium carbonate, 1% magnesium carbonate, and 1% calcium phosphate) can be additional material extracted for food minerals. The main composition of calcium carbonate in eggshells can cause environmental pollution because soil microbes can't degrade it easily **(Trilaksani, Salamah and Nabil, 2006)**.

Several studies confirmed that nanosizing increases the bioavailability of calcium **(Park et al., 2008; Seo et al., 2009; Hilty et al., 2011).** Nanocalcium powder can be used in various products and food fortification as a form of functional food that benefits people's health. Therefore, research was conducted on the effect of eggshell type (chicken and duck) and extraction time on the physicochemical characteristics of nanocalcium powder.

## Scientific Hypothesis

It is hypothesized that different types of eggshells and extraction time had significant effects on the physicochemical characteristics of eggshells' nanocalcium powder.

# MATERIAL AND METHODOLOGY

## Samples

Chicken and duck eggshells were obtained from the cake home industry in Palembang city, South Sumatera Province, Indonesia.

## Chemicals

The main chemicals used in this study were lanthanum, chloric acid, potassium hydroxide, and demineralized water.

## Instruments

The instruments used in this study were an atomic absorption spectrophotometer (AAS, Shimadzu AA-7000, Japan), a chromameter (CR-410 Konica Minolta, Japan), and X-ray diffraction (XRD, RigakuBenchtop XRDMiniflex 60, Japan).

## Description of the Experiment

The sample in this research was eggshell nanocalcium powder prepared from eggshell powder.

**Preparation of eggshell powder (Rahmawati and Nisa, 2015)**

Chicken and duck eggshells (500 g) were washed with water until clean. The cleaned eggshells were boiled at 100 $^{o}$C for 10 minutes to kill pathogenic microbes. They were drained and then dried in an oven for 2 hours at 60 $^{o}$C. Then, the eggshells were placed at room temperature. They were ground to powder and sieved with a 100 mesh sieve. The eggshell powder was included in the oriented polystyrene) plastic and stored at 4 $^{o}$C for analysis.

**Preparation of eggshell nanocalcium powder (Khoerunnisa, 2011)**

Eggshell powder was immersed in 1N HCl solvent (1:5) for 48 hours and then extracted at 90 $^{o}$C for 1, 1.5, and 2 hours. The extracts were then filtered with filter paper to obtain filtrates and sediments. The filtrate was precipitated by adding 3N NaOH. It was stirred and left until the precipitate was formed. The precipitate was then neutralized by using aquabidest to pH neutral. The solution was separated from the sediment by pouring it slowly so that the precipitate was not wasted. The sediment was dried in an oven for 3 hours at 105$^{o}$C. It was ashed in a muffle furnace at 600 $^{o}$C for 5 hours and refined with a mortar (Figure 1). The nanocalcium powder was packed in airtight plastic bags and stored at 4°C until it was used.

**Number of sample analyzed:** The research was conducted using a completely randomized factorial design with two factors, and each treatment analysis was repeated three times. The first factor (A) was the type of eggshell (A1 = chicken and A2 = duck), and the second factor (B) was the extraction time (B1 = 1hour, B2 = 1.5 hour, and B3 = 2 hours).

**Number of experiment replication:** The experiment was performed in triplicates**.**

## Analyses of physicochemical characteristics of eggshell nanocalcium powder

**Yield determination**

The nanocalcium yield was calculated using the following formula (1):

$$\text{Yield (\%)} = \frac{\text{weight of nanocalcium powder}}{\text{weight of eggshell powder}} \text{ x } 100\% \qquad (1)$$

**Color determination of nanocalcium**

Nanocalcium powder's color was measured using **Munsell (1977)**. The values were L (lightness), C (chroma), and H (hue) scales.




**Figure 1** Processing of Nanocalcium from Egg and Duck.

**Moisture content (AOAC, 2005) of nanocalcium**

A sample of approximately 1 g ($W_1$) was placed on a dish and dried at 105°C for 18 hours. After drying, a dish containing samples was transferred to a desiccator for 15 minutes. Then, the dish was reweighted until its weight was constant ($W_2$). Nanocalcium's moisture content was calculated using the following formula (2):

$$\text{Moisture content (\%)} = \frac{(W_1 - W_2)}{W_2} \text{ x } 100\% \quad (2)$$

$W_1$ = weight (g) of a sample before drying
$W_2$ = weight (g) of a sample after drying

**Ash content (AOAC, 2005) of nanocalcium**

A sample of about 1 g sample ($W_1$) was placed on a porcelain crucible. Then, it was put in a muffle furnace at 550°C until the samples turned whiteish gray. The ash content of the nanocalcium was calculated using the following formula (3):

$$\text{Ash content (\%)} = \frac{\text{weight of ash}}{\text{weight of the sample}} \text{ x } 100\% \quad (3)$$

**Calcium determination (AOAC, 2005) of nanocalcium**

The calcium content of samples was determined using an AAS (Shimadzu AA-7000, Japan) according to the AOAC (Association of Official Analytical Chemists) method **(AOAC, 2005)**. The ground sample (5 g) was placed in an ashing vessel, charred in a muffle furnace, and then ashed at 500°C overnight. The completely ashed sample was dissolved in 10 mL of concentrated hydrochloric acid. The solution was boiled and evaporated nearly to dryness. The residue was redissolved in 20 mL of 2N hydrochloric acid and boiled gently. The solution was cooled and diluted to 100 mL with distilled deionized water. Its absorbance was then measured using the AAS at 422.7 nm. The measurements were calibrated using a commercial standard solution (Merck KGaA, Germany). To eliminate phosphorus interference in the measurements, lanthanum was added to the test ash and standard solutions so that the final solution contained 1% lanthanum.

**The crystalline structure of nanocalcium powder**

XRD was used to measure the crystal structure of samples.

## Statistical Analysis

All analyses were performed in triplicates. The data were subjected to the analysis of variance followed by Fisher's least significant difference (LSD) test to compare treatment means. Differences were considered at a significant level of 95% ($p$ <0.05) by using SPSS v.19 software.

## RESULTS AND DISCUSSION

### Physical Characteristics of Nanocalcium Powder

**Yield**

The yield of nanocalcium powder ranged from 11.62% to 15.27% for all samples. The lowest average yield was found in the $A_1B_1$ treatment (chicken eggshells, 1 hour extraction time), while the highest yield value was found in the $A_2B_2$ treatment (duck eggshell, extraction time of 1.5 hours). The results showed that the type of eggshell and the extraction time had a significant effect on the yield of nanocalcium powder ($p$ <0.05). Table 1 shows the value of the average yield of nanocalcium powder.

The yield of duck nanocalcium powder was significantly higher ($p$ <0.05) than that of chicken nanocalcium powder due to the main component forming the eggshell, calcium carbonate ($CaCO_3$). Calcium carbonate is a major component of an eggshell **(Shwetha et al., 2018)**. Duck eggshells have a higher $CaCO_3$ content (96.48%) **(Sari, 2013)** than chicken eggshells (90.90%) (**Warsy et al., 2016)**. However, the result of research conducted by **Ajayan et al. (2020)** showed that the average percentage of calcium carbonate in eight varieties of chickens (89.05%) was higher than that in six varieties of ducks (84.63). **Adeyeye (2009)** stated that hen eggshells contain more calcium than duck eggshells. This is because the differences in calcium carbonate content between shells are due to differences in chicken or duck varieties. Also, calcium carbonate content depends on the ratio of the dietary calcium of poultry feed **(Lestari, Riyanti, and Wanniatie, 2015)**.

During the extraction process, higher $CaCO_3$ content and a longer extraction time caused a higher solvent penetration into the eggshell powder, resulting in more compounds diffusing out of the shell and giving a higher yield. However, a yield of 2-hour extraction time was not significantly different from that of 1.5 hours. This is because the calcium carbonate cycle has a reversible reaction so that it can allow the return of products to reactants, where carbonic acid can react again with calcium carbonate to form calcium bicarbonate **(Dewi et al., 2012)**. **Risnojatiningsih (2009)** stated that the formation of $Ca(HCO_3)_2$ occurs when the formed $CaCO_3$ continues to react with water containing $CO_2$ gas. The result also agreed with the findings of **Khoerunnisa (2011)**, showing that the yield of local mussel shell nanocalcium with 1N HCl extraction significantly increased from 1-hour extraction (5.02%) to 1.5 hours (8.53%) and decreased insignificantly from 1.5 hours (8.53%) to 2 hours of extraction (7.89%).

**Color**

The color of nanocalcium powder from chicken and duck eggshells was white. This indicates that calcium oxide (CaO) has formed. This is following the statement of **Sing et al. (2011)** and **Tangboriboon et al. (2012)** that the eggshells' change in color to white during calcinations indicates that a complete chemical transformation from calcium carbonate to calcium oxide has been achieved.

**Table 1** Physicochemical characteristics of nanocalcium powder from chicken and duck eggshells with different extraction times.

| Treatment | Yield (%) | Colour | | | Moisture content (%) | Ash content (%) |
|---|---|---|---|---|---|---|
| | | Lightness (%) | Chroma (%) | Hue (°) | | |
| $A_1B_1$ | 11.62 ±0.28[a] | 93.67 ±3.71[a] | 7.07 ±0.06[a] | 26.30 ±0.20[a] | 0.33 ±0.01[a] | 96.80 ±0.44[a] |
| $A_1B_2$ | 13.30 ±0.27[b] | 93.73 ±0.12[a] | 6.80 ±0.10[a] | 28.30 ±0.30[a] | 0.25 ±0.01[a] | 98.32 ±0.05[a] |
| $A_1B_3$ | 13.59 ±0.20[b] | 93.67 ±0.35[a] | 6.83 ±0.12[a] | 28.00 ±0.17[a] | 0.26 ±0.01[a] | 98.28 ±0.05[a] |
| $A_2B_1$ | 13.83 ±0.03[b] | 93.53 ±0.15[a] | 7.03 ±0.06[a] | 26.93 ±0.35[a] | 0.18 ±0.03[b] | 97.91 ±0.64[a] |
| $A_2B_2$ | 15.27 ±0.32[c] | 93.37 ±1.01[a] | 6.73 ±0.12[a] | 28.87 ±0.46[a] | 0.18 ±0.03[b] | 98.69 ±0.10[a] |
| $A_2B_3$ | 15.20 ±0.20[c] | 93.87 ±0.21[a] | 6.77 ±0.06[a] | 28.27 ±0.23[a] | 0.17 ±0.03[b] | 98.67 ±0.10[a] |

Note: Data are presented as mean ± standard deviation of triplicate determinations.
Means for each characteristic followed by the same superscript within the same row are not significantly different at $p < 0.05$ by the LSD test.
$A_1B_1$ = chicken eggshell; 1-hour extraction time
$A_1B_2$ = chicken eggshell; 1.5-hour extraction time
$A_1B_3$ = chicken eggshell; 2-hour extraction time
$A_2B_1$ = duck eggshell; 1-hour extraction time
$A_2B_2$ = duck eggshell; 1.5-hour extraction time
$A2B_3$ = duck eggshell; 2-hour extraction time

The lightness value of nanocalcium powder ranged from 92.00% to 94.03%. The lowest average lightness value was found in $A_1B_1$ (chicken eggshell, extraction time of 1 hour), while the highest lightness value was found in treatment $A_2B_2$ (duck eggshell, extraction time of 1.5 hours). Chroma value of nanocalcium powder ranged from 6.73% to 7.07%. The lowest average chroma value was found in $A_2B_2$ (duck eggshell, extraction time of 1.5 hours), while the highest chroma value was found in $A_1B_1$ (chicken eggshell, 1 hour extraction time). The hue value of nanocalcium powder ranged from 26.30° to 28.87°. The lowest hue value was found in the $A_1B_1$ treatment (race chicken eggshell, extraction time of 1 hour), while the highest hue value was found in the $A_2B_2$ treatment (duck eggshell, extraction time of 1.5 hours). Table 1 shows the average lightness, chroma, and hue values of nanocalcium powder.

The value of lightness is the degree of brightness of a product. Chicken and duck eggshells produced high lightness values of nanocalcium powder. This was because the preparation of nanocalcium powder through HCl caused pigment deposition in each eggshell so it degraded easily and the color became brighter. Duck eggshells contain biliverdin pigments,hence their greenish-blue color. On the other hand, chicken eggshells contain porphyrin pigments, hence their brownish shells **(Mushawwir and Latipudin, 2013; Yonata et al., 2017)**.

The immersion process with HCl and extraction can cause the greenish color of the duck eggshells to degrade easily, resulting in a whiter color, while the brownish color of the chicken eggshell produces a darker color than the duck egg nanocalcium powder. The difference in the components of the constituent minerals in the eggshell also affects the lightness value of the nanocalcium powder. The main component of the constituent minerals of nanocalcium powder is calcium, which generally has a white color. Therefore, the lightness value of nanocalcium powder was also high.

Chroma is a parameter used to determine the color intensity of a product. The chroma value is inversely proportional to the lightness value because if a product has a dark color, the intensity of the resulting color will be stronger.If the lightness value is high, then the chroma value produced from a product will be lower. The longer the extraction, the more $CaCO_3$ hydrolyzed by HCl will produce a whiter color, resulting in a decreased chroma value.

Hue is a value to determine the dominant wavelength of the color in a product. The hue of nanocalcium powder was red (R), with a range of 18°–54°. The longer the extraction, the more the hue of nanocalcium powder turned yellowish red. This was because the longer the extraction, the more calcium was extracted from the eggshell. The constituent elements of calcium from the flame color test are brick red **(Permata et al., 2018)**. Therefore, the more calcium in eggshell powder, the more the hue of the nanocalcium powder turned yellowish red.

## Chemical Characteristics of Nanocalcium Powder

### Moisture content

The moisture content of nanocalcium powder ranged from 0.16% to 0.33% for all samples. These values were very small due to drying in the preparation of nanocalcium powder. The moisture content of nanocalcium of all samples is shown in Table 1. That of nanocalcium powder of chicken eggshells was significantly higher than that of duck eggshells ($p < 0.05$). This must be because the initial moisture content of duck eggshells (1.43 ±0.04%) was significantly lower than that of chicken eggshells (1.99 ±0.01%), decreasing the water content in the calcination stage of the nanocalcium preparation.

The extraction time significantly affected the moisture content of nanocalcium powder ($p < 0.05$). The longer the extraction time, the lower the moisture content of nanocalcium powder.

**Table 2** Calcium content of microcalcium and nanocalcium powders from chicken and duck eggshells.

| Treatment | Calcium content (%) |
|---|---|
| Microcalcium powder (149 μm) | |
| Chicken | 11.41 ±0.03 |
| Duck | 12.32 ±0.14 |
| Nanocalcium powder | |
| $A_1B_1$ | 40.80 ±0.06 |
| $A_1B_2$ | 24.78 ±0.04 |
| $A_1B_3$ | 24.52 ±0.05 |
| $A_2B_1$ | 49.39 ±0.08 |
| $A_2B_2$ | 27.38 ±0.05 |
| $A_2B_3$ | 25.42 ±0.04 |

Note: $A_1B_1$ = chicken eggshell; 1-hour extraction time
$A_1B_2$ = chicken eggshell; 1.5-hour extraction time
$A_1B_3$ = chicken eggshell; 2-hour extraction time
$A_2B_1$ = duck eggshell; 1-hour extraction time
$A_2B_2$ = duck eggshell; 1.5-hour extraction time
$A_2B_3$ = duck eggshell; 2-hour extraction time

This was due to the evaporation of water in the filtrate so that the water content of the nanoparticle powder became free water. The decrease in water content was also due to the protein content in eggshell powder being hydrolyzed when it is mixed with chloric acid and the heating process resulting in decreased water binding. This observation was in agreement with the result found by **Trilaksani et al. (2006)** and **Agustini et al. (2011)**, who stated that the low water content in tuna bone and clamshell powder was due to protein hydrolysis during heating in powder preparation.

**Ash content**

The ash content of nanocalcium powder ranged from 96.80% to 98.69% for all samples. Table 1 shows the ash content of all samples' nanocalcium. The extraction time significantly affected the ash content of nanocalcium powder ($p$ <0.05). The ash content increased as the extraction time increased. This may be because the longer the extraction time, the longer the contact of nanoparticle powder and solvent (chloric acid). This, in turn, increased the chance of hydrolysis reaction. This result was supported by the findings by **Widyastuti et al. (2015)**, who concluded that the ash content of chicken eggshell nanocalcium with a 1N NaOH solvent and 1-,2-, and 3-hour extraction gave the highest value of ash content in the 3-hour extraction time: 98.07%, 98.01%, and 98.03% for 1-,2-, and 3-hour extraction, respectively.

**Calcium content**

The calcium content of microcalcium and nanocalcium of all samples is shown in Table 2. Microcalcium content (149 μm) of chicken eggshells was higher than that of duck eggshell. This was because $CaCO_3$ of duck eggshell (96.48%) was higher than that of duck eggshell (90.90%), resulting in a higher amount of extracted calcium. Moreover, the calcium content of nanocalcium was higher than that of microcalcium both from chicken and duck eggshells. This was due to demineralization, precipitation, and calcination processes during nanocalcium preparation, maximally opening eggshell spores and extracting more calcium extracted. The steps of the reactions are as follows:

Demineralization:

$$CaCO_{3(s)} + 2HCl_{(aq)} \rightarrow CaCl_{2(aq)} + H_2O_{(l)} + CO_{2(g)} \quad (1)$$

Precipitation:

$$CaCl_{2(aq)} + 2NaOH_{(aq)} \rightarrow Ca(OH)_{2(s)} + 2NaCl_{(aq)} \quad (2)$$

Calcination:

$$Ca(OH)_{2(s)} + Heat \rightarrow CaO_{(s)} + H_2O_{(l)} \quad (3)$$

The observation supported by previous research **(Navarro et al., 2009; Mohamed et al., 2012; Mosaddegh et al., 2014; Zuhra et al., 2015)** concluded that $CaCO_3$ can be converted to CaO through thermal decomposition (calcination).

Moreover, Table 2 shows that the calcium content of nanocalcium from chicken and duck eggshells decreased with increasing extraction time. This may be due to $CaCO_3$ reformation, resulting from the reversible reaction of the calcium carbonate cycle. The steps are as follows:

$$CO_{2(g)} + H_2O_{(l)} \rightarrow H_2CO_3 \quad (4)$$

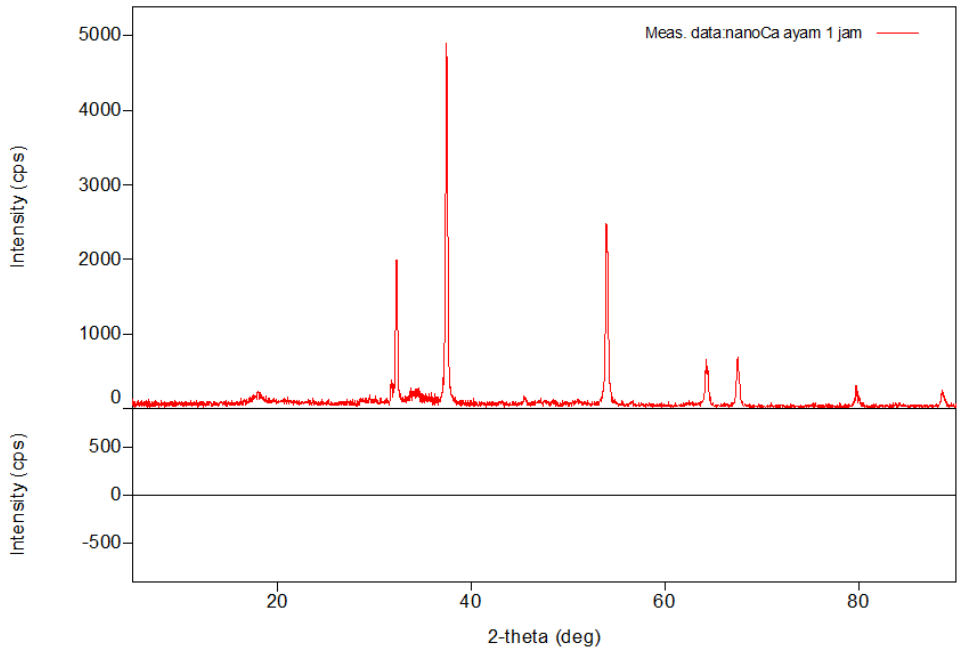
$$CaCO_{3(s)} + H_2CO_{3(aq)} \rightarrow Ca(HCO_3)_{2(aq)} \quad (5)$$

$$Ca(HCO_3)_2 \rightarrow CaCO_{3(s)} + H_2O_{(l)} \quad (6)$$

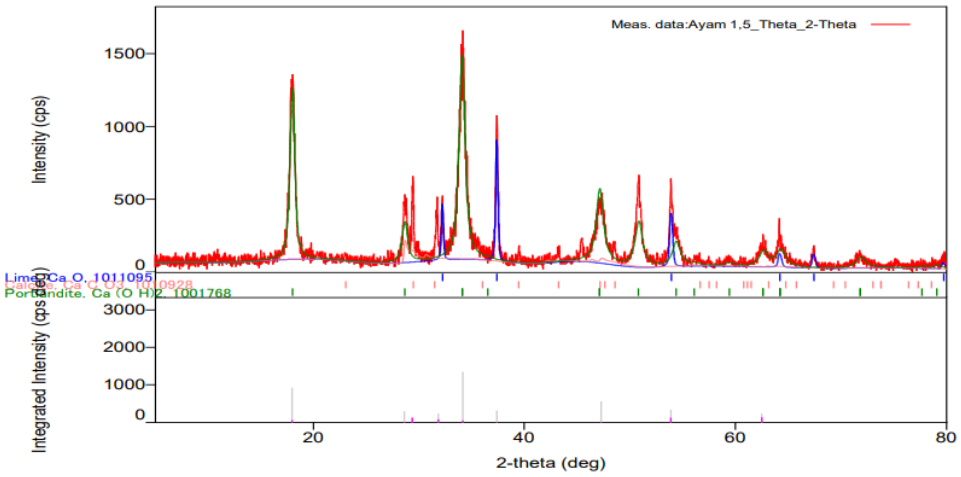
## X-Ray Diffraction

The XRD results are used to determine the crystalline structure of nanocalcium powder. The XRD is monitored at $2\Theta = 5^{\circ} - 90^{\circ}$. The results of the XRD analysis of nanocalcium from chicken eggshells with extraction times of 1, 1.5, and 2 hours are presented in Figure 2. Figure 3 shows the result of the XRD analysis of nanocalcium from duck eggshells with extraction times of 1, 1.5, and 2 hours.

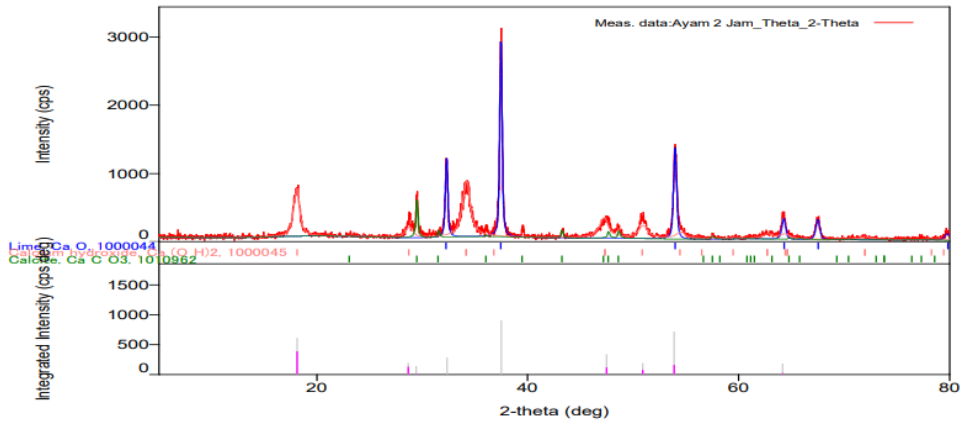
The main peak appeared at $2\Theta = 37.44°$ of nanocalcium from chicken eggshells and $2\Theta = 37.36°$ of nanocalcium from duck eggshells. Peaks of nanocalcium oxide (CaO) from chicken eggshells appeared at $2\Theta = 32.28°$, 37.44°, 53.94°, 64.21°, and 67.44°. Moreover, peaks of nanocalcium oxide (CaO) from duck eggshells appeared at $2\Theta = 32.15°$, 37.36°, 53.85°, 64.10°, and 67.32°.




(a)




(b)




(c)

**Figure 2** XRD of nanocalcium chicken eggshells.
Note: from (a) 1-hour extraction, (b) 1.5-hour extraction, and (c) 2-hour extraction.

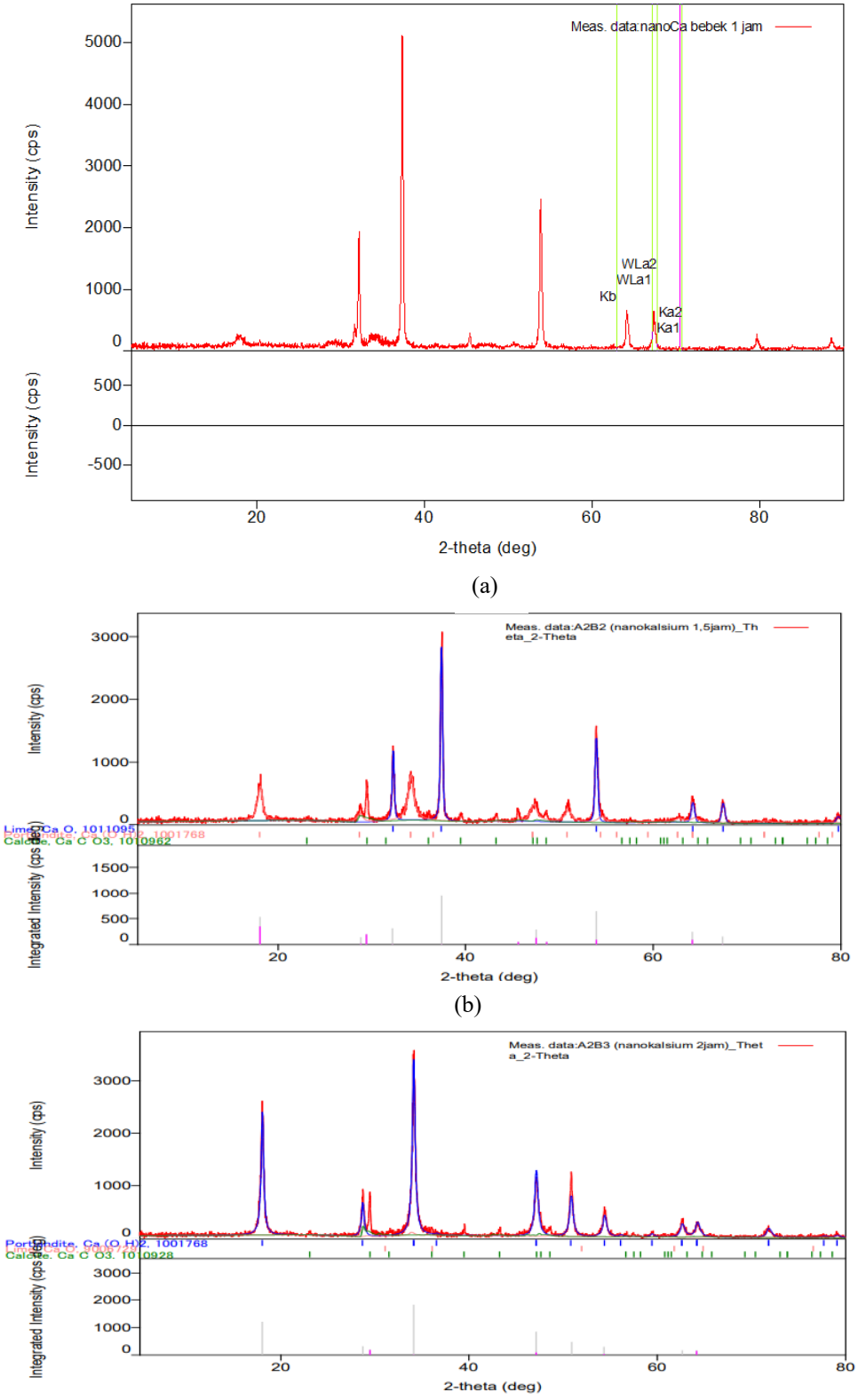



**Figure 3** XRD of nanocalcium duck eggshells from (a) 1-hour extraction, (b) 1.5-hour extraction, and (c) 2-hour extraction.

The peaks that appeared were all identified and corresponded to the database International Center for Diffraction Data (ICDD) of calcium oxide (CaO) (PDF No. 99-0070). This result was supported by **Taufiq_Yap et al. (2011)**, and **Habte et al. (2019)** stated that the peaks at 2Θ = 32.22°, 37.36°, 53.8°, 64.1°, and 67.3° were assigned to planes of pure CaO phase. Nanocalcium oxide powder of chicken and duck eggshells had a polycrystalline structure **(Habte et al., 2019; Khan et al., 2018)**.

The XRD patterns displayed diffractograms of CaO, $Ca(OH)_2$, and $CaCO_3$. The XRD patterns of nanocalcium from chicken eggshells have peaks of CaO (100%) at 1-hour extraction time; $Ca(OH)_2$ (77.4%), $CaCO_3$ (11.0%), and CaO (11.6%) at 1.5-hour extraction time; and $Ca(OH)_2$ (52.2%), $CaCO_3$ (9.0%), and CaO (38.8%) at 2-hour extraction time. Moreover, the XRD patterns of nanocalcium from duck eggshells have peaks of CaO (100%) at 1-hour extraction time; $Ca(OH)_2$ (48.1%), $CaCO_3$ (6.9%), CaO (45.1%) at 1.5-hour extraction time; and $Ca(OH)_2$ (87.3%), $CaCO_3$ (11.2%), and CaO (1.5%) at 2-hour extraction time.

The nanocalcium oxide (CaO) content from chicken and duck eggshells at 1 hour of extraction was formed 100%. This indicated that the transformation of chemical composition from $CaCO_3$ to CaO was completely achieved after the whole process took place. This observation was supported by **Dasgupta et al. (2004)**, who stated that $CaCO_3$ turned to CaO at 540°C. The appearance of peak $Ca(OH)_2$ of nanocalcium from chicken and duck eggshells at extraction time 1.5 and 2 hours due to the hydration reaction between hygroscopic CaO and water vapor. In the same condition, the appearance of peak $CaCO_3$ must be due to the incomplete decomposition of $CaCO_3$ to CaO.

Based on the XRD analysis, the crystallite size and density of nanocalcium oxide from chicken and duck eggshells were 41.54 nm and 3.36 g/cm$^3$ and 24.90 nm and 3.34 g/cm$^3$, respectively. Previous studies showed that the crystallite sizes of nanocalcium from chicken eggshells were 10.46 nm **(Sunardi, Krismawati, and Mahayana, 2020)** and50–198 nm **(Habte et al., 2019)**, and the crystallite size of nanocalcium from duck eggshells was 262 nm **(Prayitno, Prasetyo, and Sutirtoadi, 2020)**.

These values were different from the values of nanocalcium crystallite sizes from this study. This was due to differences in raw material since the substances contained in eggshells depend on the breed, feed, and environment of the chicken.

The density values of nanocalcium oxide from chicken and duck eggshells were 3.361 g/cm$^3$ and 3.342 g/cm$^3$, respectively. These values were lower than density values of the chicken eggshells (2.16 g/cm$^3$) and duck eggshells (2.84 g/cm$^3$) calcinated at 900 °C for 1 hour **(Tangboriboon et al., 2012).** The differences are caused by differences in temperature and calcination times; the species and the feeding of the chickens and ducks also influenced the nutrient content during the egg formation.

## CONCLUSION

The results of this study indicated that extraction time influenced yield, moisture content, ash content, and color in terms of chroma and hue of nanocalcium powder. Moreover, the type of eggshells influenced yield, moisture content, and lightness of nanocalcium powder. The XRD showed that CaO was formed from nanocalcium powder of chicken and duck eggshells at 1 hour of extraction. This showed that transformation of chemical composition from $CaCO_3$ to CaO was completely achieved after the calcination and precipitation processes process took place. The size of nanocalcium oxide crystals from chicken and duck eggshells were 41.54 nm and 24.90 nm.

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# THE COMPARISON OF BIOCHEMICAL COMPOSITION OF *ACTINIDIA KOLOMIKTA* AND *ACTINIDIA POLYGAMA* FRUITS

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**ABSTRACT**
The demand for natural products, which are rich in biologically active compositions, grows constantly. The choice and production of such products can minimize the deficit of importance for human organism components, which are contained only in plant food. The paper contains the laboratory studying results of the chemical composition of the fruits of two *Actinidia* Lindl. cultivars of Federal State Budgetary Scientific Institution Federal Horticultural Research Center for Breeding, Agrotechnology, and Nursery (FSBSI FSC for Horticulture) genetic collection: *Actinidia kolomikta* (Rupr. et Maxim.) Maxim. and *Actinidia polygama* (Siebold et Zucc.) Maxim. All the presented samples are grown in field conditions. The fruits were picked up in the phase of harvest maturity while ripening. The data on antioxidant activity of water and methanol extracts, the content of phenolic compounds sum, soluble solids, and titratable acids in the fruits, and on qualitative composition of secondary metabolites (organic acids, fatty acids, mono-, di- and polysaccharides) are given in the paper. The variation limits of the parameters under study depending on the sample are presented. As a result of the laboratory studies, it was stated that *A. kolomikta* fruits 10 times exceed *A. polygama* fruits on all the stated parameters. Only the results on the soluble solids content in the fruits of both cultivars are approximately at the same level (*A. kolomikta* > *A. polygama* on 1.16%). The positive correlation between antioxidant activity and the general content of polyphenols is confirmed at both cultivars. *Actinidia kolomikta* genotypes Chempion and Lakomka and *Actinidia polygama* ones Tselebnaya and Uzorchataya showed the best results. The correct individual choice of actinidia fruits that are the best ones at the biochemical composition and the content of micronutrients allows supplying the consumers with food products.

**Keywords:** *Actinidia kolomikta, Actinidia polygama*, antioxidant activity, biochemical composition, fruits secondary metabolites

## INTRODUCTION

The demand for natural products, which are rich in biologically active compositions, grows constantly. The most useful products are the fruits that contain the complex of biologically active compositions **(Yeomans, Linseisen and Wolfram, 2005)**. In various researches **(Wang, Cao, and Prior, 1996; Zulueta, Esteve and Frígola, 2009)** it is stated that the usage of fruits in the ration makes an essential contribution to the provision of the human organism with antioxidants and useful substances: carotenoids, phenolic compounds, sugars, acids, and others. Fruits and berries consumption decreases the development of chronic and heart diseases **(Tzulker et al., 2007; Xu et al., 2010; Liu et al, 2010)**, reduces the risk of cancer and has a positive effect on comparison with chemotherapy and hormone treatment **(Liu and Dong, 2008; Liu et al, 2010)**.

Nontraditional horticultural crops including actinidia plants play an essential role in the provision of a human organism with micronutrients. Representatives of *Actinidia* Lindl. species become more and more popular in the world thanks to a wide spectrum of possibilities of its fruits usage **(Titlyanov, 1969; Kolbasina, 2007)**. The most frost-resistant species of actinidia: *Actinidia kolomikta* (Rupr. Et Maxim.) Maxim., *Actinidia. arguta* (Siebold et Zucc.) Planch. ex Miq., *Actinidia polygama* (Siebold et Zucc.) Maxim, are of the utmost interest. They outstrip the most famous on the market species of Actinidia: *Actinidia deliciosa,* also known as the kiwi, in all the biochemical parameters **(Kim et al., 2009; Krupa et al., 2011; Zuo et al., 2012; Lee et al, 2015; Leontowiczet al., 2016; Wang et al., 2018).** The advantage of these fruits is not only in bright taste but also in the possibility of eating them with the skin in contrast with actinidia species with tomentose fruits. There are the data of the registered pharmaceutical composition from the extracts of *A. arguta*, *A. kolomikta,* and *A. polygama* for prophylaxis and treatment of some immune and non-allergic inflammatory diseases **(Latocha, 2017)**.



A



B

**Figure 1** Actinidia plants and fruits.
Note: A – Actinidia plantations. B – *Actinidia Polygama* fruiting, Perchik sample.

On the territory of Russia *Actinidia* Lindl. species are still considered as a rare and nontraditional small-fruit crop. They are not introduced in industrial culture, but at the same time, they are some of the most perspective crops thanks to rich biochemical composition, longevity, and low maintenance of the plants **(Kozak and Imamkulova 2018)**. Ivan Vladimirovich Michurin received the first domestic cultivars thanks to the primary introduction of lianas. Further on, Ella Ioganovna Kolbasina made an essential contribution thanks to her *Actinidacea in situ* and breeding studies **(Kolbasina et al., 2007)**. At present, FSBSI FSC for Horticulture is one of the leading research centers of the Russian Federation that keeps in live conditions and breeds the samples collection of Far Eastern species of actinidia **(Kozak et al., 2017; Burmenko, et al., 2018)**.

Despite the studies of actinidia fruits' biochemical composition, the question of the secondary metabolites content and identification get increased attention only in the last decades. However, there is no enough publicly available information about the biochemical parameters of *A. polygama* fruits. Local and industrial cultivars are mostly studied. The comparative data of the biochemical composition of *Actinidia kolomikta* and *Actinidia polygama* fruits, grown in the Central region of Russia, are fragmentary.

Our work aims to compare the biochemical composition of *A. kolomikta* and *A. polygama* fruits from FSBSI FSC for Horticulture genetic collection to specify the best sources with the highest biochemical potential for further breeding.

## Scientific hypothesis

The study of the biochemical composition of rare fruit and small-fruit crops fruits is actual. Actinidia is a valuable material for studying thanks to the rich content of biologically active substances and unique organoleptic characteristics of its fruits. We suppose that *Actinidia kolomikta* and *Actinidia polygama* fruits should have different cross-species and intraspecific biochemical compositions.

We planned to reveal the differences in the chemical composition of two Actinidia species: *A. kolomikta* (Rupr. Et Maxim.) Maxim. and *A. polygama* (Siebold et Zucc.) Maxim., grown in the conditions of the Moscow region, on six parameters that form the quality and the nutritional value of the fruits. Based on the held laboratory experiments the best samples of the quality content will be revealed.

## MATERIAL AND METHODOLOGY

The research were held in 2019-2020 on *Actinidia* Lindl. experimental plantings of (Federal Horticultural Research Center for Breeding, Agrotechnology, and Nursery (FHRCBAN), Moscow region, Michnevo).

The collection was formed on argillaceous sod-podzolic hard soil. The total area of the plantation is 2 ha, it was planted on the 4 x 2 m (Figure 1).

### Samples

The studied objects were the fruits of six cultivars of *A. kolomikta* (Rupr. Et Maxim.) Maxim.: Chempion, Lakomka, Sestra, Vinogradnaya, Uslada, Prazdnichnaya and the fruits of six cultivars of *A. polygama* (Siebold et Zucc.) Maxim.: Ostropryanaya, Celebnaya, Osennyaya, Perchik, Uzorchataya, Krasna Devica.

The samples were taken at the fruits ripeness stage.

The biochemical researches were held at the Laboratory of Physiology and Biochemistry of Federal State Budgetary Scientific Institution Federal Horticultural Research Center for Breeding, Agrotechnology, and Nursery.

### Chemicals

All chemical substances chosen for the analysis were of an analytical sort and were bought from Sigma Aldrich (USA).

### Instruments

Homogenizer IKAA11 basic (Germany), centrifuge Sigma 2-16P (Germany), pH meter HI 2211 HANNA (Germany), shaker Lab-PU-01 (Russia), GC-MS chromatograph JMS-Q1050GC (JEOL Ltd, Japan) with capillary column DB-5HT (Agilent, USA), spectrophotometer Helios ɣ (Thermo scientific, England).

### Laboratory Methods

SSC was determined via refractometric method according to **GOST ISO 2173 (2013)**. TTA was estimated via the potentiometric method according to **GOST ISO 750 (2013).**

The total phenolics amount was determined according to the method described by **Velioglu et al. (1998).**

The scavenging activity on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically according to the method described by **Brand-Williams et al., (1995).**

We used a method described by **Robbins (2003)** for the derivation of samples**.**

The substances identification was done according to NIST-5 National Institute of Standards and Technology (USA).

### Sample preparation

300g of fruits were prepared from the representative not less than 500g probe. The mass was homogenized using the analytical homogenizer. Then it was extracted by double distilled water and by pure methanol and centrifugated at 4000 g within 10 min. The supernatant was used for measurements purposes. To study the fruits' metabolic profile a methanol extract was used.

#### *Basic chemical analyses*

General biochemical parameters, i.e. soluble solids content (SSC) and total titratable acidity (TTA) were studied. SSC was determined via the refractometric, method, the values were expressed in%. TTA was estimated via the potentiometric method by pH meter via titrating with 10 N. NaOH and expressed in the equivalent of apple acid, %.

#### *Total phenolic compounds analysis*

The total phenolics amount was determined with Folin–Ciocalteu reagent according to the method**.** A standard curve with gallic acid was used. Different concentrations of gallic acid were prepared in distilled water, and absorbance was recorded at 750 nm. 100 μL of a diluted sample (1:10) was dissolved in 500 μL of Folin–Ciocalteu reagent and

1000 µL of distilled water. The solutions were mixed and incubated at room temperature for 1 min. After 1 min, 1500 µL of 20% sodium carbonate ($Na_2CO_3$) solution was added. The final mixture was shaken and then incubated for 2 h in the dark at room temperature. The absorbance was measured at 750 nm using a Helios Y UV–vis spectrophotometer and the results are expressed in mg of gallic acid (GEA) calculated on the wet weight of plants.

***Total antioxidant capacity*** scavenging activity on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically according to the method**.** The principle of the analysis was based on the color change of DPPH solution from purple to yellow as the radical was quenched by antioxidants. The homogenized leaves were mixed with distilled water and methanol. The samples were put on the shaker Lab-PU-01 (Russia) for 6 hours, and then they were filtered and the antioxidant activity was measured in 10 minutes after interaction between the extract and the reagent. The absorbance was recorded at 515 nm to determine the concentration of the remaining DPPH. All measurements were performed in triplicate. The radical-scavenging activity was calculated as a percentage as follows:

DPPH radical-scavenging (%)= [(AC - AAt) / AC]· 100,

where;:

AC – DPPH solution absorption;

AAt – absorption at the antioxidant presence.

The lower absorbance of the reaction mixture indicates a higher level of free radical scavenging activity.

***Metabolic analysis by gas chromatography-mass spectrometry***

The metabolites analysis was fulfilled using the method of gas chromatography-mass spectrometry (GC-MS) via GCMS chromatograph. Capillary column DB-5HT; length 30 m, inner diameter – 0.25 mm, the film thickness – 0.52 um, and gas-carrier – helium) was used. The temperature gradient during the analysis was within 40 – 280°C, the injector and interface temperature – 250°C, the ionic source – 200°C. Gas flow in the column was equal to 2.0 mL/min, split-flow injection mode, sample injected in volume 1 – 2 mcl of the evaporated extract. The analysis was held for 45 min. The derivation was held using silylation reagent N,O bis (trimethylsilyl) trifluoracetamide (BSTFA). The substances identification was done according to NIST-5 National Institute of Standards and Technology (USA) retention behavior and mass spectra the scanning range was 33-900 m/z. The substance identification credibility was within 75-98%.

## Statistical Analysis

All the analyses were performed in triplicate. The results were expressed as mean values (n = 3) in standard deviation (SD). Statistical analyses were carried out through the Excel package (Microsoft Excel, v. 2016).

## RESULTS AND DISCUSSION

The laboratory studies showed that the difference of SSC in the fruits of the species under study was not big. On average, SSC depended on the species and varied from 17.78% (Uzorchataya) to 20.25% (Krasna Devica) at *A. polygama* (Figure 2.) and from 15.97% (Prazdnichnaya) to 22.50% (Chempion) at *A. kolomikta* (Figure 3).

On average, the SSC value of the fruits was 19.80% at *A. kolomikta* and 18.64% at *A. polygama*.

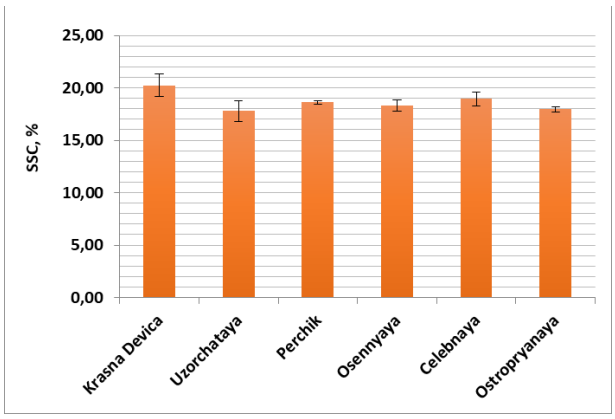


**Figure 2** Soluble solids content in *Actinidia polygama* (Siebold et Zucc.) Maxim. fruits at average in 2019 – 2020; each value represents the mean of three independent experiments (±SD).

*A. kolomikta* fruits samples Chempion and Sestra showed the highest values (Figure 3).

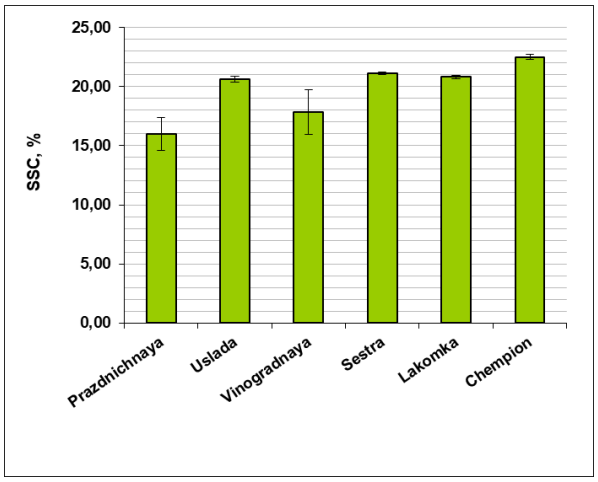


**Figure 3** Soluble solids content in *Actinidia kolomikta* (Rupr. et Maxim.) Maxim. fruits at average in 2019 – 2020; each value represents the mean of three independent experiments (±SD).

It is known that the fruits characteristic taste is determined by the ratio of the sugars and organic acids content **(McBride, Johnson, 1987)** that is considered to be especially useful as an index of acceptability for a lot of kinds of fruit **(Esti et al., 1998)**. The sugar content, acidity, the content of vitamin C, and other nutrients are essential parameters to evaluate the quality and taste; high levels of SSC, TAC, and SAR often indicate the best taste of the fruits **(Esti et al., 1998**; **Wu et al., 2003; Ivanovaet al., 2021)**.

Citric and Quinic acids are indicated in the fruits of several actinidia species, and their content predominates over Apple acid **(Nishiyama et al., 2008)**.

**Table 1** Antioxidant activity (AA) and total phenolic compounds (TPC) of *Actinidia* Lindl fruits. Values represent the mean of three replicate ± SE.

| Species, sample name | AA of ethanol extraction, % | AA of water extraction, % | TPC, mg gallic acid (GEA) / 1g fruit weight |
|---|---|---|---|
| *Actinidia kolomikta* | | | |
| Vinogradnaya | 95.15 ±0.174 | 95.39±0.192 | 7.747±0.166 |
| V, % | 0.45 | 0.49 | 5.23 |
| Prazdnichnaya | 94.99±0.214 | 95.76±0.290 | 9.727±1.102 |
| V, % | 0.55 | 0.74 | 27.76 |
| Uslada | 94.23±0.465 | 94.92±0.555 | 8.228±0.946 |
| V, % | 1.21 | 1.43 | 28.17 |
| Sestra | 94.06±0.395 | 94.06±0.450 | 8.166±0.160 |
| V, % | 1.03 | 1.17 | 4.79 |
| Lakomka | 93.19±0.080 | 92.17±0.929 | 10.253±0.520 |
| V, % | 0.21 | 2.47 | 12.41 |
| Chempion | 94.80±0.172 | 94.78±0.125 | 9.956±0.341 |
| V, % | 0.44 | 0.32 | 8.39 |
| *Actinidia polygama* | | | |
| Ostropryanaya | 25.35±1.924 | 8.33±0.723 | 0.836±0.122 |
| V, % | 18.59 | 21.26 | 35.68 |
| Celebnaya | 33.52±3.406 | 10.30±1.523 | 0.916±0.201 |
| V, % | 24.89 | 36.23 | 53.76 |
| Osennyaya | 25.58±1.214 | 7.57±0.674 | 0.733±0.096 |
| V, % | 11.63 | 21.82 | 32.02 |
| Perchik | 31.39±3.098 | 11.81±2.177 | 1.193±0.311 |
| V, % | 24.17 | 45.14 | 63.85 |
| Uzorchataya | 28.30±5.607 | 9.53±2.117 | 1.179±0.391 |
| V, % | 48.53 | 54.42 | 81.29 |
| Krasna Devica | 20.27±0.811 | 7.85±0.366 | 0.561±0.128 |
| V, % | 9.80 | 11.43 | 55.17 |

A low concentration of acids in *A. polygama* fruits in combination with a rather high content of Ascorbic acid and carotenoids make them unique and indispensable in dietetic nutrition in particularly for people with hyperpeptic stomach diseases **(Kozak et. al., 2018)**.

The content of titratable acids in the samples under study depends on the species and varies within 0.57 to 2.98% (Figure 4).

TTA of *A. kolomikta* fruits was 1.75% at average that notably higher in comparison to the average TTA value of *A. polygama* fruits (in 2.13 times). In the fruits of *A. kolomikta* samples, the maximum content of TTA was stated at Sestra and Prazdnichnaya cultivars, the minimal one – at Vinogradnaya cultivar. Among *A. polygama* fruits Ostropryanaya sample showed the high result on TTA (1.03%), the minimum value was stated at Uzorchataya genotype fruits (0.57%).

Antioxidant activity of water and ethanol extracts of *A. kolomikta* fruits is at a high level and does not show essential differences depending on extraction type. On average on *A. kolomikta* species in 2019-2020 AA of methanol extracts was 94.40%, AA of water ones – 94.51%. An essential difference in AA values depending on extraction type was stated at *A. polygama* fruits. On average AA of methanol extract was 2.5 – 3.5 times higher than water one that can be connected with quantitative differences of substances, extracted by water and methanol.

AA values of *A. polygama* fruits vary from 20.27% (Krasna Devica cultivar) to 33.52% (Celebnaya cultivar) at methanol extraction.

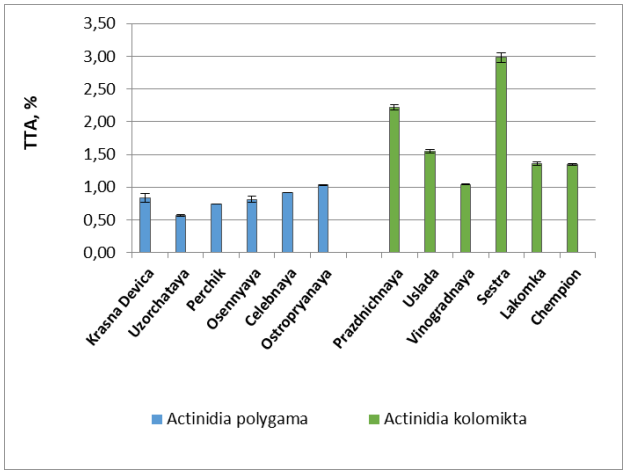


**Figure 4** Titratable acidity content in actinidia fruits at average in 2019 – 2020; each value represents the mean of three independent experiments (±SD).

The AA values of water extraction – from minimal 7.57% at Osennyaya cultivar to maximum 11.81% at Perchik cultivar. On average on the species in 2019-2020 AA of methanol extract at *A. polygama* fruits was 27.40%, AA of

water extract – 9.23%, that is 3.5 and 10 times lower than the values of *A. kolomikta* fruits relatively. On average the minimal values of AA were stated at *A. polygama* fruits of Krasna Devica cultivar (Table 1).

The results variability (V, %) during the studies of the fruits AA for *A. kolomikta* genotypes was insignificant, for *A. polygama* ones – for the methanol extracts of Krasna Devica sample and the methanol extracts of Osennyaya and Ostropryanaya samples fruits at an average level. For the rest genotypes, the variability of AA was essential (Table 1)

The majority of literary sources explain the essential difference of total antioxidant activity between species and samples by a high correlation of the vitamin C content and phenolic compounds thanks to the quick interaction of Ascorbic acid with DPPH.

Polyphenolic compounds, contained in fruits, vegetables, and grain crops, are secondary metabolites and are famous for their curative properties **(Biglari et al., 2008; Gan and Latiff, 2011; Gong et al., 2012**). Despite polyphenol's wide-spreading in plants, researchers and manufacturers of food products have started to be interested in polyphenols only in recent years. This interest is due to a wide spectrum of pharmacological properties, antimicrobial and anti-inflammatory activity of polyphenols **(Cai et al., 2004; Scalbert et al., 2005)**, and their strong effect in preventive actions to reduce various diseases associated with oxidative stress **(Manach et al., 2004; Rasmussen et al., 2005; Darvesh et al., 2010)**.

In our studies, *A. kolomikta* fruit's TPC was almost 10 times (9.98) higher than the average *A. Polygama* species TPC. Among the studied *A. kolomikta* genotypes the highest TPC value in 2019-2020 was stated at Lakomka sample fruits (10.25 mg gallic acid (GEA)/1g fruit weight), the lowest one – at Vinogradnaya (7.75 mg gallic acids (GEA)/1g fruit weight). Among the studied *A. polygama* genotypes the best TPC results were registered at Perchik and Uzorchataya samples fruits. The essential variability of the results is characteristic for these samples as well (Tab. 1). Krasna Devica fruits showed the minimum TPC value – 0,56 mg gallic acid (GEA)/1g fruit weight.

The received results can be explained by the differences of bioactive compounds concentrations that vary depending on ecological factors such as climatic and soil conditions **(Pavarini et al., 2012)**, and on cultivar differences as well **(Kim et al., 2005; Usenik et al., 2008)** and the degree of fruit ripeness **(Manach et al., 2004; Pandey, Rizvi, 2009; Babou et al., 2016)**.

We conducted the metabolic profiles identification of fruits methanol extracts of 2 Actinidia species samples received via GC-MS analysis. In this paper, we do not give the full list of the identified secondary metabolites of the studied samples but describe three groups in detail, i.e. organic acids, fatty acids, and carbohydrates (sugars in particular).

Organic acids together with sugars are the basic soluble components of ripe fruits cause an essential influence on sourness-sugariness redounding the aroma formation **(Neri, Pratella and Brigati, 2003)**. A lot of fruits on certain stages of ripening accumulate organic acids in their flesh. The main part of this content is one or two acids. Even though the most widely spread organic acids in fruits are Citric and Malic acids, the variety depends on the studied crop**.** The

**Table 2** Comparative composition of acids and carbohydrates *Actinidia* Lindl. fruits.

| Species, sample name | Organic acids | Fatty acids | Ketoses and their derivates | Aldoses and their derivates | Disaccharides | Substances quantity in all the studied groups, total |
|---|---|---|---|---|---|---|
| *Actinidia kolomikta* | | | | | | |
| Vinogradnaya | 5 | 1 | 5 | 4 | 1 | 16 |
| Prazdnichnaya | 6 | - | 5 | 4 | 1 | 16 |
| Uslada | 6 | - | 5 | 5 | 1 | 17 |
| Sestra | 5 | - | 5 | 5 | 1 | 16 |
| Lakomka | 7 | - | 5 | 6 | 1 | 19 |
| Chempion | 7 | 1 | 6 | 5 | 2 | 21 |
| *Actinidia polygama* | | | | | | |
| Ostropryanaya | 8 | - | 5 | 4 | 2 | 19 |
| Celebnaya | 14 | 4 | 6 | 11 | 7 | 42 |
| Osennyaya | - | 1 | 3 | 3 | 1 | 8 |
| Perchik | 10 | 1 | 4 | 8 | 3 | 26 |
| Uzorchataya | 17 | 3 | 7 | 9 | 7 | 43 |
| Krasna Devica | 8 | 1 | 5 | 7 | 2 | 23 |

main part of the present acids in fruits flesh is formed from the synthesized sugars **(Sweetman et al., 2009; Etienne et al., 2013)**. The basic acids are formed and take part in breathing processes, gluconeogenesis, fermentation till ethanol, amino acids synthesis/interconversion, and pigments synthesis **(Famianiet al., 2000; Famiani et al., 2005; Famiani et al., 2007; Famiani et al.,2014; Sweetman et al., 2009; Etienne et al., 2013)**.

The total and an individual number of identified components in methanol extracts of the actinidia studied samples fruits is presented in Table 2. According to the data of **Heatherbell (1975)** *Actinidia chinensis* fruits contain the essential amount of Chinic acid in ripe fruits. Small amounts of Phosphoric, Ascorbic, Glucuronic, Galactouronic, Oxalic, Amber, and p-Coumaric acids can be determined as well. **Wojdyło et al., (2017)** identified 24 polyphenolic compounds in *A. arguta* fruits. **Lim et al. (2006)** wrote about the presence of several compounds in *A. arguta* fruits, i.e. Protocatechic and Caffeic acids, β-D-glucopyranoside, Esculin, Coumarin, and three flavonoids. In the paper of **Ren, Han and Chung (2007)** it is said about the extraction and identification of polyunsaturated fatty α-Linolic acid in *A. polygama* fruits. **Motyleva et al., (2018)** identified ten organics and three fatty acids in water extracts of actinidia fruits.

However, the number of papers on metabolomic profiling of actinidia fruits is not enough, the main problem of many authors is orientation on local cultivars. Also, there is not much data on metabolomic sugar content in actinidia fruits. In the papers of **Heatherbell (1975)** and **Latocha (2017)** it is mentioned about only several sugars, such as Glucose, Fructose, and Sucrose.

In our paper, the basic common components of actinidia fruits metabolomic profiles are: acids – Pyruvic, Lactic, Itaconic, Fumaric, Malic, Erythronic, Citric, and Erythro-Pentonic; one fatty acid – Acrylic acid, thirteen monosaccharides (four of which belong to ketoses, and nine ones – to aldose) and two disaccharides (Turanose, Sucrose). Various secondary metabolites identified in the samples of this paper are found individually and can serve as cultivar markers. Thus, for example, Erythrose is found only in *Actinidia polygama* fruits of Celebnaya sample, as well as Lyxoses are found only in *Actinidia polygama* fruits.

## CONCLUSION

The fruits component composition and biochemical parameters depend not only on actinidia species, but on the sample as well. As a result of the studies it can be concluded that Chempion and Lakomka genotypes of *Actinidia kolomikta*, as well as Celebnaya and Uzorchataya ones of *Actinidia polygama* are the most perspective genotypes for breeding from the point of view of fruits qualitative properties (SSC, TAA and TPC, metabolomics analysis of secondary metabolites). These samples are also worth noticing from the point of view of using in functional nutrition.

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# CHANGES IN ORGANOLEPTIC, MICROBIOLOGICAL AND BIOCHEMICAL PROPERTIES OF KEFIR WITH IODINE ADDITION DURING THE STORAGE

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## ABSTRACT

Iodine is a vital trace element that must be constantly and daily supplied with food to the organism. Currently, the amount of food that can provide the organism with the required amount of iodine is insufficient. The purpose of the study was to investigate changes in organoleptic, microbiological, and biochemical parameters of kefir made with the addition of iodine during its refrigeration storage. It was found that during the storage of samples of kefir with iodine there is a slowdown in the reproduction of lactic acid bacteria, compared with the control sample. In particular, the number of lactic acid bacteria during the first two days of storage increased 1.3 times in the experimental sample and 1.5 times in the control sample of kefir. After 12 days of storage, the number of lactobacilli in the test sample of kefir increased 2.5 times, and in the control 3.2 times, compared with the amount in fresh kefir. Similar patterns were observed in determining the development of yeast. In particular, the reproduction rate of yeast in the control sample of kefir was, on average, 1.3 times faster (8 – 12 days), compared with yeast in the experimental sample. It was also found that during the 12-day storage period at a temperature of +6 °C, the titrated acidity in kefir with iodine increased 1.4 times, and in the control 1.6 times and was 130.5 °T and 154.1 °T, respectively. At such acidity values, the test sample of kefir still met the requirements of the standard, and the control was 24.1 °T higher. In this case, kefir containing iodide had better organoleptic characteristics during 12 days of storage. Produced kefir with the addition of iodine can be considered a functional product to provide the population with sufficient iodine.

**Keywords:** kefir; iodide concentrate; titrated acidity; lactic acid microorganisms; storage temperature

## INTRODUCTION

Iodine is a vital trace element that must enter the organism constantly and daily with food **(Hynes, et al., 2013; Abel, et al., 2017)**. Iodine is the most important component in the formation of thyroid hormones: thyroxine and triiodothyronine, which in turn regulate metabolism and several vital functions of the organism **(Kopchak, et al., 2018a)**. At insignificant earnings of iodine in an organism, adaptive processes directed on effective use of this element work are triggered. However, prolonged lack of iodine leads to iodine deficiency diseases, accompanied by various pathologies **(Abel, et al., 2017; Kopchak and Pokotylo, 2018b)**. The problem of iodine deficiency is especially relevant for regions that are far from the sea and have mountainous relief **(Ryzhkova, et al., 2017)**. Ukraine is an iodine-deficient country with varying degrees of deficit, depending on the region.

The main natural source of iodine is seafood. However, according to research **(Nerhus, et al., 2018)**, the iodine content in marine fish ranged significantly from 18 μg/100g of wet mass (Atlantic halibut) to 1210 μg/100g (pollock). There were significant differences between individuals within one species and between fish of one species from different geographical areas. For example, Atlantic cod from the North Sea had lower concentrations of iodine than cod from the Barents or Norwegian seas. It is reported that even in such developed marine countries as Norway, Finland, Iceland, England, fish an,d fish products provide only 20-30% of the daily requirement of iodine **(Dahl, et al., 2004; Bath, et al., 2013; Nystrom, et al., 2016)**. The main products in these countries that provide the organism with iodine include dairy - 20 – 64% of demand and iodized salt **(Rasmussen, et al., 2008; Van der Reijden, et al., 2017; Nerhus, et al., 2018)**.

Researchers **(Nerhus, et al., 2018)** indicate that in Norway, the average concentration of iodine in milk ranged from 12 to 19 μg/100 g, and the concentration of iodine in eggs ranged from 23 to 43 μg/100 g. In Ukraine, the average

iodine content in milk was 9.3 μg/100 g **(Mikláš, et al., 2021)**. In determining iodine in dairy products **(Haldimann, et al., 2005)** was found that the highest concentrations were in serum with a concentration of 100 to 450 μg/100 g. It was also found that during cheese production, approximately 75% of iodine from milk enters the whey, about 25% remains in the cheese **(Van der Reijden, et al., 2019)**. At the same time, it was found that heat processing does not affect the concentration of iodine in milk **(Wheeler, et al., 1983; Norouzian, et al., 2011; Soriguer, et al., 2011)** whereas defatting increased the amount of iodine in milk by only 1 – 2 μg/dm$^3$ **(Jahreis, et al., 2007; Rasmussen, et al., 2014)**. In semi-hard and soft cheeses, iodine was detected in the range of 83 – 101 μg/kg, in yogurt – 156 – 169 μg/kg **(Haldimann, et al., 2005; FCN, 2013)**. However, researchers believe that the concentration of iodine in milk can reach up to 300 μg/dm$^3$ with an additional introduction into the ratio of cows **(Van der Reijden, et al., 2019)**. Even though dairy products supplement the human ratio with iodine, its concentration is rarely determined and is not always taken into account for the prevention of iodine deficiency.

For a long time, many countries around the world have tried to solve the problem of iodine in the daily ratio by providing the population with iodized salt containing the usual 25 g of potassium iodide per 1 ton of salt. However, potassium iodide (KJ) has now been found to be unstable to light and moisture **(WHO, 2007)**. In addition, the use of inorganic compounds (KJ, $KJO_3$) in iodine enrichment of rations, from the point of view of the theory of functional nutrition is not perspective, because they are a chemical unnatural compound and cannot be considered a source of functional ingredients. The best way to provide the population with stable iodine is the use in iodine-deficient regions of organic iodine compounds – iodine-casein, iodine-active, "Iodis concentrate", seafood – natural sources of iodine, in particular, sea fish, mussels, shrimp, squid and, especially, brown seaweed and products of their processing (salads, jams, pasta).

One of the well-recommended products with biologically active iodine is "Iodis-concentrate" **(Story, et al., 2016; Mikláš, et al., 2021)**. "Jodis-concentrate" is mineral artesian water with iodine, stable during storage and heat processing, recommended for use in the food industry for iodine enrichment of water, bakery products, meat, dairy, and sour-milk products **(Kopchak, et al., 2017; Mikláš, et al., 2021)**.

Because Ukraine does not have enough functional food products that can provide consumers with the necessary level of iodine. The use of fermented milk products as a basis for the introduction of biological iodine-containing preparations is quite real and perspective. In addition, such products. as kefir is considered to be often used because, in addition to specific taste qualities, they provide the organism with useful lactic acid microflora **(Kukhtyn, et al., 2018a)**.

Therefore, given the urgency of the problem and the literature, it is currently promising to create functional dairy products with biologically active iodine, which would allow its use for prophylactic purposes to prevent thyroid dysfunction.

The purpose of the work was to investigate changes in organoleptic, microbiological, and biochemical parameters of kefir produced with the addition of iodine during its refrigeration storage.

**Scientific hypothesis.** It consists of the possible production of iodized fermented milk products, which are enriched with the help of water "Iodis-concentrate".

## MATERIAL AND METHODOLOGY

The work was carried out at the Ternopil Ivan Puliuj National Technical University in the laboratories of the Department of Food Biotechnology and Chemistry.

**Samples**

10 samples of kefir with the content of "Jodis-concentrate" were investigated - experimental samples and 5 samples of kefir were in control (without "Jodis-concentrate").

**Chemicals**

Kefir leaven (Good food, Italia) was used to make kefir, and Jodis-Concentrate solution (Yark-Kyiv, Ukraine) was used as a source of iodine.

Artesian mineral water "Jodis-concentrate" of the following chemical content (Table 1) **(TC, 2001)** was used in the experiment.

Before the conducting of the study the kefir samples had been mixed for one minute with a sterile spatula, heated in a water bath at +37 ±1 ° C, and prepared ten-fold dilutions according to the standard (**DSTU, 2003**).

Determination of the number of lactic acid microorganisms in experimental and control samples of kefir was carried out on *MRS-agar* medium (HiMedia, India) for this purpose, the sowings were incubated at a temperature of 37 ±1 °C for 48 hours. Yeast and molds were determined on Saburo medium (HiMedia, India) at a temperature of 25 ±1 °C for 3-5 days. The titer of coliform bacteria was determined in Kessler medium (Pharmactive, Ukraine) incubation of crops at a temperature of 37 ±1 °C for 24 hours. Determination of Staphylococcus aureus in kefir was carried out on Baird-Parker agar medium (Merck KGaA, Germany).

**Laboratory Methods**

The titrated acidity of milk and kefir was determined by the classical titrimetric method (**IS 2004**). Chemically pure KOH was used for titration (Khimreaktiv, Ukraine).

**Description of the Experiment**

**Sample preparation:** The process of making kefir with iodine included the following technological operations: 1) assessment of quality and safety of raw materials (microbiological and physicochemical research); 2) normalization and homogenization of the mixture for fat content up to 2.5% on the laboratory homogenizer (Microtron MB 800, Kinematica, Germany); 3) pasteurization at a temperature of 80 – 85 °C for 15 s on a mini pasteurizer (SP12, Motor Sich, Ukraine); 4) cooling to a temperature of 25 ±1 °C, adding "Jodis concentrate" in the amount of 7.5 mL per 1 liter of raw material; 5) making of kefir leaven and fermentation for 6 h to a titratable acidity of 85 – 90 °T; 6) cooling to +4 to +6 °C; 7) packing and storage.

**Number of samples analyzed:** This technology was used to make 10 samples of kefir with adding of "Iodis-concentrate" and 5 samples of kefir without adding of iodine (control). Each sample was divided into 5 parts and placed in a sterile container with a lid and stored in a refrigerator at

+4 – +6 °C for 12 days. The first part (freshly made kefir) was analyzed immediately after cooking, the second part was examined after 2 days of storage, the third – after 5 days of storage, the fourth – after 8 days, the fifth – after 12 days.

**Number of experiment replication:** Each portion of kefir was analyzed three times and subjected to statistical processing.

**Number of experiment replication:** A total of 15 samples of kefir were analyzed for microbiological, biochemical and organoleptic parame.

**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

At the first stage of the research, the chemical content of "Iodis-concentrate" was analyzed (Table 1) and it was found that 1 mL of solution contains 40.0 μg/mL of iodine. That is, to ensure the minimum daily requirement of an adult for iodine (200-300 mcg recommendation) (**WHO, 2007**), it is necessary to consume an average of 7.5 mL of "Iodis concentrate". This amount was calculated and kefir was made from cow's milk so that 1 liter contained at least 300 mcg of iodine.

Organoleptic, physicochemical, and microbiological indices were determined in freshly made kefir with iodine (experimental sample) and without it (control) for compliance with the requirements of the normative document (**DSTU, 2005**). According to organoleptic indices, the experimental and control samples of kefir had a homogeneous, viscous, with an intact clot, a semi-liquid without gas formation, appearance and consistency, and a clean, sour-milk taste and smell. According to physicochemical indices in the experimental sample, the value of titrated and active acidity was 93.2 ±0.2 °T and pH 4.6 ±0.03 units. In the control sample, the titrated acidity was 98.0 ±0.2 °T and pH 4.4 ±0.04 units. The results of microbiological research are given in table 2.

It is set (Table 2) that according to microbiological indicators freshly made samples of experimental and control kefir met the requirements provided in the standard. At the same time, it was found that the number of lactic acid bacteria in the experimental sample of kefir was 1.7 times ($p < 0.05$) less, compared with the control sample. The yeast content also in the experimental sample was 2.5 times ($p < 0.05$) less than in the control and was $2.4 \pm 0.1 \times 10^3$ CFU/g and $6.1 \pm 0.2 \times 10^3$ CFU/g, in accordance.

Therefore, freshly made samples of kefir met the requirements of the standard in organoleptic, physicochemical, and microbiological indices, despite the lower titratable acidity and lower content of lactic acid bacteria and yeast in the test sample of kefir.

Further research was sent on determining the influence of added "Jodis" on the dynamics of changes in lactic acid and yeast microflora during the storage of kefir at a temperature of + 6 ±0.1 °C for 12 days. The maximum allowed by the standard (+ 6°C) storage temperature was chosen for the more intensive reproduction of microorganisms. The results of the research of changes in lactic acid bacteria in kefir during storage are shown in Figure 1.

The classical dynamics of change of lactic acid bacteria in kefir samples was revealed (Figure 1), which was characterized by gradual growth of lactic acid microorganisms during the 12-day storage period at a temperature of +6 ±0.1 °C. At the same time, we observe a slowdown in the reproduction of lactic acid bacteria in the experimental sample of kefir, against the control. In particular, the number of lactic acid bacteria during the first two days of storage increased 1.3 times ($p < 0.05$) in the experimental sample and 1.5 times in a control sample of kefir.

After five days of storage, the number of lactic acid bacteria in kefir continued to increase. However, the intensity of reproduction of the microflora in the experimental sample was slightly lower than in the control sample of kefir, that is, the increase was 1.6 and 1.9 times ($p < 0.05$), in accordance, compared to the initial number.

The following definition of the number of lactic acid bacteria after 8 and 12 days of storage revealed a similar trend in the reproduction of the microflora as during the five-day studies. In particular, the number of lactobacilli after 12 days of storage in the test sample of kefir increased 2.5 times ($p < 0.05$), and in the control 3.2 times ($p < 0.05$), comparing with the amount in fresh kefir.

Data on the change of yeast in kefir containing "Jodis", compared with the control sample are shown in Figure 2.

It was found (Figure 2) that the presence of Jodis in the experimental sample of kefir influenced the intensity of yeast reproduction. In particular, during the two days of kefir storage, the amount of yeast increased on average 1.2 times in the experimental sample, against 1.3 times ($p < 0.05$) in the control. That is, there is a similar regularity as for the development of lactic acid bacteria. During further storage, the reproduction rate of yeast in the control sample of kefir was on average 1.3 times ($p < 0.05$) faster (8 – 12 days), compared with yeast in the experimental sample.

Therefore, the research indicates that "Jodis" added to the milk mixture for the production of kefir inhibits the development of lactic acid and yeast microflora in the product during storage.

The results of the research of the change in titratable acidity in kefir samples during storage are shown in Figure 3.

The results (Figure 3) indicate that the growth curve of the values of the titrated acidity had a regularity to the fastest increase in the control sample of kefir. In particular, during eight days of storage, the titrated acidity in the control sample of kefir increased 1.5 times ($p < 0.05$) and was 142.9 ±0.3 °T. This value of titrated acidity by 12.9 °T exceeded the permissible level (130 °T), which is normalized by the standard (**DSTU, 2005**). At the same time, in the experimental sample of kefir with "Jodis" the value of titrated acidity reached the level of 130 °T on the 12th day of storage.

Thus, in kefir with "Jodis" the dynamics of enzymatic processes were slower compared to the control sample. This gives reason to believe that it can be stored for a longer period without violating the requirements of the standard in terms of titrated acidity of the product.

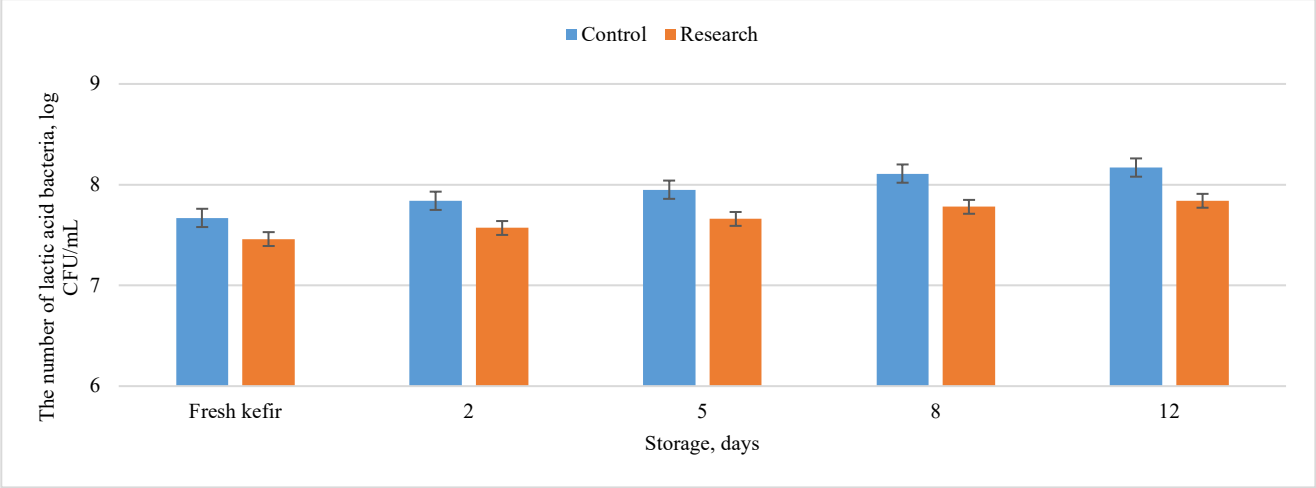


**Figure 1** Dynamics of changes in the number of lactic acid bacteria in kefir containing "Iodis" during storage at a temperature of +6 ±0.1 °C.

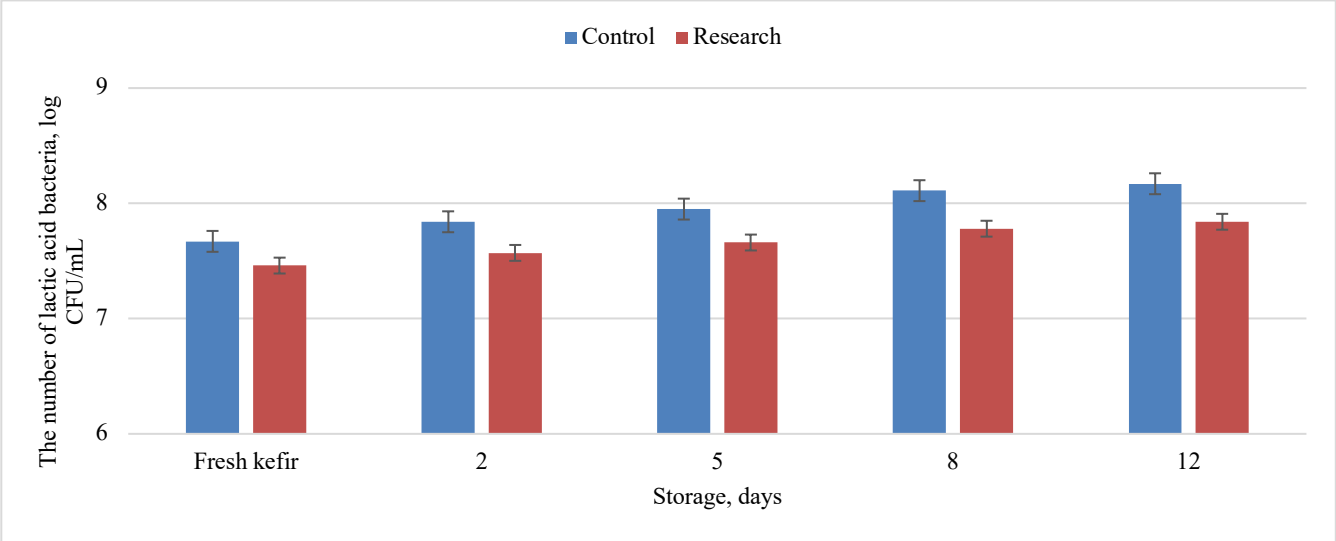


**Figure 2** Dynamics of change in the amount of yeast in kefir containing "Jodis" during storage at a temperature of + 6 ±0.1 °C.

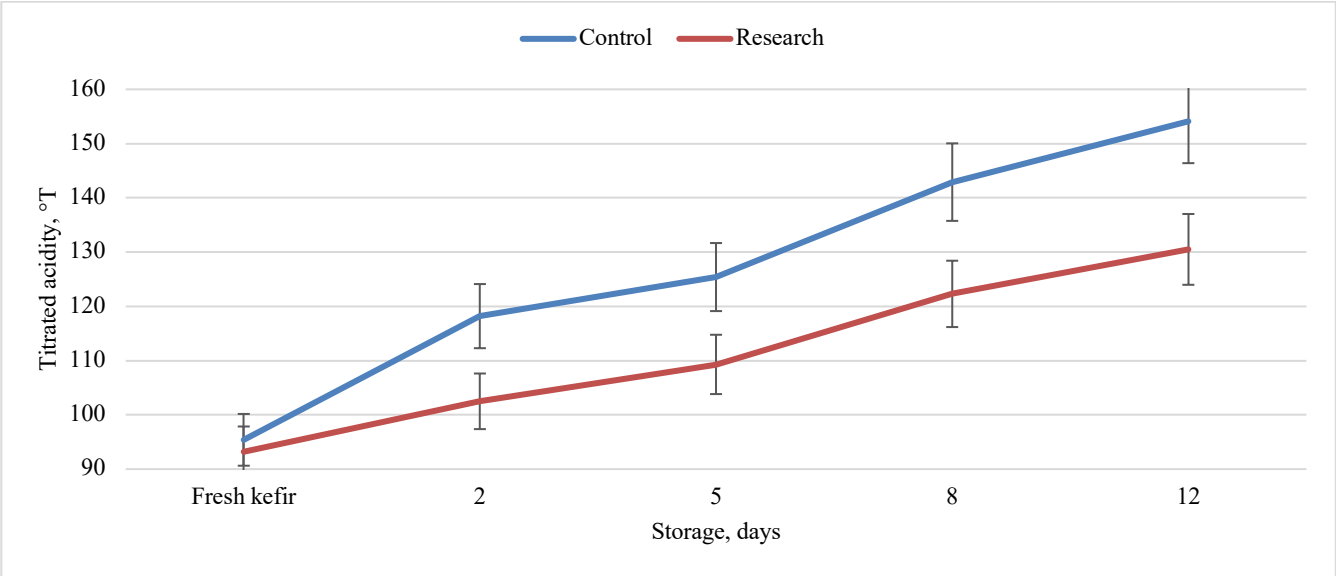


**Figure 3** Dynamics of change of titrated acidity in kefir with the content of "Jodis" during storage at a temperature of +6 ±0.1 °C.

**Table 1** Chemical composition of "Jodis concentrate".

| Indicators | Number |
|---|---|
| Total mineralization, g/dm$^3$ | 0.4 – 0.8 |
| Sodium + Potassium, mg/dm$^3$ | 10 – 100 |
| Calcium, mg/dm$^3$ | 50 – 150 |
| Magnesium, mg/dm$^3$ | 10 – 100 |
| Chlorides, mg/dm$^3$ | <50 |
| Sulfates, mg/dm$^3$ | <50 |
| Hydrocarbonates, mg/dm$^3$ | 300 – 600 |
| Iodine, μg/cm$^3$ | 40.0 |
| Organic matter content, mg/dm$^3$ | <30 |

**Table 2** Microbiological indices of freshly made experimental and control samples of kefir.

| Indicator | Search sample - kefir with iodine | Control - kefir | Permissible quantity according to DSTU 4417:2005 |
|---|---|---|---|
| The number of lactic acid microorganisms, CFU/g | 2.8 ±0.2 × 10$^7$ | 4.7 ±0.2 × 10$^7$ | No less 1 × 10$^7$ |
| Yeast, CFU/g | 2.4 ±0.1 × 10$^3$ | 6.1 ±0.2 × 10$^3$ | No less 1 × 10$^3$ |
| The titer of coliform bacteria, g | >1 | >1 | Is not allowed 0,01 |
| Molds, CFU/g | 8.3 ±0.3 | 12.1 ±0.3 | Not more 50 |
| Staphylococcus aureus, g | >1 | >1 | Is not allowed 1.0 |
| Salmonella spp., 25 g | Not found | Not found | Is not allowed 25 g |

**Table 3** Scale for organoleptic evaluation of kefir with "Jodis".

| The name of the indicator | Characteristics of the indicator | Evaluation in points |
|---|---|---|
| Taste and smell (5 points) | Pure, sour milk. Taste without extraneous tastes and smells | 5 |
| | Pure, sour milk. The taste is pungent, without foreign tastes and odors | 4 |
| | Sour milk. The taste is excessively pungent, without extraneous flavors and odors | 3 |
| | Excessively sour milk (sour) | 2 |
| | Unexpressed taste or presence of extraneous flavors | 1 |
| Appearance and consistency (5 points) | Homogeneous, viscous, with intact clot, semi-liquid, without gas formation | 5 |
| | Homogeneous, viscous, with intact clot, with slight serum separation, semi-liquid, without gas formation | 4 |
| | Homogeneous, viscous, with some broken clot, with slight serum separation, semi-liquid, with some gas formation | 3 |
| | Homogeneous, with a broken clot and serum separation, liquid, with gas formation | 2 |
| | Homogeneous, with significant serum separation, liquid, with gas formation | 1 |
| Color (2 points) | Milky white, uniform throughout the mass | 2 |
| | Milk white, not uniform for the whole mass | 1 |
| General perception (3 points) | The harmonious combination of taste and smell | 3 |
| Overall maximum score | | 15 |

**Table 4** Organoleptic evaluation of kefir containing "Jodis" during storage at a temperature of + 6 ±0.1 °C.

| Product sample | Indicators | Storage for, days | | | | |
|---|---|---|---|---|---|---|
| | | fresh yogurt | 2 | 5 | 8 | 12 |
| Experimental kefir with "Jodis" | Taste and smell (5) | 5.0 | 5.0 | 4.8 | 4.6 | 4.4 |
| | Appearance and consistency (5) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| | Color (2) | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| | General perception (3) | 3.0 | 3.0 | 3.0 | 2.8 | 2.8 |
| | The total number of points (15) | 15.0 | 5.0 | 14.8 | 14.4 | 14.2 |
| Control (kefir) | Taste and smell (5) | 5.0 | 5.0 | 4.6 | 4.2 | 4.0 |
| | Appearance and consistency (5) | 5.0 | 5.0 | 5.0 | 5.0 | 4.8 |
| | Color (2) | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| | General perception (3) | 3.0 | 3.0 | 3.0 | 2.8 | 2.8 |
| | Total Points (15) | 15 | 15.0 | 14.4 | 14.0 | 13.6 |

It is known that the organoleptic properties of fermented milk products primarily depend on the quality of milk raw materials which undergo fermentation, introduced food additives, and the activity of fermentation crops. Therefore, the next part of the work was to conduct an organoleptic evaluation of kefir samples. However, before the tasting, a scale was developed to evaluate kefir samples (Table 3).

The scale for evaluating test samples of yogurt included the determination of taste and smell, which was evaluated with a maximum of 5 points; appearance and consistency - 5 points; color was evaluated at 2 points and general perception – 3 points.

According to the results of the tasting commission of kefir samples (Table 5), it was found that the shelf life of the product deteriorates its organoleptic properties. The decrease in the overall score of the experimental and control sample of kefir is mainly due to a defect in taste, in particular the appearance of a pungent sour-milk taste, which develops due to lactic acid fermentation. No exterior taste or odor was detected in the research sample. At the same time, in the research sample of kefir, a decrease in the total score by 0.2 points, compared with the initial amount was observed on the 5th day of storage. At the same time, in the control sample for this storage period, the decrease was 0.6 points. On the 12th day of kefir storage, the difference between the experimental and control samples was 0.6 points, with an overall score of 14.2 and 13.6 points, respectively.

Thus, the addition of 7.5 mL of "Iodis concentrate" per 1 liter of milk mixture for fermentation of kefir does not reduce the organoleptic characteristics of the produced product. In this case, kefir containing iodide had better organoleptic characteristics during 12 days of storage, compared with the control sample.

## DISCUSSION

Iodine is a trace element whose daily requirement ranges from 100 to 300 μg/day, depending on age **(Hynes et al., 2013; Abel et al., 2017; Walther et al., 2018)**. Many countries, including Ukraine, are in the zone of endemic iodine deficiency **(Dahl et al., 2004; Bath et al., 2013; Nystrom et al., 2016; Ahvanooei, et al., 2021)**. Unfortunately, in Ukraine, there is a lack of food that can provide the organism with the required amount of iodine **(Ryzhkova et al., 2017; Kopchak and Pokotylo, 2018b)**. On the other hand, dairy products are a good basis for the production of many functional products **(Dal Bello et al., 2017**; **Kukhtyn et al., 2021; Albracht-Schulte et al., 2018; Yukalo et al., 2019; Lialyk et al., 2020)**. This research shows that the problem of iodine deficiency can be solved by producing high-quality kefir enriched with biological iodine - a solution of "Iodis-concentrate". In particular, it was found that freshly made kefir with iodide in organoleptic, physicochemical, and microbiological indicators met the requirements of the standard **(DSTU, 2005)**, despite 2.3 °T lower titratable acidity and 1.7 times lower content of lactic acid bacteria than in the control sample of kefir.

During the storage of samples of kefir with Jodis, a slowdown in the reproduction of lactic acid bacteria was detected compared to the control sample. In particular, the number of lactic acid bacteria during the first two days of storage increased 1.3 times in the experimental sample and 1.5 times in the control sample of kefir. After 12 days of storage, the number of lactobacilli in the test sample of kefir increased 2.5 times, and in the control 3.2 times, compared with the amount in fresh kefir. Analogical patterns were observed in determining the development of yeast. In particular, the rate of reproduction of yeast in the control sample of kefir was, on average, 1.3 times faster (8 – 12 days), comparing with yeast in the experimental sample. This gives reason to consider that "Jodis" added to the milk mixture for the production of kefir inhibits the development

of lactic acid and yeast microflora during production technology and in the finished product during its storage. The presence of antibacterial action in iodine concentrate has been reported by other researchers **(Mikláš, et al., 2021)**. Despite the influence of added Jodis concentrate on the development of microflora in the technology of production and storage of kefir, the researchers **(TC, 2001; Mikláš, et al., 2021)** report that overdose and negative action on consumers from this preparation is not possible. It was also found that during the 12-day storage period at a temperature of +6 °C, the titrated acidity in kefir with iodine increased 1.4 times, and in the control 1.6 times and was 130.5 °T and 154.1 °T, respectively. For such values of acidity, the test sample of kefir still met the requirements of the standard **(DSTU, 2005)**, and the control was 24.1 °T higher. In general, we can confirm that in this case, iodine in kefir acts in part as a biological preservative, due to which it is extended the expiration date of the product. Thus kefir with iodine on organoleptic indicators exceeded 0.6 points of the test sample, generally because of less sour taste.

Thus, we agree with the data of researchers **(Haldimann, et al., 2005; Rasmussen, et al., 2008; Norouzian et al., 2011; Nerhus et al., 2018; Van der Reijden, et al., 2019)**, who recommend increasing the concentration of iodine in dairy products by introducing iodine into the ration of cows or immersion of teats in iodine-containing drugs (**Reijden et al., 2017; Walther et al., 2018; Ahvanooei, et al., 2021**). At the same time, we consider that the priority direction of overcoming iodine deficiency is the production of traditional fermented milk products enriched with biological iodine from natural sources. It is Jodis concentrate that belongs to such food additives **(Stori, et al., 2016; Kopchak & Pokotylo, 2018b; Mikláš, et al., 2021)**.

## CONCLUSION

It was found that freshly produced kefir with iodide in organoleptic, physicochemical, and microbiological indicators met the requirements of the standard, despite 2.3 °T lower titratable acidity and 1.7 times lower content of lactic acid bacteria than in the control sample of kefir.

Therefore, the produced kefir with the addition of iodine was characterized by good microbiological, biochemical indicators, and organoleptic properties and can be considered a functional product to normalize the intake of stable iodine.

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# MODIFICATION OF THE PROPERTIES OF MILK-FAT EMULSIONS WITH THE PHASE STRUCTURE OF "OIL IN WATER" IN THE DEPENDENCE ON THE MASS PART OF THE LIPOID AND THE STABILIZING SYSTEMS

*Igor Stadnyk, Oksana Bodnarchuk, Kateryna Kopylova, Pylyp Petrov, Larysa Bal-Prylypko, Sergiy Narizhnyy*

## ABSTRACT

The composition and properties of cream with fat levels from 30% to 70% were investigated. It has been established that the decrease of fat level and, accordingly, the increase of plasma level in the cream leads to significant changes in the physicochemical parameters of the fat emulsion. Accordingly, the production of low-fat dairy products requires adjustment of the cream properties. It has been shown that using different doses of structure stabilizers: QNA colloid as a consistency stabilizer in the amount of from 1 to 2% and the Dimodan emulsifier U/G – from 0 to 1 % (Danisco, Denmark) changes the physicochemical properties (effective viscosity, sedimentation stability) and organoleptic properties (consistency, taste) of cream with fat level 40%. It has been conducted mathematical modeling, aimed to calculate dosing for improving the properties of milk-fat emulsions, structure formation and further ensuring of the necessary consistency of cream pastes. Optimal doses of colloid QNA as a consistency stabilizer and a U/G Dimodan emulsifier have been determined. It has been established that their content should be 1,0% and 0,5%, relatively, for pastes with a fat level of 40%. The effectiveness of the joint action of consistency stabilizers and emulsifiers in obtaining milk-fat emulsions were defined by the level of effective viscosity. Moreover, the use of the emulsifier had less effect on the effective viscosity of the milk-fat emulsions. It has been found that using the structure stabilizers could improve the formation of low-fat products and the formation of the desired paste-like consistency of high-fat cream with a fat level of 70%. Thus, it is possible to adjust the composition and properties of raw material as a basis for milk-fat emulsions by optimizing the ratio of structure stabilizers. Comparative evaluation of the physicochemical properties of milk-fat emulsions and high-fat cream makes it possible to predict their potential for conversion into a creamy paste with a given consistency.

**Keywords:** cream; milk and fat emulsions; stabilizers; emulsifiers; effective viscosity

## INTRODUCTION

In the current conditions of the current shortage of milk raw materials and the full utilization of the secondary raw material potential of the dairy industry and dairy enterprises of Ukraine today are focusing on the creation of a new group of dairy products. This group is based on the low content of milk fat, the effectiveness of various conditions of thermomechanical treatment for the conversion of milk fat emulsions into a quality product.

In particular, oil and cream pastes are promising for improving the efficiency of the dairy industry and for providing various consumer needs. According to world standards **(CAS 279, 1971; CAS 253, 2006)**, these products are called dairy spreads or semi-fat oils. Considering the increased content of milk plasma in these products, the formation of the proper structural-mechanical properties and their stability during storage requires the involvement of functional-technological ingredients, in particular stabilization systems. These systems combine well with the milk base and allow the most effective use of the properties of their constituents for the formation of stable complex emulsions. In the writings of scientists **(Krasulya and Zaitseva, 1986; Ipsen, 2017)**, such approaches are revealed that all us to obtain products with a given paste-like structure, which is close to the structure of classical butter.

The development of low-fat dairy products with a fat content of 30-40% is complicated by the deterioration of the thermomechanical treatment of low-fat milk-fat emulsions. The complication is caused by a change in their physical and chemical properties **(Mollakhalili, et. al., 2014; Nguyen, et. al., 2015; Rios, et. al., 2014).** his was especially noted in the works **(Vyshemirsky, Topnikova, Kuchnova 1997; Gulyaev-Zaitsev 1988)**, which revealed the essential properties of reducing viscosity and instability. Several researchers **(Bayés-García, et. al., 2015; Patel, et. al., 2010; Fredrick, et. al., 2009)** consider that the stability of

the emulsion is impeded by the processes of coalescence, delamination, and flocculation.

Therefore, the manufacture of a high-quality emulsion product with a high content of milk plasma, first of all, involves the choice and the right combination of stabilization systems: consistency stabilizers and emulsifiers. The action of emulsifiers is due to their ability to accumulate at the boundary of two liquid phases, reducing interfacial tension and creating a protective layer around the droplets that prevents coagulation and coalescence. We agree with the authors (**Topnikova, 2004; Topnikova, 2005)** that the type of emulsifier and its amount depend on the mass fraction of fat in the emulsion product, the modes of technological process, the type of raw material used, the degree of milk fat replacement, the conditions and shelf life of the product.

Typically, to stabilize low-fat emulsions, it is necessary to use mixed-type emulsifiers that combine hydrophilic and hydrophobic compositions. They have maximum functionality and allow them to create a wide range of emulsion products with specified properties. The most common emulsifiers include mono- and diglycerides of dietary fatty acids, lecithin and phospholipids, emulsifiers based on polyglycerol, and others. In the writings of the authors **(Zobkova, 2007; Dorozhkina and Buhmet, 2012; Ipatova, Zadorozhnaya and Malchenko, 1999)** is noted that due to hydrophilicity and stronger moisture retention at elevated phospholipid temperatures, their additional attraction improves the functional properties of the emulsifiers.

Also, they must meet certain requirements: be physiologically safe, promote the retention of moisture in the product during machining in the production process, stabilize high- and low-dispersion emulsions, increase their stability, and ensure the stability of the product during storage. When choosing an emulsifier take into account the melting point, iodine number, characterizing the degree of saturation. For example, in studies **(Zobkova, 2007; Dorozhkina and Buhmet, 2012; Ipatova, Zadorozhnaya and Malchenko, 1999; Fedyakina, Gorshkova and Rubina, 2007)** state that monoglycerides with an iodine value of 50 – 90 g $I_2$/100 g are used in the production of low-fat emulsions.

The authors **(Vyshemirsky, Topnikova and Kuchnova 1997; Topnikova, 2004**, **Topnikova, 2005**) have found that the use of structure stabilizers causes changes in the physicochemical properties and structural and mechanical parameters of milk-fat emulsions. Their nature of change depends on the type and amount of stabilizer used.

Several authors **(Vyshemirsky, Topnikova and Kuchnova, 1997; Topnikova, 2004; Bogdanova, 2013)** suggest the use of gelatin, cellulose derivatives, pectin, complex stabilization systems Hamulsion, Palsgaard to stabilize the structure of low-fat products. They include hydrocolloids – carrageenan, xanthan and guar gums, modified starch, etc., which allows you to get pastes with a different structure.

### Scientific hypothesis

Ensure the stability of the direct type emulsion needs to use surfactants, in which there is a dispersion of the fat phase, increase of the organoleptic and physicochemical characteristics of the finished product.

The process of forming the properties of milk-fat emulsions with the phase structure of "fat in the water" under the influence of structure stabilizers for cream pastes is aimed at the creation of the new technologies of low-fat butter-making products. Direct control of the composition and properties of the raw material – cream with a fat content of 40%, depending on the dose of the structure stabilizers could ensure a stable structure.

It has been determined physical and chemical properties of milk-fat emulsions as a basis for the production of cream pastes for the realization of this hypothesis and achievement of this goal.

## MATERIAL AND METHODOLOGY

The objects of this study were milk fat emulsions with a fat level of 30% with adding of the structure stabilizers and cream with fat level from 30% to 70% and, accordingly, a solids level from 2.5% to 9.3%. High-fat cream was obtained by the method of double separation.

The physicochemical properties of cream were determined according to the recommendations of the author (**GOST 3626, 1973)** Determination of the mass fraction of milk solids non-fat (MSNF) was conducted by GOST 3626-73, the mass fraction of fat **(GOST 5867, 1990),** the mass fraction of total nitrogen by the Kjeldahl method – by DSTU ISO 8968:2005-part 2 **(ISO 8968-2, 2005)** ("Fisher Bioblock Scientific", France).

The physicochemical properties of cream and cream with added structure stabilizers were investigated by the sedimentation resistance of the emulsions to delamination, according to the method **(Vaitkus, Kazlauskaite and Malakauskiene, 1981)** of fat settling.

The effective viscosity of cream and cream with added structure stabilizers was determined by the rotary viscometer "Rheotest II" with a cylinder/cylinder measuring system (S/S1, S/S2, S/S3) for different viscosities according to the instructions. In the gap between the cylinders made cream, in which under the influence of the rotation force of the rotor was shifting one layer of the bunch relative to another. The rheological characteristics of the product were determined by the speed of rotation of the rotor and the force of resistance of its rotation. The measurements were performed at a strain rate of 48.6 $s^{-1}$. The parameters of the instrument were translated into the shear stress (PA) at a given strain rate by the formula:

$$\tau = Z \cdot \alpha,$$

where:

$\tau$ – shear stress, Pa;

$\alpha$ – indicators of the device;

Z – cylinder constant, Pa / unit. scale instrument.

The effective viscosity of the test samples was determined by the formula:

$$\eta_{eff} = \tau/Dr .$$

Where:

$\eta_{eff}$ - effective viscosity, Pa

Dr – shear stress, $Pa^2$/sec. Dr=constfor each mode of clot destruction.

Milk-fat emulsions were prepared based on skim milk and high-fat cream with a fat content of 70% by weight. For this purpose, the stabilizer was separately dissolved in skim milk, heated at (45 ±5 ºC), and the emulsifier in high fat

cream at temperature. The components in the quantities determined in the experimental conditions were combined to obtain normalized cream with a mass fraction of fat, respectively, 30% and 40%. The resulting mixture was heated to (80 ±1°C) with continuous stirring, followed by cooling to (15 ±1°C). Component ratios were varied to normalize cream 30% and 40%.

A software package STATISTICA© v 5 for Windows (StatSoft Inc., USA) was used to processing of the obtained experimental data according to the recommendations of the author **(Lapach, Chubenko and Babich, 2001)**.

The results of the studies were evaluated according to the significance level of *p*-value. The replication of the experiments was threefold. The results were considered credible at the trust level p <*0.05*.

Graphic processing of the results is done using the software Microsoft Excel 2010. Inc, Mathsoft Mathcard Enterprise Edition ON V11. A.

## Statistical analysis

Also, a multivariate regression analysis of the experimental data was performed to evaluate the effect on the studied performance of each of the factors introduced into the model at a fixed position at the average of the other factors. An important condition is the lack of a functional link between the factors. For this purpose, a mathematical model was constructed in the form of an analytical expression that best reflected the relationship of factor traits with the resultant one. To obtain stable milk-fat emulsions, mathematical modeling was performed using a two-factor linear regression model, which in general looks like:

$$Y = b_0 + b_1 \cdot x_1 + b_2 \cdot x_2 + \varepsilon$$

Where:

$b_0$ – a free member that defines values Y, when all independent variables $x_i$ equal 0.

$b_1$, $b_2$ – shows how much the result will change if you change the unit of measurement of each independent factor $x_1$ and $x_2$.

To obtain stable milk-fat emulsions:

$x_1$ – a mass fraction of consistency stabilizer in cream in the range from1.0 to 2.0 %;

$x_2$ – a mass fraction of emulsifier in cream in the range from 0 to 1.0 %;

$Y_1$ – effective viscosity; $Y_2$ – taste, and smell; $Y_3$ – consistency; $Y_4$ – sedimentation stability;

$\varepsilon$ – a random variable characterizing the deviation of the factors $x_1$, $x_2$ from regression line (residual variable).

In the study, the mathematical expectation of random deviation $\varepsilon_i$ is 0 for all observations (M ($\varepsilon_i$) = 0).

To evaluate unknown parameters $b_0$, $b_1$, $b_2$ the least squares method. According to the method, unknown function parameters are chosen such that the sum of the squares of the deviations of the experimental (empirical) values $Y_i$ from their calculated (theoretical) $Y_{ip}$ values were minimal, that is:

$$S = \sum_{i=1}^{n} (Y_i - Y_{ip})^2 = \sum_{i=1}^{n} (Y_i - \varphi(X_i, b_0, b_1, ..., b_k))^2 \rightarrow \min$$

The modeling and processing of the experimental data were performed using a mathematical package MathCad. For emulsions with a mass fraction of fat 40% the equations of mathematical models have the form:

1) The equation that describes quantitatively effective viscosity:

$$Y_1 = 2{,}71 + 23{,}89 \cdot x_1 + 8{,}61 \cdot x_2$$

2) An equation that quantitatively describes taste and smell:

$$Y_2 = 5{,}99 - 0{,}46 \cdot x_1 - 0{,}87 \cdot x_2$$

3) The equation that describes quantitatively consistency:

$$Y_3 = 3{,}89 - 0{,}02 \cdot x_1 + 0{,}21 \cdot x_2$$

4) Equation quantitatively describing sedimentation stability:

$$Y_4 = 89{,}22 - 3{,}09 \cdot x_1 - 0{,}65 \cdot x_2$$

The adequacy of the models was verified by the coefficients of determination $R^2_{Y1}$=96 %, $R^2_{Y2}$=98 %, $R^2_{Y3}$=91 %, $R^2_{Y4}$=92 %, which testify to the high-quality character of the correlation of the system coefficients, as well as the F-test (Fisher's F-test) and the Student's t-distribution to evaluate the reliability of the correlation coefficients.

For a comprehensive analysis of the change in the properties of cream with a fat content of 40%, depending on the dose of the stabilizers of the structure and to determine the rational ranges of the stabilizers, a multidimensional regression analysis of the experimental data was performed.

Based on the results of the experimental studies, a two-dimensional approximation of the mathematical dependence was performed f = φ(x,y) – a mathematical model that reproduces with sufficient accuracy the studied regularity y = f(x,y) – experimental data.

Functional dependencies in the form of two-dimensional second-degree polynomials are described, which describe the technological process fairly accurately. The simulation results are presented in Figure 1.

Analysis of the studies showed that the optimal indicators for $Y_1$ are 50.36 Pa·s; for $Y_2$ is 4,7 points; for $Y_3$ is 5.0 points and $Y_4$ is 82.2 % by making 1.0 % of stabilizer and 0.5 % of emulsifier.

Equations that adequately describe the result of the experiment for a 40% mass fraction of fat have the following form:

$$Y1(x,y) := 0.07 + 27.47 \cdot x - 1.05 \cdot x^2 + 8.98 \cdot y - 0.05 \cdot y^2 - 2.13 \cdot x \cdot y$$

$$Y2(x,y) := 0.13 + 6.63 \cdot x - 2.28 \cdot x^2 - 0.11 \cdot y - 0.16 \cdot y^2 - 0.39 \cdot x \cdot y$$

$$Y3(x,y) := -0.12 + 4.86 \cdot x - 1.47 \cdot x^2 + 1.67 \cdot y - 2.45 \cdot y^2 + 0.67 \cdot x \cdot y$$

$$Y4(x,y) := 2.7 + 110.09 \cdot x - 35.2 \cdot x^2 + 21.02 \cdot y - 4.6 \cdot y^2 - 11.5 \cdot x \cdot y$$

The results of the researches made it possible to determine the influence of all independent factors on the formation of physical-structural and organoleptic parameters.

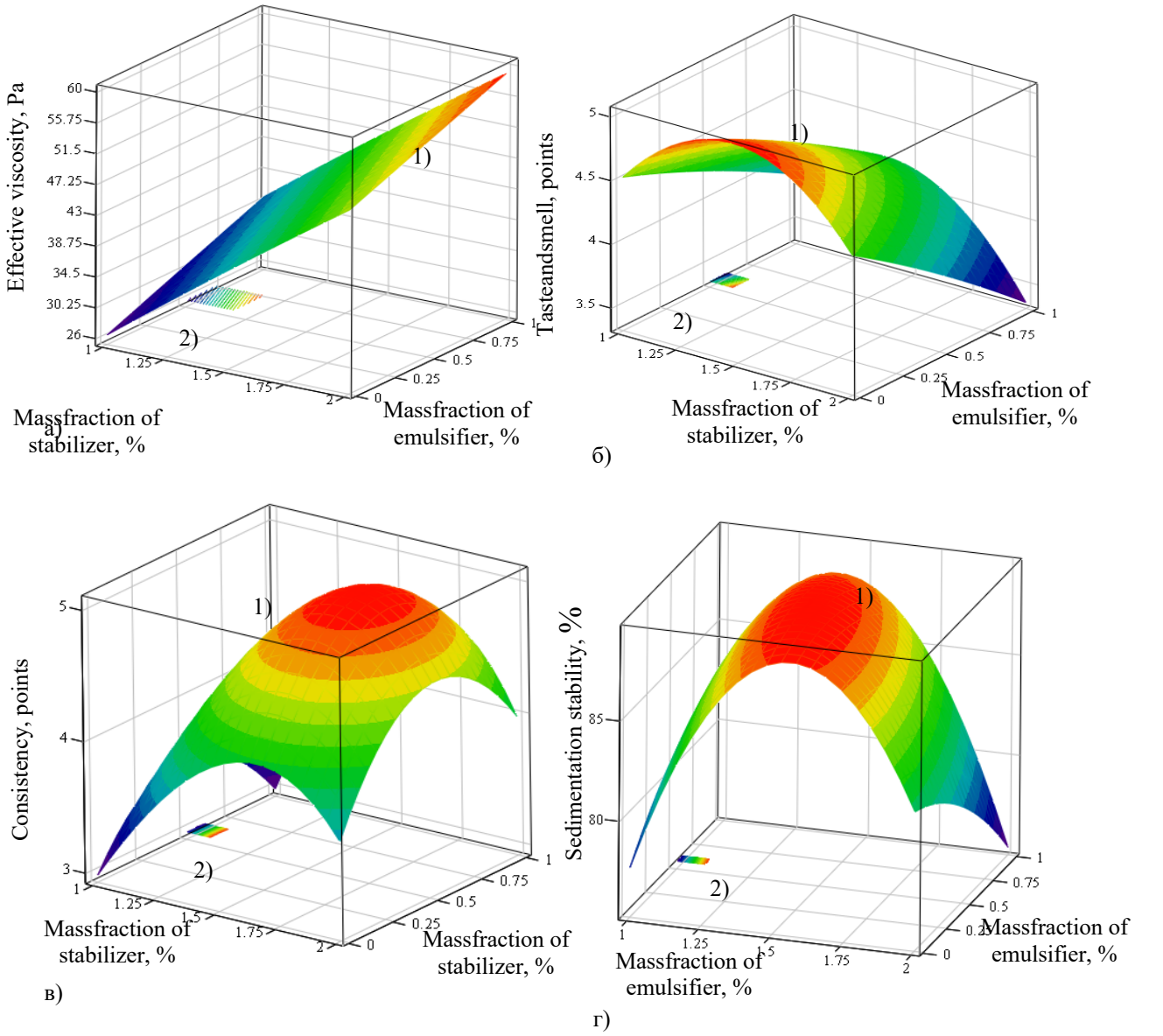


**Figure 1** Graphic 3D models (a, b, c, d – 1) and rational ranges (a, b, c, d – 2) of the influence of independent factors x and y on the basic physical-structural and organoleptic parameters of milk-fat emulsions with a fat content by weight of 40%.

## RESULTS AND DISCUSSION

The decrease in the fat level in the production of low-fat pastes (30 – 40% of fat) causes a decrease in their viscosity, the stability of structuring, regardless of the hardware design process. Several researchers **(Vishemirsky, et. al., 1997; Gulyaev-Zaitsev, 1986)** report that the reason for this is a change in the composition and physicochemical properties of cream **(Vyshemirsky, Topnikova and Kuchnova, 1997; Gulyaev-Zaitsev, 1986)**. Studies on the composition and physicochemical properties of cream with the fat level of 30-50% as the basis for milk-fat emulsions in the production of cream pastes, showed that a decrease in the fat level leads to a decrease of the ratio fat level milk solids non-fat (MSNF) in cream, effective viscosity and sedimentation resistance (Table 1).

In particular, a decrease in the fat mass from 70 to 30% in the cream resulted in a decrease in their viscosity from 13.789 to 0.036 Pa·s. Cream with a mass fraction of fat 30-40% by this indicator approached each other and was in the range of 0.036-0.072 Pa·s. The stability of fat emulsions is the inverse of the amount of fat and directly proportional to the ratio in them Fat/ DSMR. Sedimentation resistance with mass fraction of fat 50%, 40%, 35% and 30% was lower respectively on 10.5%, 11.4%, 22.4%, 33.3%, than in cream with a mass fraction of fat 70% (Table 1).

The data obtained indicate that a decrease in the fat content of the cream and, consequently, an increase in the plasma, leads to significant changes in the indicators, which impair the ability of the cream to turn into oily products and stabilize their structure To improve the ability of the cream to machining and the formation of a homogeneous stable structure of the products of low-fat content requires changes in the properties of the original cream **(Rybak, 1997; Ronholt, Mortensen and Knudsen, 2013; Leal-Calderon, 1997)**.

**Table 1** Physico-chemical indicators of cream of different fat content as raw material for paste production.

| Mass fraction of fat cream, % | The ratio of mass fraction of fat / MSNF | Precipitate of fat, % | Effective viscosity, Pa·s |
|---|---|---|---|
| 70 | 27.91 | 96.0 ±1.0 | 13.789 ±0.50 |
| 50 | 11.96 | 85.9 ±0.8 | 0.818 ±0.009 |
| 40 | 7.97 | 85.1 ±1.0 | 0.072 ±0.008 |
| 35 | 4.99 | 74.5 ±1.0 | 0.054 ±0.006 |
| 30 | 3.22 | 64.0 ±0.9 | 0.036 ±0.006 |

**Table 2** The change in the properties of cream with a mass fraction of fat 40% depending on the dose of the stabilizers of the structure.

| Mass part of the stabilizer, % | Mass fraction of emulsifier, % | Effective viscosity, Pa·s | Taste and smell, points | Consistency, points | Sedimentation stability,% |
|---|---|---|---|---|---|
| 0.5 | 0 | 24.3 ±0.5 | 4.5 | 3.0 | 85.1 |
| 0.5 | 0.5 | 32.3 ±0.9 | 4.5 | 3.7 | 86.0 |
| 0.5 | 1 | 35.5 ±0.9 | 3.5 | 3.0 | 86.4 |
| 1 | 0 | 48.6 ±0.8 | 5.0 | 4.5 | 83.2 |
| 1 | 0.5 | 50.4 ±1.6 | 5.0 | 5.0 | 82.2 |
| 1 | 1 | 56.1 ±1.6 | 4.0 | 2.5 | 88.8 |
| 1.5 | 0 | 52.4 ±1.6 | 4.5 | 4.2 | 82.7 |
| 1.5 | 0.5 | 56.1 ±1.6 | 4.5 | 4.0 | 82.3 |
| 1.5 | 1 | 68.9 ±1.6 | 3.0 | 3.0 | 80.6 |

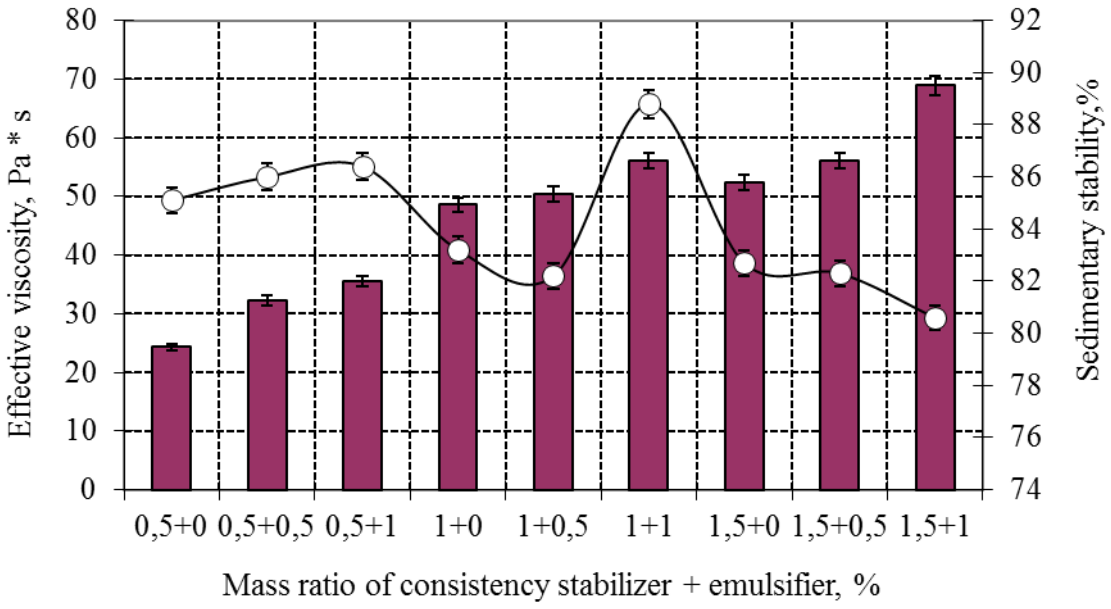


**Figure 1.** Dependency of change in effective viscosity and sedimentation resistance to increasing the dose of stabilizers in milk-fat emulsions.

In this regard, for direct control of the properties of milk-fat emulsions (mass fraction of 40% fat as a basis for the production of pastes and optimization of doses of structure stabilizers relative to the mass fraction of fat, which would provide milk-fat emulsions physical indicators close to the control cream (70% fat by weight)) were studied the effectiveness of the joint action of consistency stabilizers on the example of QNA colloidal in the amount of 1 to 2.0% and the emodulator of the dimodan U / G - from 0 to 1.0% and the dependences of changes in physicochemical and organoleptic properties were determined.

The results of the analysis of changes in the properties of cream with a fat content of 30 – 40% using a stabilizer and emulsifier are presented in Table 2.

It has been found that with increasing doses of structure stabilizers, effective viscosity increases in milk-fat emulsions. And sharing emulsifier and consistency stabilizer is more effective. It should also be noted that adding 40% fat to the emulsifier had less effect on the effective viscosity of the milk-fat emulsions. Significant changes in this indicator occurred due to the introduction of the stabilizer cream.

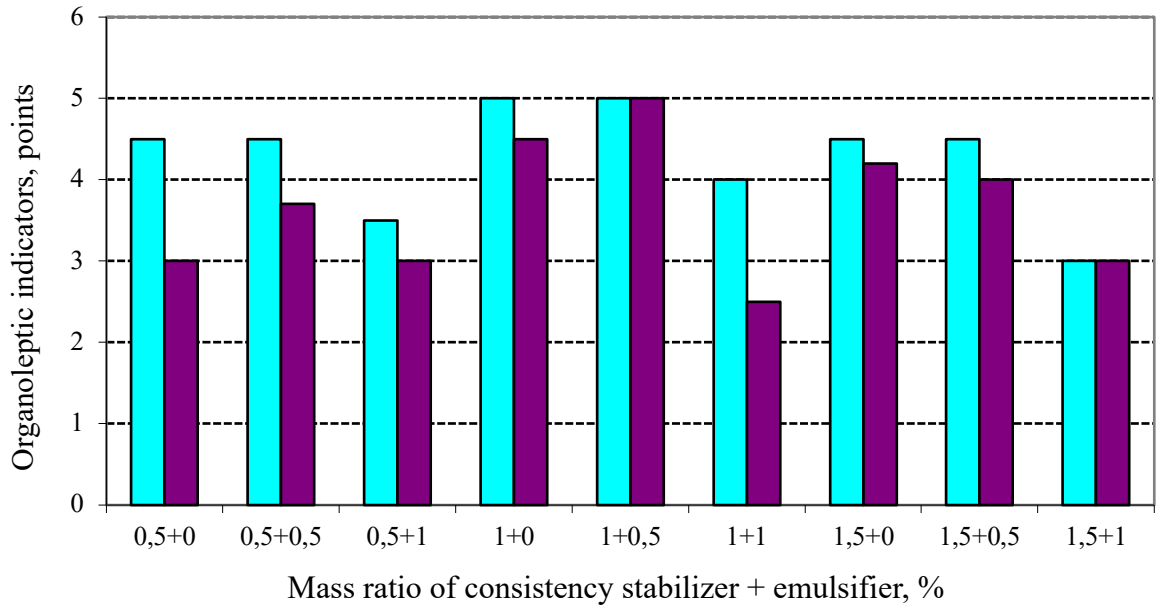


**Figure 3** Dependence of organoleptic parameters of milk-fat emulsions on mass particles of stabilizer and emulsifier: taste and smell, consistence.

**Table 3** Scale of organoleptic evaluation of milk-fat emulsions to obtain cream pastes.

| No. | Characteristic | Points |
|---|---|---|
| | **Taste and smell** | |
| 1 | Pure, creamy, without the foreign flavors and odors of structure stabilizers | 5.0 |
| 2 | The creamy taste and smell are not pronounced enough | 4.0 |
| 3 | Not expressed creamy, slightly foreign | 3.0 |
| 4 | Slightly unclean, with a foreign taste and the smell of consistency stabilizers and emulsifiers | 2.0 |
| 5 | Strange flavors and smells of stabilizers and emulsifiers of structure are pronounced, bitterness is felt | 1.0 |
| | **Consistence** | |
| 1 | Dense, plastic, homogeneous | 5.0 |
| 2 | Too tight, homogeneous, slightly powdery | 4.0 |
| 3 | Pasty, not dense, creamy, homogeneous, low fatness | 3.0 |
| 4 | Pasty, heterogeneous, sticky, refractory or sour cream, very viscous, sandy | 2.0 |
| 5 | Liquid, unstable | 1.0 |

Obtaining stable milk-fat emulsions with corresponding structural and mechanical properties has a narrow range of a mass fractioni of the stabilizer of consistency ($x_1$ ) and the emulsifier ($x_2$). The values of these ranges significantly affect the performance of viscosity, taste, and consistency. Figure 1 shows the dependence of the change in effective viscosity on the dose of stabilizers in milk and fat emulsions. Adding of the emulsifier partially affected the effective viscosity of milk-fat emulsions compared to the addition of stabilizer in the cream. The optimal ranges of values of mass fraction of the stabilizer of consistency ($x_1$) and the mass fraction of the emulsifier ($x_2$) were 1% and 1%.

The viscosity of cream with a fat level of 40%, even with 0,5% of the stabilizer addition was in 1.7 times higher than the viscosity of the control cream with a fat level of 70% and values 13.789 Pa·s (tab. 2). Instead, the addition of 1 and 2% of stabilizer resulted in a 3.5, and 3.8 times increase in the viscosity of the cream. Accordingly, compared to the control cream results were in diapason ranged within 48.6-52.4 Pa·s.

There was no clear trend in the adding of structure stabilizers in cream with 40% fat content. In particular, some samples showed an increase in sedimentation resistance of up to 4.4%, while in others a decrease in sedimentation resistance was observed.

A certain pattern in the study of the sedimentation stability of cream, depending on the doses of stabilizers and emulsifiers was not found by another researcher

**(Topnikova, 2002)**, which used in its studies other stabilizers of the structure: gelatin and monoglycerides distilled P-0291 **(Topnikova, Oborina and Vyshemirsky, 2001)**.

Taste and odor, as well as the consistency of the samples of milk and fat emulsions, were determined on a specially designed 5-point scale (Table 3) and evaluated respectively in the range of 3.0-5,0 points and 2.5-5.0 points. Graphic representation of organoleptic parameters is presented in Figure 3.

In all investigated variants of milk-fat emulsions using structure stabilizers, there was a tendency to improve the consistency due to the increase of their plasticity. The highest consistency was milk-fat emulsions with a fat content by weight of 40%, obtained with the use of stabilizer and emulsifier in quantities of 1.0% and 0.5%, respectively. Milk-fat emulsions with the only stabilizer in quantity were also highly rated 1.0 %. Milk and fat emulsions using stabilizer up to 1.0 – 1.5% and emulsifier 0.5% by taste were acceptable. Increasing the emulsifier to 1.0% worsened the taste of the emulsion due to the foreign taste of bitterness.

Thus, the results of studies on the effect of stabilizers and emulsifiers on the viscosity of milk-fat emulsions with a fat content of 40% suggest that adding stabilizers of the structure makes it possible to approximate the structural and mechanical properties of the cream 70% fat.

Thus, the data obtained by us indicate that low-fat cream with a high plasma content is significantly different in physicochemical properties from high-fat cream, which are a key factor in the formation of plastic fat bases **(Viriato, et. al., 2016, Ribeiro, et. al., 2015)**. Our findings are consistent with the results of the authors' research **(Oborina, Vyshemirsky and Topnikova, 2003)**. Such milk-fat emulsions can serve as the basis for the development of technologies for new types of fat products with necessary properties. Other researchers are inclined to this opinion **(Halukh, et. al., 2016)**.

## CONCLUSION

It has been established the dependence of changes in the physicochemical properties of cream with fat level of 40% by the dose of the stabilizers of the structure. In particular, with increasing doses of stabilizers of the structure and mass fraction of fat in milk-fat emulsions, the effective viscosity is increased and the consistency score is increased by increasing their plasticity. It should be noted that common use of emulsifiers and consistency stabilizers are more effective. According to the research results, it has been established that the selected parameters affect on the efficiency of the milk-fat emulsion. However, the formation of the structure is more influenced by consistency stabilizers than emulsifiers.

Thus, optimized doses of structure stabilizers will provide physicochemical and organoleptic indicators close to the control cream in the original low-fat cream. In particular, for milk-fat emulsions with a fat level of 40%, the QNA colloid stabilizer and the dimodan U/G emulsifier are respectively in the amounts of 1.0% and 0.5%.

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# ASSESSMENT OF THE EFFECT OF BIOACTIVE NUTRIENTS AND PROBIOTIC MICROORGANISMS ON THE PARAMETERS OF LIPID METABOLISM IN THE BODY

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## ABSTRACT

The aim of the work is a comparative study of the effect of vegetable nutrients - wheat germ oil (WGO), flour from wheat germ cake (WGC), and animal - concentrated tissue fish oil, origin on lipid metabolism indicators of students and university teachers, as well as an assessment of the probiotic factor in increasing the effectiveness of the target biologically active substances. The data obtained confirm the relation between the nutritional sufficiency of the body and the increase in lipid metabolism effectiveness and atherosclerosis risk reduction in students and teachers of an engineering university with the daily use of bioactive nutrients: WGO, WGCF, CTFO and their combinations with the biomass of lactobacilli and bifidobacteria consortium. The lipid metabolism was evaluated based on the analysis of indicators of the total cholesterol (TC) concentrations, high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TG), atherogenic index (AI). The values of these indicators were recorded before and after daily consuming the study foods for 30 days. The data obtained were analyzed for 3 age groups: 16 – 24, 25 – 44, and 45 – 65. It was found that WGO has the most effective antiatherosclerotic effect; the decrease in the TC level was 6.4%, the increase in the HDL concentration was 13.7%, the decrease in the LDL concentration was 11.3%, the decrease in the TG concentration was 17.1%, and the AI decreased by 22.6%, respectively. With WGCF, the changes in the studied parameters were 6.0, 12.3, 13.1, 13.1, and 22.1%, respectively. Together with probiotics WGO effectiveness increased in terms of the reduced TC level (1.9%), increased HDL concentration (8.2%), decreased LDL concentration (2.5%), reduced TG concentration (5.7%), and decreased AI (6.3%) for CTFO by 9.1, 26.1, 14.7, 24.1 and 31.2%, respectively. Thus, the dynamics of lipid metabolism indices objectively testifies to the positive effect of bioactive nutrients on human health indicators.

**Keywords:** bioactive nutrient; lipid metabolism; LDL cholesterol; HDL cholesterol; triglyceride;

## INTRODUCTION

Currently, the need for timely diagnosis of cardiovascular diseases, interpretation of changes in the blood lipid profiles, and effective correction of disorders is beyond dispute. In this regard, it is relevant to search for and assess alimentary factors that provide energy needs of the body utmost normalize its functional state, and implement non-drug methods for correcting lipid metabolism disorders **(Arabidze and Tebloev, 2008; Popov et al, 2016; Bouillon and Antonio, 2020; Longo, Ferrari and Zocchi, 2017; Mohammed Farugue, 2018)**.

Natural vegetal and animal-based resources obtained in the process of their deep processing and probiotic microorganisms, including their consortia, which enhance the effect of target biologically active substances, are the most relevant, effective, and promising means for correcting nutritional status and preventing pathological conditions **(Chugh and Kamal-Eldin, 2020; Rodionova et al, 2019a)**.

A good therapeutic effect in normalizing blood parameters that characterize lipid metabolism for different groups of patients was established when taking biologically active food supplements containing policosanol, which is a mixture of several long-chain alcohols **(Rodionova, Popov, and Sokolova, 2016; Rodionova and Alekseeva, 2015; Arruzazabala and Carbajal, 2012; Rodionova et al, 2019b)** and omega 3 polyunsaturated fatty acids, in particular eicosapentaenoic and docosahexaenoic acids **(Meerson et al, 1993; Rodionova et al, 2016; Isaev and Simonenko, 2016; Heshmati, 2019; Spector and Kim, 2019)**.

Its mechanism of action policosanol is based on the modulation of 3-hydroxy-3-methyl-glutaryl-CoA reductase, ab-sorption of bile acids, and partial utilization

of lactic acid. Policosanol increases receptor-dependent processing of low-density lipoproteins (LDL) by enhancing the LDL-receptor binding affinity, improving the LDL transport to liver cells, thereby significantly increasing the cleavage of LDL cholesterol. In addition to lowering the LDL levels, policosanol has been found to increase high-density lipoprotein (HDL) levels, protect HDL from the damaging effects of free radicals, and inhibit excessive platelet aggregation **(Arruzazabala and Carbajal, 2012; Arruzazabala, Valdes and Mas, 2013; Canetti, Morera, and Illnait, 2012; Carbajal, Arruzazabala and Mas, 2012)**.

## Scientific hypothesis

It was clinically proven that probiotic products increased the body's resistance to various external and internal factors **(Angelin and Kavitha, 2020; Cenit, 2014; Roobab et al, 2020; Teneva-Angelova et al, 2018, Zuo, Chen and Marcotte, 2020)**. Concerning such a wide range of positive clinical effects, a hypothesis was put forward about the feasibility of studying the biomass influence on the homeostasis in the human body when introduced into the diet.

## MATERIAL AND METHODOLOGY

### Samples

Wheat germ is a valuable source of policosanol **(Vishnyakov et al, 2020; Boukid et al, 2018)**. This by-product of flour milling includes up to 15% oil with a policosanol content of 1.5-8.0 mg/100 g (Rodionova et al, 2016; Popov et al, 2016). When separating wheat germ oil by cold pressing (at 150 MPa), the protein-carbohydrate component (cake) remains with a residual oil content of 6-8%; this cake is processed into flour, resulting in Vitazar dietary supplement. This flour contains up to 35% of protein, including all essential amino acids, up to 47% of carbohydrates, represented by glucose, maltose, sucrose, oligosaccharides, pentosans, polysaccharides. It is a source of biologically active substances: vitamins (B1, B2, B3, B6, B9, PP), macro- and microelements (Zn, Mn, Mg, Ca, K, Fe, Na, Se, P) and policosanol content of 0.1-0.7 mg/100 g. The amount of cake in oil production is up to 95% of the feedstock; it is an inexpensive food bioresource with valuable functional properties **(Rodionova et al, 2016; Rodionova, Popov and Sokolova, 2016)**.

Concentrated tissue fish oil (CTFO), the Eikonol dietary supplement, is a source of polyunsaturated fatty acids of the omega-3 class, including eicosapentaenoic and docosahexaenoic acids. It is an active bioregulator of metabolic processes, obtained with the use of gentle modes of the physical impact that preserve the native properties of the lipid fraction to the uttermost **(Isaev and Simonenko, 2016)**. Installed impact of CTFO on the normalization of lipid metabolism indicators, immunological parameters in cardiac ischemia, cardiac muscle contractile function and arrhythmias, rheological parameters of blood, and disseminated sclerosis correction **(Jamshidi et al, 2020; Yang et al, 2020; Zamroziewicz et al, 2017)**.

The biomass of lactobacilli and bifidobacteria, introduced additionally into the diet, having a viable cell concentration of 109 CFU/g enables to solve a wide range of tasks on selective stimulation of the immune system, influencing the main links of disease pathogenesis, enhancing the essential drug action. Additional effects were also revealed, such as hypolipidemic, antihistaminic properties, gastrointestinal motility regulation, and reinfection prevention.

Male and female students and university members of the University voluntarily participated in these four experimental groups aged 16 to 65. The participants spent at least 6 hours daily in the same conditions – the premises of the university. All the subjects had not previously used the bioactive nutrients under study. They had no concomitant diseases impairing lipid metabolism (liver and kidney diseases, hypothyroidism, obesity) and were not subject to regular medical check-ups. The study was carried out in the autumn to exclude changes associated with the seasonality of nutrition and physical activity. Smoking and pregnancy were exclusionary criteria for study entry **(Grigoriev, 2015)**. Each member of the experimental group signed informed consent to participate in the trial.

### Chemicals

The following supplements were investigated as correctors of lipid metabolism: WGO and WGCF (manufactured by Pulat LLC, RF); CTFO (manufactured by NPP Trinita LLC, RF).

### Animals and Biological Material:

The biomass of the lactobacilli and bifidobacteria consortium – Streptococcus thermophilus, Caseisubsp. Rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium adolescentis, with a viable cell content of n 109 CFU/g.

### Instruments

Cobas 6000 diagnostic immunoassay analyzer (produced by F. Hoffmann-La Roche Ltd, Switzerland; distributed by LLC MedTechnic, Russia).

### Laboratory Methods

The reliability of the obtained parameters was assessed according to the Mann-Whitney nonparametric U-test **(Grigoriev, 2015)**. Volunteer patients were divided into four groups. The experimental groups amounted to 180 persons, of which 92 are men and 82 are women; the same number of subjects (180 persons) was in the control groups that did not consume the test products (Table 1).

**Table 1**Membership and size of the experimental groups.

| Bioactive nutrients | Patients' age, years | | | | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | 16-24 | | 25-44 | | 45-65 | | | |
| | male | female | male | female | male | female | male | female |
| WGO | 9 | 9 | 7 | 7 | 7 | 6 | 23 | 22 |
| WGCF | 9 | 9 | 7 | 7 | 7 | 6 | 23 | 22 |
| WGO-containing probiotic emulsion | 9 | 9 | 7 | 7 | 7 | 6 | 23 | 22 |
| CTFO-containing probiotic emulsion | 9 | 9 | 7 | 7 | 7 | 6 | 23 | 22 |

The first group was given 3.5 g of WGO, the second group consumed WGCF in an amount providing 3.5 g of oil, which corresponded to 50 g in the composition of a confectionery **(Rodionova, Popov and Rodionova, 2019)**. The third group consumed a probiotic emulsion containing 3.5 g of WGO, and the fourth group took a probiotic emulsion containing 6.5 g of CTFO combined with 10 g of biomass of probiotic microorganisms **(Zuo, Chen and Marcotte, 2020)**.

**Description of the Experiment**

**Sample preparation:** To study the fermentation processes, consortia of probiotic microorganisms were activated, in 100 ml sterilized (temperature 121 ±2 ºC, exposure 13 ±2 min) and cooled to a temperature of (39 ±1 ºC) skim milk, 0.7 g of dry sour-dough was added. To activate microbial cells, the concentrate was thoroughly mixed and kept for 4.0 h at a temperature of (37 ±1 ºC). After 1 and 2 h after the start of activation, the bacterial suspension was stirred again (by shaking) to evenly distribute the bacterial cells throughout the mass. The resulting activated concentrate (without the formation of a clot) immediately after activation was introduced with stirring into 900 g of pasteurized (92 ±2 ºC, exposure 2 – 8 min) and chilled (37 – 42 ºC) milk. Fermentation was carried out at temperatures in the range (37 – 42 ºC), with an interval of 1 hour, the titratable and active acidity of the medium was monitored until the formation of a clot, the standard fermentation time, taking into account the acid-forming properties of the strains of Lacto- and Bifido-bacteria included in the consortium was 12 – 14 hours. The titratable acidity of the test samples was 80 – 100 ºT, pH = 4.61 – 4.65.

**Number of samples analyzed:** 736.

**Number of repeated analyses:** 36*3=2208.

**Number of experiment replication:** 1.

The amount of added wheat germ oil (3.5 g / day) corresponded to the satisfaction of 50% of the body's daily need for vitamin E. Contraindications to the use of wheat germ oil are the period of exacerbation of cholecystitis, gastritis, hepatitis, or cholelithiasis, as well as individual intolerance.

The mass of the injected concentrated tissue fish oil (6.5 g / day) was determined by 50% of satisfying the daily requirement of the body in omega-3 polyunsaturated fatty acid. Contraindication to admission is a pronounced exacerbation of chronic cholecystitis and pancreatitis, individual intolerance.

The stability of emulsions was at least 70 – 75% because of the high exopolysaccharide activity of the biomass **(Rodionova, Popov and Rodionova, 2019; Zuo, Chen and Marcotte, 2020)**. These food forms were consumed by volunteers regardless of food intake, without correction of the basic diet. The duration of the experimental studies was 30 days; at the beginning and the end of the intake of bioactive nutrients, the studied parameters were controlled. On the day of sampling, the investigated bioactive nutrients were not given to the subjects; the time interval between using the investigated products and sampling was no less than 24 hours.

Blood was analyzed by a hardware automatic method using a Cobas 6000 diagnostic immunoassay analyzer (F. Hoffmann-La Roche Ltd, Switzerland), which is based on the electrochemiluminescence method. The atherogenic index (AI) was determined by calculation. Blood was collected from the cubital vein at least 12 hours after the last meal. The lipid metabolism parameters were monitored before and after 30 days of taking the test product **(Grigoriev, 2015)**.

### Statistical Analysis

The reliability of the data presented is confirmed by the values of the Mann-Whitney test (Table 3). Staitistica 13 software (developed by StatSoft Russia) was used for statistical analysis.

The results obtained by the authors agree with experimental data on the study of the impact of policosanol, isolated from the sugar cane wax mass, on the stabilization and subsequent approximation to the norm of the lipid metabolism parameters in the human body.

A comparative assessment of the biocorrective influence of WGO and WGCF on the lipid metabolism parameters, in the case of an identical concentration of the target biologically active substance, showed that WGCF has a more intense effect on the studied parameters.

## RESULTS AND DISCUSSION

All performed calculations show $p<0.05$.

Analysis of lipid metabolism indicators for three age groups before the start of the experiment showed that the TC level in the first two groups of the surveyed participants was within the borderline level – 5.03 mmol/l; in the third group, it exceeded the norm: 5.82 mmol/l (norm <5.2 mmol/l). The HDL content in the subjects' blood serum was below the norm: 1.21 mmol/l (norm >1.42 mmol/l) in all groups. The LDL content was within the norm: 3.12 mmol/l in the first two groups, and the third group, it exceeded the norm: 3.61 mmol/l (norm <3.37 mmol/l). The TG concentration was within the normal range: 1.66 mmol/l in the first group; while in the second and third group it exceeded the norm: 1.98 mmol/l (norm ranging within 0.14-1.82 mmol/l). In the first age group, the atherogenic index (AI) was within the normal range (2.44 units), while in the second and third age groups it exceeded the norm amounting to 3.71 units (norm ranging within 2.0-3.0 units). Thus, certain lipid metabolism disorders of varying degrees were recorded practically in all age groups. It is known that the significance of the HDL concentration in the body is primarily determined by their participation in the cholesterol homeostasis in blood serum and tissues due to their involvement in the so-called reverse cholesterol transport **(Meerson et al, 1993; Jamshidi et al, 2020; Longo, Ferrari and Zocchi, 2017)**.

Evaluation of changes in lipid metabolism after 30 days of taking WGO and WGCF showed that the TC level decreased by 6.4 and 6.0%, the HDL concentration increased by 13.7 and 12.3%, the LDL concentration decreased by 11.3 and 13.1 %, the TG level decreased by 17.1 and 13.1% with a corresponding decrease in AI by 22.6 and 22.1%, respectively (Figure 1).

Previous experimental studies on the correction of lipid metabolism disorders in patients with arterial hypertension showed that after 30 days of taking CTFO, there was a fixed decrease in TC concentration by 7.5%, TG by 23%, AI by 23.6%, LDL by 12.9% -and an increase in HDL by 15.6% **(Meerson et al, 1993; Jamshidi et al, 2020)**.

**Table 2** Calculated values of the Mann-Whitney test.

| Indicator assessed for reliability | After the WGO intake course | | | After the WGCF intake course | | |
|---|---|---|---|---|---|---|
| | Group (Ut) | | | | | |
| | I (99) | II (55) | III (45) | I (99) | II (55) | III (45) |
| TC | 71.5 | 43.0 | 34.5 | 85.5 | 49.0 | 43.0 |
| HDL | 73.5 | 44.0 | 33.0 | 81.5 | 52.0 | 42.5 |
| LDL | 75.0 | 47.5 | 30.5 | 79.5 | 48.5 | 39.5 |
| TG | 72.5 | 43.0 | 36.5 | 80.0 | 51.5 | 42.0 |
| AI | 74.0 | 41.0 | 34.5 | 82.5 | 49.0 | 37.5 |

**Table 3** Calculated values of the Mann-Whitney test.

| Indicator assessed for reliability | After the CTFO-containing probiotic emulsion intake course | | | After the WGO-containing probiotic emulsion intake course | | |
|---|---|---|---|---|---|---|
| | Group (Ut) | | | | | |
| | I (99) | II (55) | III (45) | I (99) | II (55) | III (45) |
| TC | 72.5 | 45.0 | 33.5 | 82.0 | 47.5 | 44.5 |
| HDL | 75.0 | 43.5 | 35.5 | 82.5 | 51.5 | 44.0 |
| LDL | 77.5 | 46.5 | 31.0 | 78.0 | 46.5 | 40.5 |
| TG | 73.0 | 44.5 | 37.0 | 78.5 | 50.0 | 44.5 |
| AI | 72.5 | 44.5 | 36.0 | 80.0 | 48.5 | 40.5 |

When comparing the results obtained with the patients' age as a result of the reception WGO and WGCF, a 6.8% decrease in the TC level was recorded in the first age group with the LDL concentration decreasing by 6.9%, TG by 18.2%, AI by 22.9%, and the HDL concentration increasing by 13.3%. In the second group, the HDL concentration increased by 12.2%, TC, LDL, TG decreased by 5.9, 13.9, and 16.5%, respectively, the AI value decreased by 21.5%. In the third group, there was a decrease in the TC, LDL, and TG concentration by 6.1, 15.1, 16.6%, respectively, an increase in the HDL concentration by 13.8%, and a positive correction of the AI values by 22.4% (Figure 2 and Figure 3).

The calculated values of the Mann-Whitney test do not exceed its critical parameters for each age group, which indicates the reliability of the revealed differences in the values of the studied indicators and suggests the adequate effectiveness of the WGO and WGCF influence on the lipid metabolism parameters in the human body (Table 2).

The possibility of increasing the performance of target biologically active substances through the use of probiotic components that perform the functions of transfer and targeted delivery in various body systems made it possible to proceed to the next stage of the study – a comparative assessment of the corrective action of WGO- and CTFO-containing probiotic emulsions **(Roobab et al, 2020; Teneva-Angelova et al, 2018; Zuo, Chen and Marcotte, 2020)**.

It was found that the combined use of the investigated bioactive nutrients with probiotic microorganisms is specific for each bioactive nutrients under study (Figure 4). The CTFO-containing probiotic emulsion showed a more pronounced impact on lipid metabolism compared to the WGO-containing emulsion. **(Isaev and Simonenko, 2016;Rodionova et al, 2016; Vishnyakov et al, 2020; Boukid et al, 2018; Arabidze and Tebloev, 2008; Popov et al, 2016)**. In the case of WGO- and CTFO-containing probiotic emulsion intake, TC level decreased by 8.3 and 9.1%, HDL concentration increased by 21.9 and 26.1%, LDL concentration decreased by 13.8 and 14.7%, TG level decreased by 22.8 and 24.1%, positive AI correction was 28.9 and 31.2%, respectively, which indicates an increase in the nutrient correction effectiveness in the presence of probiotic microorganisms **(Meerson et al, 1993; Jamshidi et al, 2020; Longo, Ferrari and Zocchi, 2017)**.

The WGO and CTFO effectiveness increased in terms of the decreased TC level by 1.9 and 1.6 %, increased HDL con-centration by 8.2 and 10.5 %, decreased LDL concentration by 2.5 and 1.8 %, decreased TG concentration by 5.7 and 1.1 %, and reduced AI values by 6.3 and 7.6 %.

The results of experimental studies allow for the statement of the vital need to expand the range of products containing combinations of bioactive nutrients and probiotic microorganisms in a viable form. .**(Meerson et al, 1993; Jamshidi et al, 2020; Grigoriev, 2015; Rodionova et al, 2019c; Angelin and Kavitha, 2020; Cenit, 2014; Yang et al, 2020; Zamroziewicz et al, 2017)**. The development of formulations and technologies for these products is an urgent task at the present stage of developing food technologies that have the properties of pharmaceuticals intended for preventing pathological conditions, providing health preservation, and improving the quality of life. **(Rodionova, Popov and Sokolova, 2016; Arruzazabala and Carbajal, 2012) (Arruzazabala, Valdes and Mas, 2013; Canetti, Morera, and Illnait, 2012; Carbajal, Arruzazabala and Mas, 2012).**

The results of experimental studies illustrate the dependence of the patients' age and the achieved antiatherosclerotic effect on the intake of the probiotic forms under study (Figure 5 and Figure 6).

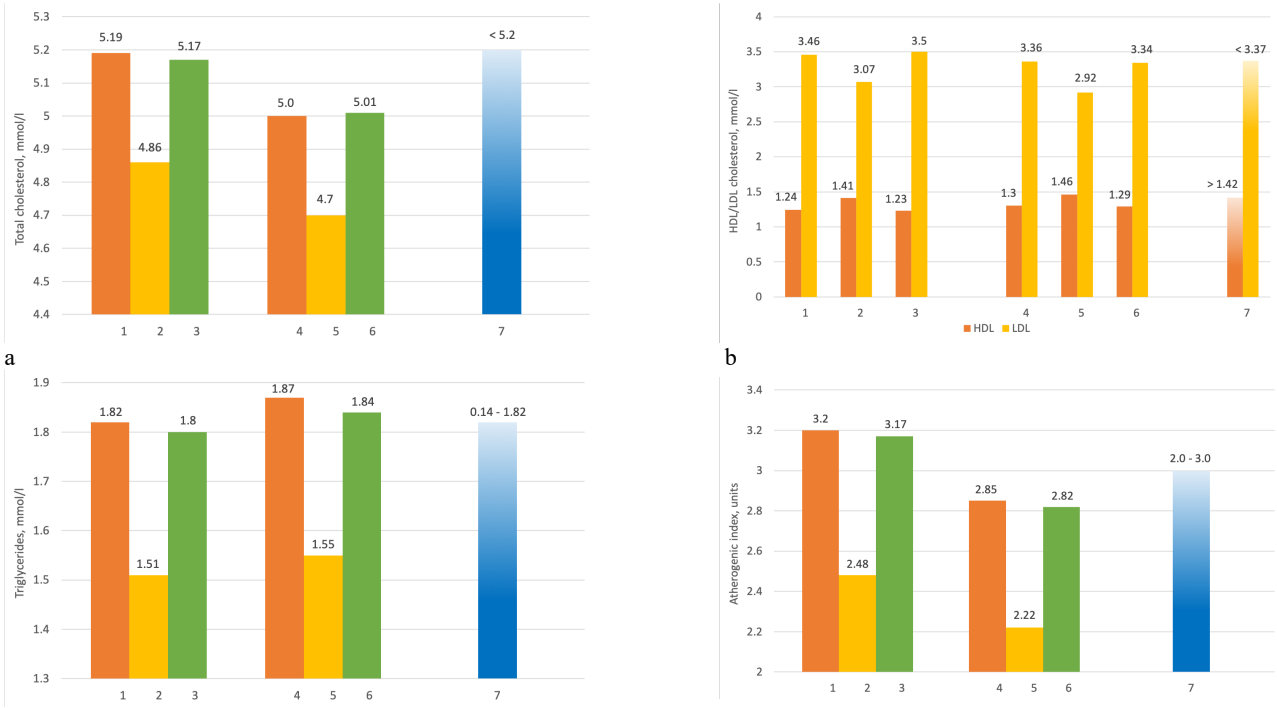


**Figure 1** Concentrations in blood: a –blood TC level, mmol/l; b - HDL/LDL cholesterol level, mmol/l; c – TG level, mmol/l; d - AI, units. 1 (4) – prior to WGO (WGCF) intake; 2 (5) –30 days after WGO (WGCF) intake; 3 (6) – control group (during the entire period); 7 – reference interval.

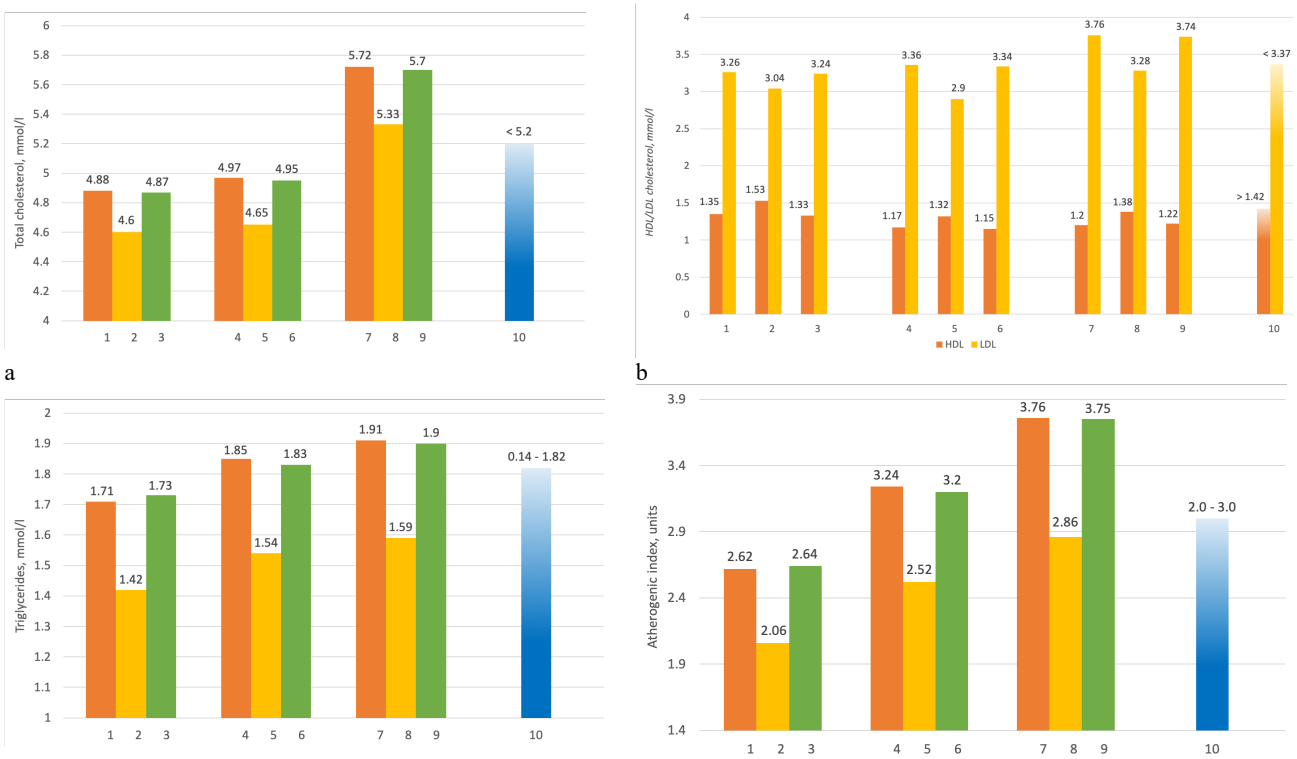


**Figure 2** Concentrations in blood: a – blood TC level, mmol/l; b - HDL/LDL cholesterol level, mmol/l; c – TG level, mmol/l; d – A I, units. 1, 4, 7 – prior to WGO intake in the 1st, 2nd, and 3rd age groups; 2, 5, 8 – 30 days after WGO intake in the 1st, 2nd, and 3rd age groups; 3, 6, 9 – control group (during the entire period); 10 – reference interval.

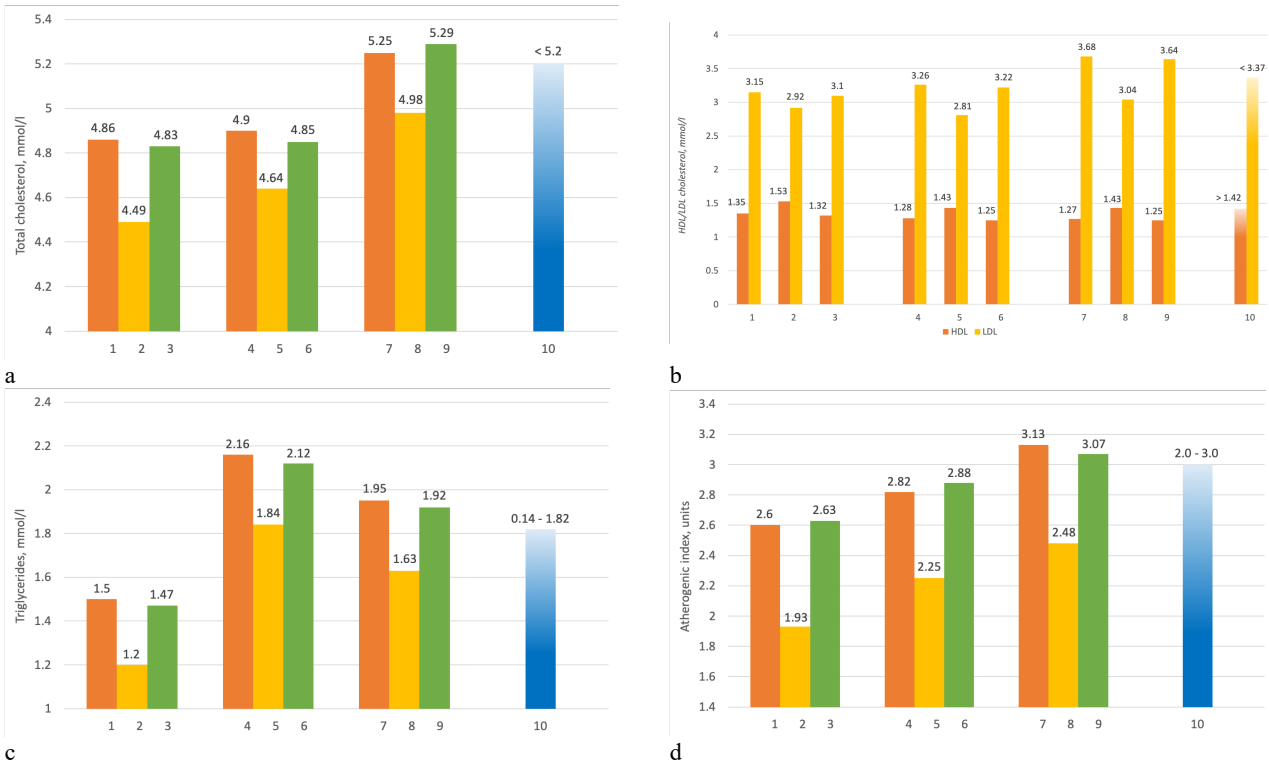


**Figure 3** Concentrations in blood: a – blood TC level, mmol/l; b – HDL/LDL cholesterol level, mmol/l; c – TG level, mmol/l; d – AI, units. 1, 4, 7 – prior to WGCF intake in the 1st, 2nd and 3rd age groups; 2, 5, 8 – 30 days after WGCF intake in the 1st, 2nd, and 3rd age groups; 3, 6, 9 – control group (during the entire period); 10 – reference interval.

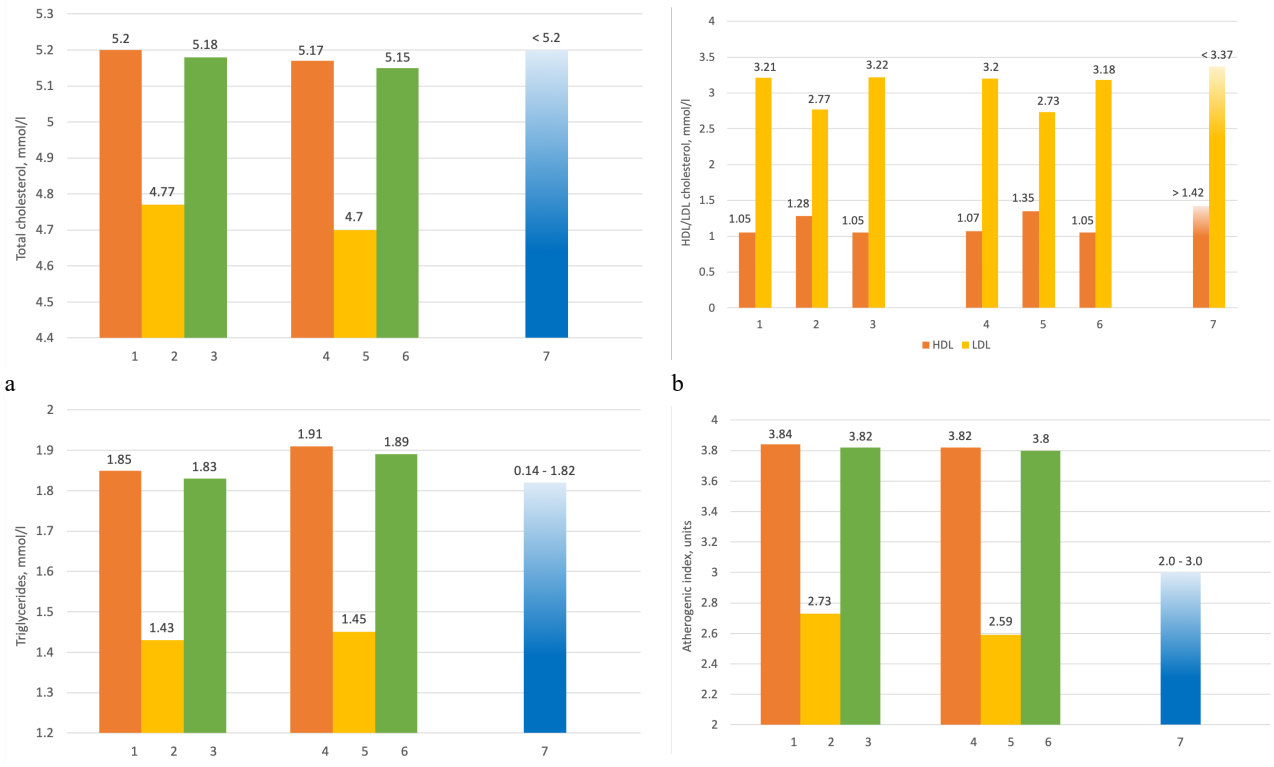


**Figure 4** Concentrations in blood: a –blood TC level, mmol/l; b – HDL/LDL cholesterol level, mmol/l; c – TG level, mmol/l; d – AI, units. 1 (4) – prior to WGO (CTFO) intake; 2 (5) – 30 days after WGO (CTFO) intake;.3 (6) – control group (during the entire period); 7 – reference interval.

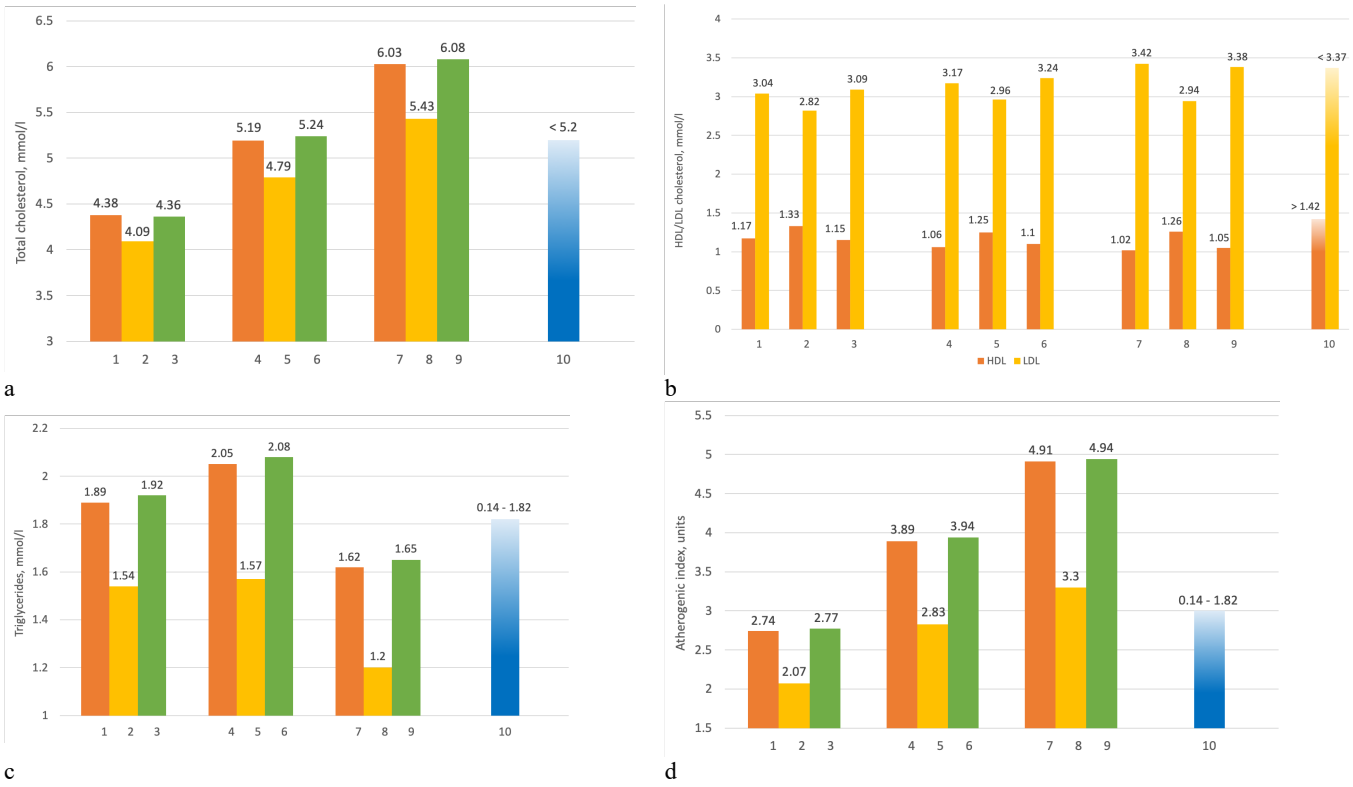


**Figure 5** Concentrations in blood: a – blood TC level, mmol/l; b - HDL/LDL cholesterol level, mmol/l; c – TG level, mol/l; d – AI, units. 1, 4, 7 – prior to the WGO-containing probiotic emulsion intake course in the 1st, 2nd and 3rd age groups; 2, 5, 8 – 30 days after the WGO-containing probiotic emulsion intake course in the 1st, 2nd, and 3rd age groups; 3, 6, 9 – control group (during the entire period); 10 – reference interval.

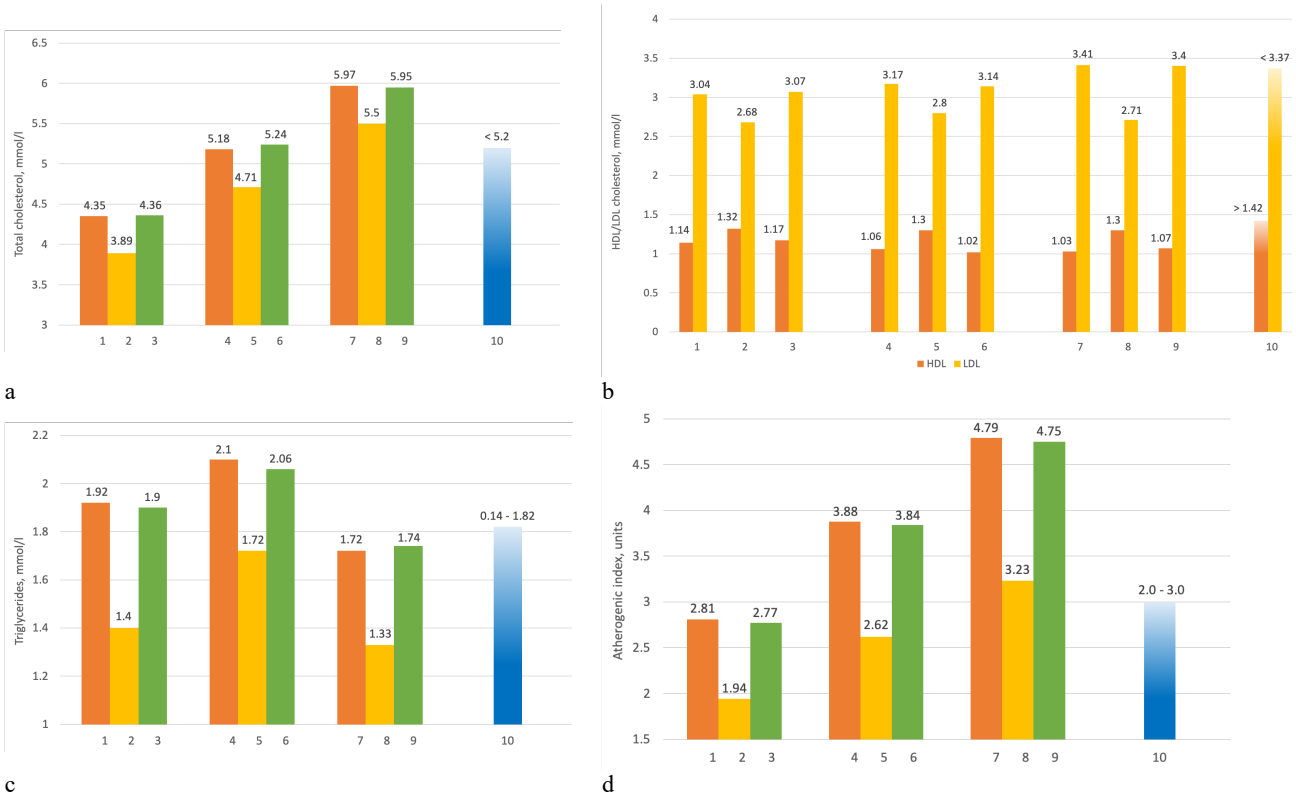


**Figure 6** Concentrations in blood: a –blood TC level, mmol/l; b - HDL/LDL cholesterol level, mmol/l; c – TG level, mmol/l; d – AI, units. 1, 4, 7 – prior to the CTFO-containing probiotic emulsion intake in the 1st, 2nd and 3rd age groups; 2, 5, 8 – 30 days after the CTFO -containing probiotic emulsion intake in the 1st, 2nd and 3rd age groups; 3, 6, 9. – control group (during the entire period); 10 – reference interval.

## CONCLUSION

The results of experimental studies illustrate the dependence of the patients' age and the achieved antiatherosclerotic effect on the intake of the probiotic forms under study. As a result of the reception WGO and CTFO in the first age group, the outcomes demonstrated a decrease in the TC level, LDL concentration, TG level, and AI values by 9.2, 9.6, 23.1, 27.8%, respectively, and an increase in the HDL concentration by 18.2%. The second group showed a decrease in the TC level by 8.4%, an increase in the HDL concentration by 19.8%, a decrease in the LDL concentration by 15.5%, a decrease in the TG concentration by 20.8%, and a positive correction of AI values by 30.2%. In the third group, the TC level decreased by 8.9%, with a decrease in the LDL concentration by 17.7%, the TG level by 24.6%, AI values by 22.6%, and an increase in HDL concentration by 23.4%.

The results of experimental studies illustrate a decreased level of TC, an increased concentration of HDL, a decreased concentration of LDL, a decreased concentration of TG, and AI reduction in all age groups when WGO, WGOM, and CTFO are added to the main diet. Evaluation of the combined influence of the viable forms of probiotic microorganisms together with the studied bioactive nutrients on the lipid metabolism indicators showed a synergistic effect in all age groups. It is fair to state that the bioactive nutrients under study, WGO, WGCF, and CTFO, can be attributed to alimentary factors of non-entropic action, ensuring the restoration of the equilibrium state of the body systems responsible for lipid metabolism.

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# THE CONTENT OF SILVER, ALUMINUM, AND ZINC IN WILD EDIBLE MUSHROOM *MACROLEPIOTA PROCERA*

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## ABSTRACT

The content of selected elements (Ag, Al, and Zn) in wild edible Parasol mushroom (*Macrolepiota procera* Scop. Singer) collected from five sites in Slovakia – Lazy pod Makytou, Lozorno, Nemečky, Tesáre, and Zbyňov was investigated. The element analysis was determined using the ICP-OES method. The average concentrations of Ag, Al, and Zn in *M. procera* caps ranged as follows: 0.41 – 3.23, 16.6 – 113, and 73.4 – 111 mg $kg^{-1}$ dry weight, respectively. Also, Spearman's correlation test was used to determine the correlations between Parasol mushroom caps and stems in the content of Ag, Al, and Zn. Subsequently, the obtained data on the content of the monitored elements in *M. procera* caps were used for the evaluation of health risks arising from the consumption of *M. procera*. Although mushrooms are an important part of the diet, they are consumed mainly as a seasonal delicacy therefore, the intake of the monitored elements from the consumption of *M. procera* may be limited. Regular and long-term consumption of *M. procera* caps from investigated sites does not pose any health risks to the consumers.

**Keywords:** Parasol mushroom; potential risk elements; essential element; consumption; health risk

## INTRODUCTION

Mushrooms are one of the most diverse and economically important species on Earth. They are a significant part of the ecosystem and important inclusions for the human diet **(Painuli, Semwal and Egbuna, 2020; Singh, Lallawmsanga and Passari, 2018)**. The consumption of edible wild-grown mushrooms is widespread in many European countries, mainly due to the diversity of species and their culinary characteristics **(Kalač, 2016)**. Mushrooms are popular not only for their texture and taste but also for their chemical and nutritional composition **(Mallikarjuna et al., 2013)**. They are considered as a rich source of proteins, carbohydrates, minerals, and vitamins while they are low in fat and energy value **(Painuli, Semwal and Egbuna, 2020; Atri et al., 2019; Singh, Lallawmsanga and Passari, 2018; Chatterjee et al., 2017)**.

However, wild-grown mushrooms may contain high concentrations of both essential and risk elements to humans **(Singh, Lallawmsanga and Passari, 2018; Stefanović et al., 2016; Falandysz and Borovička, 2013; Kalač, 2010; Kalač, 2009)**. Many previous studies have revealed the high ability of edible wild-growing mushrooms to accumulate various elements present in the biosphere even at trace levels, especially risk elements and radionuclides **(Slávik et al., 2016; Záhorcová et al., 2016; Árvay et al., 2015a; Miššík et al., 2015)**. In some cases, a certain mushroom species can accumulate specific trace elements, whereas the concentrations in fruiting bodies being at least 100-times higher than concentrations of the respective element in other plant species on the same substrate **(Chatterjee et al., 2017)**. Risk elements are not biodegradable, and even in trace amounts, they tend to accumulate in wild edible mushrooms and subsequently enter the food chain **(Singh et al., 2008)**. Their presence in the food chain poses a risk to human health. Long-term exposure and accumulation of risk elements in the body can lead to harmful effects on human health **(Árvay et al., 2017; Dixit et al., 2015)**.

In general, the accumulation of elements in mushrooms is influenced primarily by internal factors (species-dependent) and soil factors (soil geochemistry, soil pollution) **(Falandysz et al., 2017; Mallikarjuna et al., 2013; Giannaccini et al., 2012)**. Wild-growing mushrooms are involved in biogeochemical processes - element cycle and transformation of inorganic and organic substrates. The ability to accumulate elements in fruiting bodies of mushrooms is very important from an ecological point of view – cycling the elements, transformation of inorganic and organic substrates **(Kuldo et al., 2014; Falandysz and Borovička, 2013)**. Although the ability to accumulate risk elements in wild edible mushrooms finds application in the process of bioremediation and environmental pollution monitoring, it can also pose a potential health risk to

consumers **(Chatterjee et al., 2017; Falandysz and Borovička, 2013)**.

*Macrolepiota procera* (Scop.) Singer, also known as Parasol mushroom (Figure 6), is a wild edible mushroom widespread in temperate and sub-tropical regions such as Thailand, India, and Europe **(Falandysz et al., 2017; Stefanović et al., 2016; Kuldo et al., 2014)**. *M. procera* is one of the most favourite wild edible mushrooms for consumers in Central Europe, including Slovakia **(Kalač, 2016)**. Especially caps of *M. procera* are highly valued due to their taste and aroma in both cooked and fresh states **(Falandysz et al., 2017)**. Many previous studies reported bioaccumulation property of *Macrolepiota procera* for Ag, Cd, Cu, Hg, Mg, Na, K, and Zn **(Falandysz et al., 2017; Stefanović et al., 2016; Árvay et al., 2015a; Kuldo et al., 2014; Giannaccini et al., 2012; Gucia et al., 2012a)**. Due to its high bioaccumulation capacity, a long-term and regular consumption of wild edible mushrooms, especially from sites affected by anthropogenic contamination, represents the highest health risk to consumers **(Árvay et al., 2015b; 2014)**.

This study is focused on monitoring the element's contents (Ag, Al, and Zn) in different morphological parts of Parasol mushroom (*Macrolepiota procera* Scop. Singer) collected from selected five sites in Slovakia – Lazy pod Makytou, Lozorno, Nemečky, Tesáre, and Zbyňov. Selected elements belong to 3 different groups of elements: Ag – element with detrimental health effects, Al – nutritionally non-essential element, and Zn – essential trace element. Higher concentrations of the monitored elements may have adverse effects on human health, therefore, the potential health risks arising from regular and long-term consumption of *M. procera* caps from selected localities were evaluated based on the obtained contents of monitored elements.

## Scientific hypothesis

Increasing age of the mycelium and a prolonged interval between fructifications significantly elevate the contents of many elements in fruiting bodies, and higher levels usually occur in caps than in stems of wild-growing mushrooms.

Caps of wild-grown mushrooms are collected and eaten most often than stems therefore, they may represent a potential source of risk elements in the human diet in terms of long-term consumption.

## MATERIAL AND METHODOLOGY

### Samples

The samples of Parasol mushroom (*Macrolepiota procera* Scop. Singer) (n = 50) were collected from five selected sites in Slovakia – Lazy pod Makytou, Lozorno, Nemečky, Tesáre, and Zbyňov (Figure 1) during harvesting season (September – October) in 2018.

During the collection of mushroom samples, several principles were followed. The whole fruit body was cleaned up from any organic and inorganic debris and placed into a paper bag for transportation.

In the Slovak republic, five levels of environmental quality are determined, from the high-quality level of the environment to the highly disturbing level. Investigated localities, Lazy pod Makytou and Tesáre belong to the 1st environmental quality – regions with the undisturbed environment. The three other locations, Lozorno, Nemečky, and Zbyňov, belong to the 2nd environmental quality - regions with moderately disturbed environments in Slovakia **(Ministry of Environment of the Slovak Republic, 2016)**. Each sampling point was identified by using GPS coordinates (Table 1).

### Chemicals

$HNO_3$ (nitric acid, 69%, Sigma Aldrich, Germany; trace purity); $H_2O_2$ (hydrogen peroxide, 30%, Sigma Aldrich, Germany; trace purity).

### Biological material

Parasol mushroom (*Macrolepiota procera* Scop. Singer).

### Instruments

Venticell 111st (BMT, Czech Republic), ABT-120/5 DW (Kern & Sohn, Germany), EthosOne (Milestone, Italy), Agilent ICP-OES 720 (Agilent Technologies, USA).

### Laboratory Methods

ICP-OES **(Árvay et al., 2019)**.

### Description of the Experiment

**Sample preparation:** In laboratory conditions, the mushroom samples were divided into two parts – cap and stem and dried at 40 °C in Venticell 111st (BMT, Czech Republic). Thoroughly dried mushroom samples were homogenized in porcelain mortar (cap and stem separately) and stored in PE bags until analysis.

**Number of samples analyzed:** 50

**Number of repeated analyses:** 1

**Number of experiment replication:** 2

### ICP-OES

Before the analysis of risk elements, the samples of biological material were mineralized. Approximately 0.15 – 0.20 g of homogenized sample was weighted on analytical balances ABT-120/5 DW (Kern & Sohn, Germany) and put into PTFE mineralization tubes. Mineralization was performed by a pressure microwave digestion on EthosOne (Milestone, Italy) using 5 mL concentrated nitric acid (69%) (Sigma Aldrich, Germany; trace purity) and 1 mL of 30% hydrogen peroxide (Sigma Aldrich, Germany; trace purity) with the addition of 1 mL of deionized distilled water ($ddH_2O$). Subsequently, the digestate was filtered through a quantitative filter paper Filtrak 390 (Munktell&Filtrak, GmbH, Bärenstein, Germany) and filled with $ddH_2O$ to 50 mL.

**Table 1** Characteristics of sampling sites.

| Locality | n | Coordinates (VGS 84) | | Altitude (m) |
|---|---|---|---|---|
| Lazy pod Makytou | 12 | 49.244400 | 18.225650 | 542 |
| Lozorno | 8 | 48.335600 | 17.063083 | 220 |
| Nemečky | 6 | 48.683833 | 18.105333 | 298 |
| Tesáre | 15 | 48.604333 | 18.073000 | 251 |
| Zbyňov | 9 | 49.124650 | 18.639466 | 378 |

Note: n – number of samples.

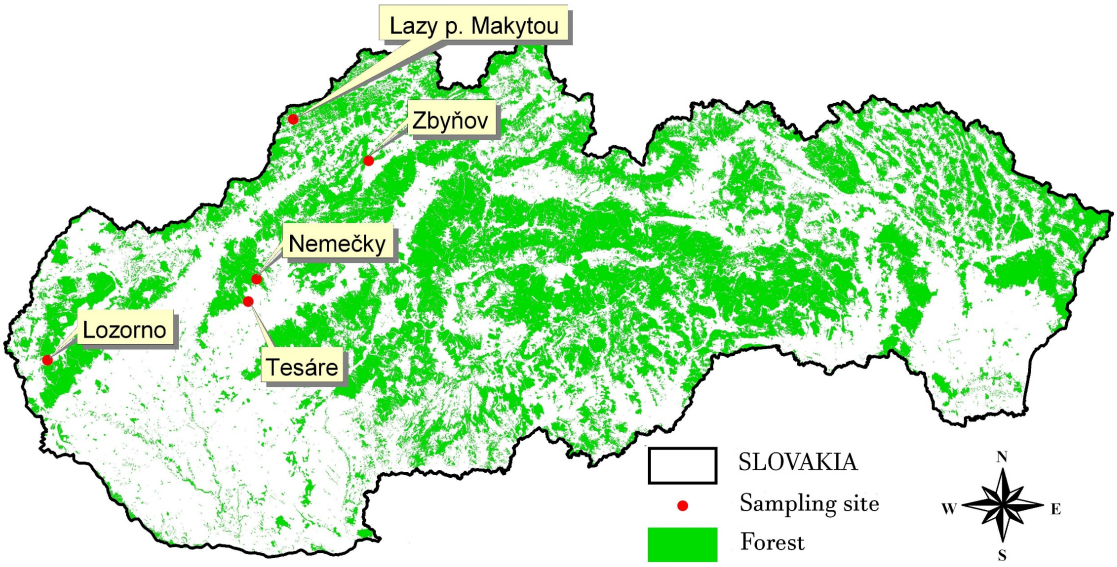


**Figure 1** Map of monitored sites in Slovakia.

In samples thus prepared, the analysis of the content of risk elements was performed by inductively coupled argon plasma emission spectrometry (ICP-OES) on Agilent ICP-OES 720 (Agilent Technologies, USA) **(Árvay et al., 2019)**.

## Health risk assessment

Potential health risks from the consumption of the Parasol mushroom were evaluated based on Provisionally Tolerable Weekly Intake of Al (140 mg person$^{-1}$ week$^{-1}$) **(JECFA, 2012)** per person weighed 70 kg. Because no limit is set up for Ag, this element was not included in the health risk assessment.

Obtained data of concentrations of Ag, Al, and Zn were calculated to fresh matter **(Kalač, 2009)**. Because the caps of *M. procera* are consumed most often, the contents of monitored elements in caps were included in the calculation.

According to the **Statistical Office of the Slovak Republic (2020)**, the consumption of "Other vegetables including mushrooms" is at the level of 0.26 kg person$^{-1}$ week$^{-1}$, which was taken into account in calculation the exceedance of the PTWI value for Al according to the following equation (1):

$$PTWI(\%) = \frac{element\ content\ (FW) \times 0.26}{PTWI_{Al}} \times 100 \qquad (1)$$

where:
PTWI(%) – a percentage of the provisional tolerable weekly intake; element content (FW) – the content of the element (caps) in mg kg$^{-1}$ of fresh weight; 0.26 – average weekly consumption of mushrooms in Slovakia (kg person$^{-1}$ week$^{-1}$); $PTWI_{Al}$ – the value of provisional tolerable weekly intake of Al (140 mg person$^{-1}$ week$^{-1}$).

In the case of Zn, the value of recommended dietary allowance (RDA) – 10 mg person$^{-1}$ day$^{-1}$ **(Act No. 1169/2011)** per person weighted 70 kg was considered. The percentage value of RDA of Zn was calculated using the average consumption of "Other vegetables including mushrooms" according to the **Statistical Office of the Slovak Republic (2020)** (0.04 kg person$^{-1}$ day$^{-1}$) based on the following equation (2):

$$RDA(\%) = \frac{element\ content\ (FW) \times 0.04}{RDA_{Zn}} \times 100 \qquad (2)$$

where:
RDA(%) – a percentage of the recommended dietary allowance; element content (FW) – the content of the element (caps) in mg kg$^{-1}$ of fresh weight; 0.04 – average daily consumption of mushrooms in Slovakia (kg person$^{-1}$ day$^{-1}$); $RDA_{Zn}$ – the value of recommended dietary allowance of Zn (10 mg person$^{-1}$ day$^{-1}$).

## Statistical Analysis

The obtained results on the content of Ag, Al, and Zn were characterized using descriptive statistics. From the data, the minimum, maximum, average value, and standard deviation (SD) were calculated. The processed data for each monitored element in Parasol mushroom (*Macrolepiota procera* Scop. Singer) from individual localities are shown in tables as average ± standard deviation (SD). The dataset was tested for normality. Subsequently, a non-parametric Kruskal-Wallis test was used to determine the statistical differences among monitored localities ($p$ <0.05). To determine the relationship between anatomical parts of Parasol mushroom, Spearman's correlation test was used. All data processing and graphical presentations were performed using **RStudio (2020)** software package.

## RESULTS AND DISCUSSION

The content of three selected elements (Ag, Al, and Zn) in wild edible mushrooms (*Macrolepiota procera* Scop. Singer) was collected from five different sampling sites in Slovakia (Lazy pod Makytou, Lozorno, Nemečky, Tesáre, and Zbyňov) was investigated. All presented results are expressed in mg $kg^{-1}$ dry weight (DW).

### Silver

Parasol mushroom (*Macrolepiota procera* Scop. Singer) has a high potential for accumulation of silver even in the unpolluted areas **(Stefanović et al., 2016; Kułdo et al., 2014; Falandysz and Borovička, 2013)**. The concentration of Ag in samples from monitored areas varied from 0.22 to 6.27 mg $kg^{-1}$ DW in caps and from 0.05 to 5.46 mg $kg^{-1}$ DW in stems of *M. procera* (Table 2). The average concentration of Ag in *M. procera* caps decreased in the order of Tesáre > Lozorno > Nemečky > Lazy pod Makytou > Zbyňov.

The highest average concentration of Ag was detected in locality Tesáre (3.23 ±1.75 and 2.46 ±1.35 mg $kg^{-1}$ DW in caps and stems, respectively). The average concentration of Ag in cap samples from Lazy pod Makytou (0.54 ±0.19 mg $kg^{-1}$ DW) is very similar to results reported by **Vukojević, Đurđić and Mutić (2019)** in samples of *M. procera* originating from the Goč Mountains in Serbia (0.53 ±0.30 mg $kg^{-1}$ DW). In general, our obtained results are comparable with many previous studies **(Mleczek et al., 2020; Falandysz et al., 2017; Kojta et al., 2016; Mleczek et al., 2016; Stefanović et al., 2016; Kułdo et al., 2014; Gucia et al., 2012a; Gucia et al., 2012b; Jarzyńska et al., 2011)**. The content of elements depends on the element content in the soils, but the bioavailability of elements is significantly influenced by other factors such as soil pH, Eh, etc. **(Stefanović et al., 2016)**. Higher concentrations in mushroom samples from locality Tesáre can be related to higher content of Ag in soil substrate and soil properties, which were not studied in this research.

To compare the concentrations of Ag in individual anatomical parts of *M. procera*, the stem samples from Lazy pod Makytou, Lozorno, and Zbyňov contained higher amounts of Ag than caps. These results agree with some previous studies **(Stefanović et al., 2016; Kułdo et al., 2014; Gucia et al., 2012a, Falandysz et al., 2008)**. Although the biological significance of Ag accumulation in *M. procera* is unclear, it can be attributed to a defensive effect against pathogenic microscopic fungi, bacteria, insect larvae, or gastropods. High concentrations of Ag can be found in *M. procera*, even from unpolluted areas **(Stefanović et al., 2016; Falandysz and Borovička, 2013)**.

The statistically significant differences ($p$ <0.05) were observed between locality Tesáre and all other monitored localities. In samples from Tesáre, the highest content of Ag was detected. All statistical differences are shown in Figure 2.

### Aluminum

In some species of wild edible mushrooms a wide range of Al was recorded **(Kalač, 2016)**. According to the values presented in Table 2, the Al concentration in *Macrolepiota procera* ranged from ND (not detected) to 335 mg $kg^{-1}$ DW in caps and from 8.93 to 2441 mg $kg^{-1}$ DW in stems. The average concentration of Al in caps from the monitored sites decreased in the order of Nemečky > Tesáre > Lazy pod Makytou > Zbyňov > Lozorno.

**Table 2** Content of Ag, Al, and Zn (mg $kg^{-1}$ DW) in *Macrolepiota procera* caps and stems from selected localities.

| Element | Ag | | Al | | Zn | |
|---|---|---|---|---|---|---|
| | Average ±SD<br>range | | | | | |
| **Locality** | **Cap** | **Stem** | **Cap** | **Stem** | **Cap** | **Stem** |
| **Lazy pod Makytou** | 0.54 ±0.19<br>0.28 – 0.85 | 1.30 ±0.47<br>0.61 – 2.21 | 46.8 ±50.9<br>ND – 191 | 31.5 ±16.7<br>9.97 – 69.4 | 77.0 ±16.3<br>44.5 – 102 | 51.3 ±8.1<br>38.4 – 65.2 |
| **Lozorno** | 1.58 ±1.58<br>0.35 – 5.42 | 1.96 ±1.40<br>0.55 – 4.76 | 16.6 ±6.9<br>7.88 – 29.3 | 91.4 ±69.4<br>21.2 – 231 | 111 ±31.7<br>62.0 – 170 | 61.0 ±11.7<br>32.7 – 75.2 |
| **Nemečky** | 1.57 ±0.88<br>0.28 – 3.06 | 1.03 ±0.58<br>0.05 – 1.94 | 113 ±103<br>39.9 – 335 | 453 ±491<br>51.1 – 1412 | 78.4 ±33.7<br>33.7 – 139 | 42.2 ±8.2<br>26.3 – 52.9 |
| **Tesáre** | 3.23 ±1.75<br>0.75 – 6.27 | 2.46 ±1.35<br>0.96 – 5.46 | 73.5 ±52.5<br>21.6 – 213 | 428 ±583<br>68.5 – 2441 | 73.4 ±16.7<br>49.4 – 105 | 47.2 ±8.0<br>29.2 – 62.3 |
| **Zbyňov** | 0.41 ±0.16<br>0.22 – 0.67 | 0.76 ±0.40<br>0.31 – 1.63 | 19.7 ±24.9<br>0.56 – 74.7 | 35.7 ±30.3<br>8.9 – 84.2 | 90.9 ±11.8<br>80.2 – 120 | 55.6 ±5.2<br>47.2 – 61.6 |

Note: ND – Not Detected.

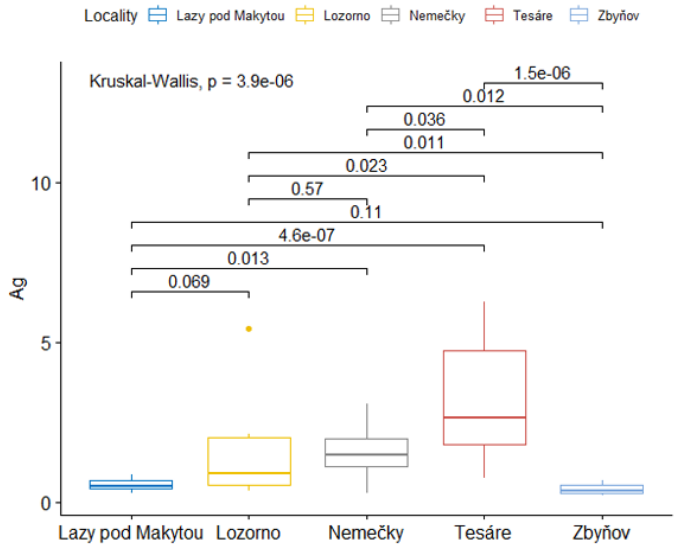


**Figure 2** Statistical differences in the content of Ag (mg kg$^{-1}$ DW) in *M. procera* among monitored localities.

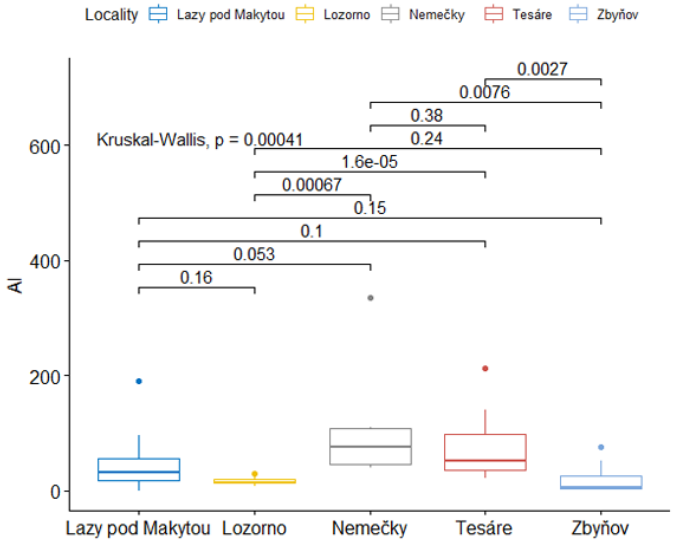


**Figure 3** Statistical differences in the content of Al (mg kg$^{-1}$ DW) in *M. procera* among monitored localities.

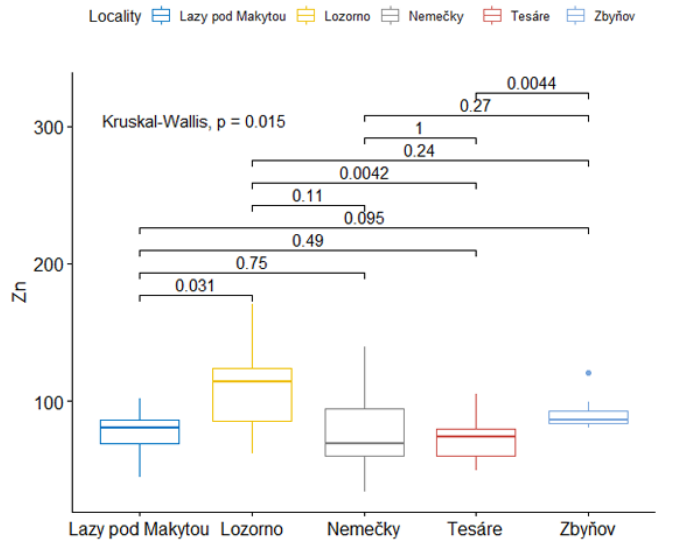


**Figure 4** Statistical differences in the content of Zn (mg kg$^{-1}$ DW) in *M. procera* among monitored localities.

**Table 3** Percentage values of PTWI (Al) and RDA (Zn) after consumption of *M. procera* from monitored sites.

| Locality | $PTWI_{Al}$ | $RDA_{Zn}$ |
|---|---|---|
| Lazy pod Makytou | 0.87 | 3.08 |
| Lozorno | 0.31 | 4.43 |
| Nemečky | 2.10 | 3.13 |
| Tesáre | 1.37 | 2.94 |
| Zbyňov | 0.37 | 3.64 |

Note: $PTWI_{Al}$ – Provisional Tolerable Weekly Intake of Aluminum; $RDA_{Zn}$: Recommended Dietary Allowance of Zinc.

The highest average concentration of Al was detected in locality Nemečky (113 ±103 and 453 ±491 mg $kg^{-1}$ DW in caps and stems, respectively). The average content of Al in *M. procera* caps in locality Tesáre (73.5 ±52.5 mg $kg^{-1}$ DW) is comparable with the results reported by **Kojta et al. (2016)** and **Kułdo et al. (2014)**. The lowest average concentrations of Al were detected in samples from locality Lozorno and Zbyňov (16.6 and 19.7 mg $kg^{-1}$ DW, respectively). A similar average concentration was reported by **Mleczek et al. (2020)** (14.7 mg $kg^{-1}$ DW).

The concentration of Al in wild edible mushrooms from unpolluted areas usually ranges from 20 to 150 mg $kg^{-1}$ DW **(Kalač, 2010)**. Some samples from the monitored sites – Lazy pod Makytou, Nemečky, and Tesáre contained higher concentrations of Al. The highest concentration of Al in the cap was measured in the sample from Nemečky (335.3 mg $kg^{-1}$ DW). However, the average concentrations of Al in *M. procera* caps from the monitored localities were not above the limit of 150 mg $kg^{-1}$ which is reported by **Kalač (2010)**. In samples from 4 sampling sites (Lozorno, Nemečky, Tesáre, and Zbyňov), higher concentrations of Al were detected in stems than in caps of *M. procera*, which is in agreement with several previous studies **(Kojta et al., 2016; Gucia et al., 2012a; Jarzyńska et al., 2011)**.

According to **Kalač (2010)**, increasing the age of mycelium and a long interval between fructifications significantly elevate the contents of many elements in fruiting bodies of wild-growing mushrooms. Moreover, most elements are unequally distributed in the fruit body **(Kalač, 2019)**. As aluminum is a widespread element in soils **(Kabata-Pendias and Mukherjee, 2007)**, relatively high content of Al may be detected in *M. procera*. However, the content of Al in soil substrate was not investigated in this research.

The statistically significant differences ($p$ <0.05) in the content of Al were observed between locality Zbyňov and two other localities (Tesáre and Nemečky) and also between locality Lozorno and localities Tesáre and Nemečky, respectively. All statistical differences are shown in Figure 3.

## Zinc

Zinc, as an essential trace element is a bioavailable element for all organisms and plays an important role in various biological reactions **(Sarikurkcu et al., 2020; Simon, 2014)**. Wild edible mushrooms are usually rich in Zn **(Falandysz and Borovička, 2013)** and their content is normally between 25 and 200 mg $kg^{-1}$ DW **(Kalač, 2016)**. In monitored samples of *M. procera*, the concentration of Zn ranged from 33.7 to 170 mg $kg^{-1}$ DW in caps and from 26.3 to 75.2 mg $kg^{-1}$ DW in stems (Table 2). The average concentration of Zn in *M. procera* caps decreased in the order Lozorno > Zbyňov > Nemečky > Lazy pod Makytou > Tesáre.

According to **Alonso et al. (2003)** zinc tends to accumulate in various anatomical parts of *M. procera*. The highest average concentration of Zn was detected in locality Lozorno (111 ±31.7 and 61.0 ±11.7 mg $kg^{-1}$ DW in caps and stems, respectively). **Mleczek et al. (2020)** (135 mg $kg^{-1}$ DW) and **Giannaccini et al. (2012)** (124.7 ±67.9 mg $kg^{-1}$ DW) reported higher concentrations of Zn in *M. procera* than in our samples. Higher content of Zn was also detected in samples of *M. procera* from Slovak Paradise National Park, where the average value of Zn was 177 ±138 mg $kg^{-1}$ DW **(Árvay et al., 2015a)**. **Sarikurkcu et al. (2020)** reported the content of Zn in *M. procera* at the level of 60 mg $kg^{-1}$ DW. High concentrations of Zn in mushrooms from uncontaminated areas may be due to its essentiality **(Árvay et al., 2014)**.

The highest average concentration of Zn in *M. procera* caps (111 ±31.7 mg $kg^{-1}$ DW) was detected in the locality Lozorno. This phenomenon may be due to the higher content of Zn in soils in this locality **(Linkeš et al., 1997)** compared to other monitored sites, which was not investigated in this research. A similar average concentration of Zn was reported in previous studies **(Mleczek et al., 2020; Kojta et al., 2016; Gucia et al., 2012a; Gucia et al., 2012b; Jarzyńska et al., 2011)**.

The content of Zn is different in individual anatomical parts of the fruiting body. The richest sources of Zn are spore-forming parts of the fruiting body **(Kalač, 2016; Borovička and Řanda, 2007)**. Some authors also reported higher concentrations of Zn in caps than in the stems of *M. procera* **(Kułdo et al., 2014; Gucia et al., 2012a; Falandysz et al., 2008)**, which is confirmed by the obtained results (Table 2).

The statistically significant differences ($p$ <0.05) in the content of Zn were observed between locality Lozorno and localities Lazy pod Makytou and Tesáre and also between locality Zbyňov and two other monitored localities (Tesáre and Nemečky). All statistical differences are shown in Figure 4.

## Correlations

The linear regression and Spearman's correlation test were used to determine the relationships of the content of Ag, Al, and Zn between Parasol mushroom cap and stem (Figure 5). By comparing the contents of the monitored elements in individual anatomical parts of *M. procera*, a strong dependence between the stem and cap was found. The he strongest correlation between cap and stem was observed in the content of Ag (R = 0.69).

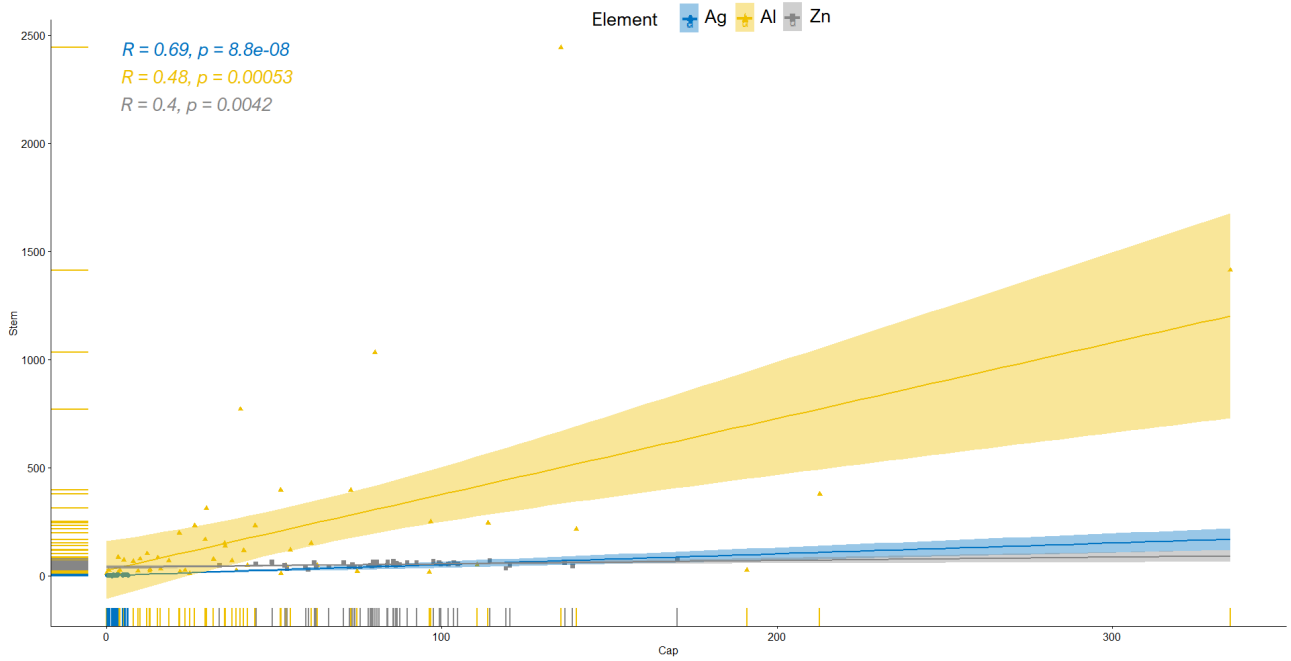


**Figure 5** Relationship between cap and stem of *M. procera* in the content of monitored elements.



**Figure 6** Parasol mushroom (*Macrolepiota procera* Scop. Singer).

### Health risk assessment

The monitored sites are popular localities for collecting mushrooms in Slovakia therefore, the potential health risk caused by regular and long-term consumption of wild edible mushroom *M. procera* was evaluated. Tolerable weekly intake in case of Al and daily intake of Zn from *M. procera* consumption was evaluated for a person of 70 kg weight that consumes 13.3 kg of "other vegetables and mushrooms" per year according to the **Statistical Office of the Slovak Republic (2020)**. As a reference data were used values of Provisional Tolerable Weekly Intake (PTWI) for Al and Recommended Dietary Allowance (RDA) for Zn. The health risk assessment of Ag from *M. procera* consumption was not evaluated, because no PTWI limit is set up for Ag. The contents of monitored elements in fresh weight were used for the calculation. The percentage values of $PTWI_{Al}$ and $RDA_{Zn}$ were calculated (Table 3).

Aluminum is a relatively abundant element in soils **(Kabata-Pendias and Mukherjee, 2007)**. The concentrations of Al in mushrooms are comparable or in some cases even higher than in many plant foods **(Kalač, 2016)**. Aluminum is one of the nutritionally non-essential elements **(Kalač, 2019)**. Its intake is through food. Aluminum is relatively easily excreted from the body, mainly in the urine, but in kidney disorders, it can be accumulated in brain cells and bones **(Liptáková, Prachar and Valík, 2015; Kabata-Pendias and Mukherjee, 2007)**.

The values of $PTWI_{Al}$ from *M. procera* consumption ranged from 0.31 (Lozorno) to 2.10% (Nemečky). Results showed that the content of Al in *M. procera* caps was not above the PTWI limit for Al. It can be stated that the health risk from consumption of *M. procera* in case of Al decrease in order Nemečky > Tesáre > Lazy pod Makytou > Zbyňov > Lozorno. However, the intake of Al from mushroom consumption may be limited due to their usually low consumption **(Kalač, 2016)**.

Zinc is an essential trace element required for the proper growth, development, and function of the human body. Despite the necessity of zinc, its excessive intake is inappropriate. At high concentrations, Zn causes toxic effects such as disorders of metabolism of Cu and Fe **(Liptáková, Prachar and Valík, 2015)**.

In the case of the trace element – Zn, its daily intake from consumption of *M. procera* was in the range of 2.94 (Tesáre) – 4.43% (Lozorno) of recommended dietary intake. The content of Zn in *M. procera* caps was not above the value of recommended daily intake of Zn. Therefore, by consuming 0.04 kg of Parasol mushroom caps from monitored localities, the consumer will not exceed the specified RDA value of Zn.

## CONCLUSION

This study provides information on the content of three elements: silver, aluminum, and zinc in wild edible Parasol mushroom (*Macrolepiota procera* Scop. Singer) collected from five different sites in Slovakia (Lazy pod Makytou, Lozorno, Nemečky, Tesáre, and Zbyňov). Caps of *M. procera* are the richest in Ag and Zn content. *M. procera* stems contained the most aluminum compared to caps. Spearman's correlation test confirmed a strong relationship in element content between cap and stem of *M. procera.* Investigated localities are popular sites for collecting mushrooms in Slovakia therefore, the health risk for mushroom consumers was evaluated. The concentrations of Al and Zn in *M. procera* caps were not above the determined limit of $PTWI_{Al}$ and $RDA_{Zn}$, respectively. Since mushrooms are not an essential component of the human diet and they are a seasonal delicacy, the intake of Ag, Al, and Zn from the consumption of *M. procera* caps may be limited. This study showed that regular and long-term consumption of *M. procera* from monitored sites does not represent any health risk to the consumers.

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# Adoption of sustainable agriculture practices by citrus farmers and its determinants in the jordan valley: the case of northern ghor

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## ABSTRACT
The agricultural sector in the Jordan Valley plays an important role in the economic system and that the region is essentially Jordan's food basket. The adoption of sustainable agricultural practices is widely recognized as essential to ensure agricultural sustainability. This study analyzed factors influencing citrus farmer's adoption of sustainable agricultural practices (SAPs) in the Northern Ghor of Jordan valley. The study used a quantitative approach. Simple random sampling was adopted to select 115 farmers in the study area. A multiple regression analysis was applied to identify factors influencing the sustainability of citrus farming practices. The study found that the largest proportion 44.4% of the citrus farmers had a fairly high adoption rate of SAPs while 13.0% of ones had high adoption of SAPs. In addition, the study revealed that age of farmer, Farm experience, primary education, and tertiary education were factors that affected farmers SAPs adoption. Policy recommendations are proposed to enhance the implementation of sustainable agriculture practices by the citrus farmers in the Northern Ghor of Jordan valley.

**Keywords:** adoption; sustainable agricultural practice; citrus farmer; Jordan valley

## INTRODUCTION
The effects of the "Green Revolution" technology on the environment and the issue of sustainability of agricultural growth received attention only recently **(Singh, 2000; Redclift, 1989; Bowonder, 1979)**. **Singh (2000)** considered the widespread adoption of green revolution technologies to be the main cause of significant soil degradation in the Haryana state of India. **Redclift (1989)** links the problem of environmental degradation to agricultural modernization processes in rural areas of Latin America. **Bowonder (1979)** in his analysis of the impact of the green revolution in India concluded that increased use of high-yielding variety technology and chemical fertilizers have some indirect effects, including faster depletion of micronutrients.

Sustainable agriculture has been identified as the best approach that not only makes to better utilization of natural goods and services for human needs without harming the environment but also reduces the utilize of external inputs; where sustainable agriculture minimize the cost of purchasing inputs by employing farming techniques such as natural biological cycles and farmers skills and knowledge **(Shiri et al., 2012; Lubell, Hillis, and Hoffman, 2011)**. It also allows for small farms to keep operating through variegation and increased gains and profits from alternative methods of marketing, like value-added products, niche markets, or direct marketing strategies **(Kheiri, 2015)**.

Sustainable agriculture contains a dynamic group of sustainable agricultural practices (SAPs). SAPs that are considered suitable for a specific area might be unsuitable to other areas due to the difference in circumstances **(Tey et al., 2012)**. In other meaning, sustainable agriculture cannot be reduced to one defined group of practices **(Pretty and Hine, 2000)**.

In the Jordan Valley, there is a small group of wealthy agriculture who own large landholdings, but also there is a large group of farmers who live close to the poverty line of JD 32.6 per person per month **(Kool, 2016)**. Today, agriculture still dominates the socio-economic reality of the Jordan Valley. Fruit trees, field crops, and vegetables are the major crops in the Jordan Valley **(Philippe, 2003)**. However, the weakness of the farmers' knowledge, experience, and capabilities were the main factors affecting the cultivation management practices in the Jordan Valley and farmers resort to using many several unsustainable cultivation techniques and bad cultivation habits. Such weakness has been reflected in several environmental problems related to fertilizer applications and irrigations such as salinity buildup, deterioration of groundwater through leaching of overflow fertilizers, and loss of water



**Figure 1** The Northern Ghor of Jordan valley.

through inappropriate network designing and operations **(Al-Qinna and Salahat, 2017)**.

Also, the weak linkage between production and consumption quantities leads to lower and fluctuating product prices. Hence, meliorating production and consumption practices toward sustainable agriculture has been considered as a new strategy to solve and address above mentioned problems in the Jordan Valley of Jordan especially in Northern Ghor of Jordan Valley.

One of the major goals for the Jordanian environmental sustainable development is to increase the awareness and realization of farmers on the issues of water savings and environmental and resource protection on the farm **(MoEnv, 2009)**. Although the Jordan government has been having efforts in promoting sustainable agriculture practices **(UNDP, 2010)**, related research on Jordan Valley on the extent to which farmers have applied SAPs and the factors that influence farmers' adoption of SAPs has been scarce.

The paper, therefore, makes a practical contribution to address the gap in the literature and helps policymakers to put better Strategies and extension programs for the Jordan Valley. The current study was carried as a case study on citrus farmers due to citrus is the main crop in Northern Ghor of Jordan Valley. The objectives of the study were to measure citrus farmers' SAPs and determine the factors influencing citrus farmers' adoption of SAPs in Northern Ghor of Jordan Valley.

## Scientific Hypothesis

This study had two hypotheses:

1. The citrus farming practices are sustainable in the Northern Ghor of Jordan valley.
2. The socio-economic factors such as farmer and farm characteristics, exogenous factors influence the sustainability of citrus farming practices.

## MATERIAL AND METHODOLOGY

### Study area

The Northern Ghor represents the area that lies below the Yarmouk River through the village of North Shounahand Addasiya to the village of Kreymeh, it is administratively affiliated to Irbid Governorate in the name of AL Aghwar AL Shamalyyah (Figure 1). It is associated with nonsaline soils of deep, medium to fine-textured Camborthids and Calciorthids of a moderate slope. The existing water resources of Northern Ghor are from the Yarmouk River, Wadi Arab dam, Wadi Jurum, Ziglab dam, and Wadi Yabis **(Al-Qinna and Salahat, 2017)**.

### Data Collection

The study employed the quantitative approach. This approach was used because of its advantage in providing a numerical description of the studied phenomenon. In addition to being useful in measuring relationships and their strength between two variables **(Apuke, 2017)**. Both primary and secondary data were gathered, the most important secondary data is the data of the Directorate of Agriculture of the Northern Jordan Valley that was used to identify the location, location, and phone numbers of citrus farmers in the study area. In addition to data from journals, documents, proceedings. Primary data were gained using a structured questionnaire. 115 respondents were selected in the research area from February to July 2020. The selection of sample farmers was based on the readiness of each farmer to participate in the interview. This was achieved with the close assistance of some staff from the Directorate of Agriculture of the Northern Jordan Valley who participated the researchers in some farms visits. In conducting the study, standard ethical considerations were observed, which guarantee obtaining consent; where farmers gave oral consent to participate in the interview after they heard a summarized explanation of the study's objective. In addition to ensuring confidentiality.

### Statistical analysis

Definitions and summary statistics of the variables used in the analysis are presented in Table 1. Twenty-three SAPs, which were applied in the study of **Tathdil et al (2008); Thanh and Yapwattanaphun (2015); Mishra et al (2018)** are considered in this study. The adoption of a sustainable agricultural practice received a score of 1 while non-adoption received a score of 0. Besides, the study considered farmer and farm characteristics; these include variables sex of farmer, age of farmer, marital status of farmer, education level of the farmer, farm experience, labor availability, employment, farm income, farm production, farm size, and land tenure. The exogenous factors include variables access to extension services, access to loans, farmer group membership, distance to input market, and Source of information. Based on the collective adoption score, the farmers were categorized into four groups according to SAPs adopting (low, fairly low, fairly high, and high), these groups were defined by mean and standard deviation as follows: A = Low: $A \leq Mean - SD$; B = Fairly low: $Mean - SD < B \leq Mean$; C = Fairly high: $Mean < C \leq Mean + SD$; D = High: $Mean + SD < D \leq Max$.

**Table 1** Definition of variables and descriptive statistics.

| Variable name | Variables: definition and codes | Mean | Std. Dev. |
|---|---|---|---|
| **Dependent variables** | | | |
| Adoption of SAPs | SAPs adoption (=1 if farmer adopted SAPs; 0 otherwise) (min. = 6, max. = 14) | 10.6783 | 1.71959 |
| **Independent variables** | | | |
| | **Farmer and farm characteristics** | | |
| Sex of farmer | Sex of farmer (=1 if male; 0 otherwise) | 1.00 | .000 |
| Age of farmer | Age of farmer (years) | 56.44 | 13.860 |
| Marital status of the farmer | Marital status of the farmer (=1 if single; 0 otherwise) | .04 | .205 |
| Education level of farmer | | | |
| No education | No education (=1 if attended; 0 otherwise) | .00 | .000 |
| primary education | Primary education (=1 if attended; 0 otherwise) | .14 | .348 |
| Basic education | Basic education (=1 if attended; 0 otherwise) | .37 | .486 |
| Secondary education | Secondary education (=1 if attended; 0 otherwise) | .30 | .462 |
| Tertiary education | Tertiary education (=1 if attended; 0 otherwise) | .18 | .388 |
| Farm experience | Farm experience (years) | 26.48 | 9.889 |
| Labor availability | Labor availability (=1 farmer used hired labor; 0 otherwise) | .90 | .295 |
| Employment | Employment (=1 if farmer work more than 200 days on the farm; 0 otherwise) | .86 | .348 |
| Farm income | Farm income (=1 if high income ; 0 otherwise) | .37 | .486 |
| Farm production | Farm production (ton/dunum) | 2.7783 | .42505 |
| Farm size | Farm size(dunum) | 28.26 | 10.337 |
| Land tenure | Land tenure status (=1 if owned; 0 otherwise) | .54 | .501 |
| **Exogenous factors** | | | |
| Access to extension services | Access to extension services (=1 if yes; 0 otherwise) | .79 | .408 |
| Access to loan | Access to loans (=1 if yes; 0 otherwise) | .10 | .307 |
| Farmer group membership | Belong to farmer group (yes=1, 0 otherwise) | .03 | .184 |
| Distance to input market | Distance to input market (km) | 7.13 | 3.631 |
| Source of information | | | |
| Reading newspaper | Reading newspaper (=1 if several times a month; 0 otherwise) | .00 | .000 |
| Listening to the radio | Listening to the radio (=1 if at least 5 h a week; 0 otherwise) | .02 | .131 |
| Watching TV | Watching TV (=1 if at least 5 h a week; 0 otherwise) | .01 | .093 |
| Participation in farming events | Participation in farming events (=1 if yes; 0 otherwise) | .12 | .328 |

After data gathering and editing, the analysis was performed by SPSS 23 software. Descriptive statistics such as frequencies, percentages, mean and standard deviations, and multiple regression were used to analyze the data.

## RESULTS AND DISCUSSION

### Characteristics of the citrus farmers

Socio-economic characteristics information of the citrus farmers which includes gender, age, marital status, educational qualifications, land tenure, farm experience, and employment status of farmers were collected. This information was pertinent because it helped the authors to obtain some insights into the background of the farmers.

The results showed that all the farmers who participated in the study were male; their ages ranged from 23 to 83 and the mean age was 56.4 years. The majority of farmers were married with a percentage of 95.7%, while the percentage of singles was 4.3%. As far as the educational qualification is concerned, no illiterate farmers were found, and 37.4 % of farmers belonged to basic education; 13.9 % of farmers to primary education; 30.4% to secondary education, and 18.3% to tertiary education. As far as farmer's experience in farming activities is concerned, farm experience ranged

from 5 to 45 and the mean was 26.4 years. The results also showed information on farmer's employment, where 86.1% of farmers work more than 200 days on the farm.

**The measurement of citrus SAPs**

The Most commonly adopted SAPs among citrus farmers are presented in Figure 2.

Table 2 displays the classification of citrus farmers according to the levels of adoption of SPAs. As shown that only 13.0% of the farmers ranked in the high adoption group of SAPs, while most farmers placed in fairly high group 44.4%, fairly low group 30.4%, and the low group only got 12.2% in terms of the adoption of SAPs.

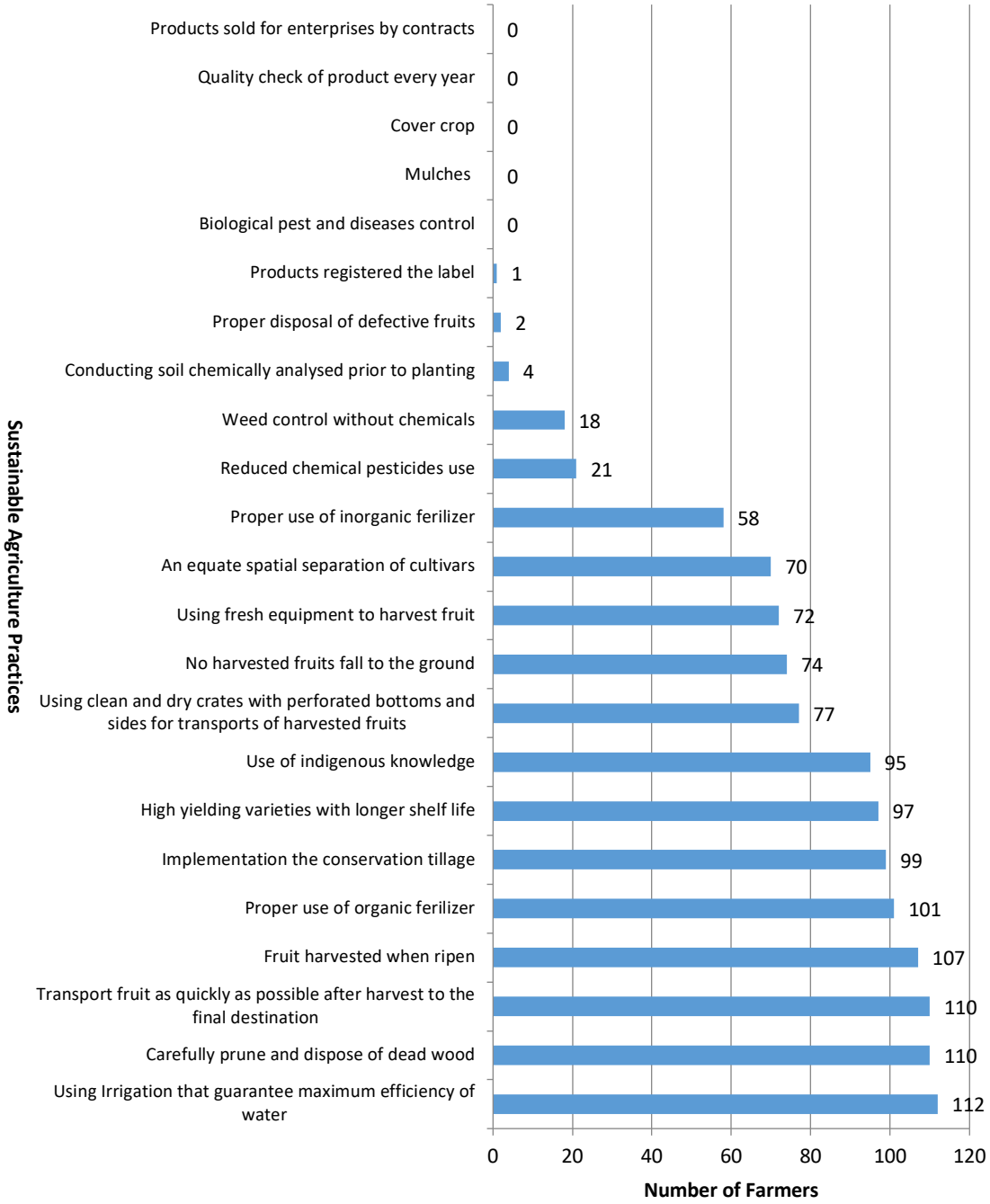


**Figure 2** The Most commonly adopted SAPs among citrus farmers.

**Table 2** Classification of citrus farmers based on SAPs.

| Groups | Frequency | Percent | Cumulative percent |
|---|---|---|---|
| Low | 14 | 12.2 | 12.2 |
| Fairly low | 35 | 30.4 | 42.6 |
| Fairly high | 51 | 44.4 | 87 |
| High | 15 | 13.0 | 100 |
| Total | 115 | 100 | |

**Table 3** Results of multiple regression analysis.

| Variables | B | Beta | T-Statistics | *p*-value |
|---|---|---|---|---|
| (Constant) | 12.266 | - | 7.311 | .000 |
| Age of farmer | 0.041 | .332 | 2.021 | .046** |
| Marital status of the farmer | 0.387 | .046 | .421 | .675 |
| primary education | -1.315- | -.266- | -2.387- | .019** |
| Secondary education | -.613- | -.165- | -1.430- | .156 |
| Tertiary education | -1.399- | -.316- | -2.338- | .021** |
| Farm experience | -.063- | -.363- | -2.151- | .034** |
| Labor availability | -1.062- | -.182- | -1.623- | .108 |
| Employment | -.842- | -.170- | -1.602- | .112 |
| Farm income | 0.246 | .069 | .653 | .516 |
| Farm production | -.065- | -.016- | -.144- | .885 |
| Farm size | 0.008 | .051 | .485 | .629 |
| Land tenure | 0.202 | .059 | .579 | .564 |
| Access to extension services | 0.184 | .044 | .455 | .650 |
| Access to loan | -.448- | -.080- | -.777- | .439 |
| farmer group membership | -1.084- | -.116- | -1.019- | .311 |
| Distance to input market | -.040- | -.085- | -.871- | .386 |
| Listening to the radio | 0.486 | .037 | .373 | .710 |
| Watching TV | -2.908- | -.158- | -1.682- | .596 |
| Participation in farming events | 0.248 | .047 | .502 | .617 |

Note: ***significance at 1% level; **significance at 5% level; * significance at 10% level. N= 115; F= 1,514; $R^2$= .232

Figure 2 shows the importance given by the citrus farmers to each of the selected SAPs. The result displayed the practices having a high rate of adoption were linked to pre-harvest and post-harvest phases, such as using irrigation that guarantees maximum efficiency of water, carefully prune and dispose of deadwood, fruit harvest when ripen, and transport fruits as quickly as possible after harvest to the final destination. Even the practices applied at a low rate or not applied were also related to pre-harvest and post-harvest phases, such as chemical analysis of the soil, mulches, and cover crop for weed and pest control, and product label establishment.

In terms of irrigation network design and installation appropriateness and the investigated irrigation efficiency, most of the tree farms are appropriate, where the farmers irrigate their trees using an open pipe network (GR 16mm) to fill mature single tree basin, and dripper Pipes (GR 16mm) to fill newly Planted trees basin. Farmers are forced to improve irrigation efficiency; since water, availability is limited and is controlled by the water user association that determines the schedule of each farm. Therefore, most farmers created lagoons (circular on-farm storage) with varying sizes. Moreover, most farmers watering trees during evening periods to reduce evaporation from the tree basins or evaporation from the lagoon itself. Besides, most respondents used high-yielding varieties with longer shelf life, are cultivated in nurseries in Northern Ghor. Also, farmers use indigenous knowledge in agricultural practices. Results showed that most farmers (93%) harvesting mature fruits. Immature fruits have low quality because of high water loss and mechanical damage. The maturity of fruits is an excellent condition for consumption (Rahman, 2007). In terms of the uses of inputs, about 87.8% of citrus farmers used organic fertilizer. Fertilizers particularly organic fertilizers are used to recover soil fertility and prevent land degradation when the nutrient supply of soil is insufficient **(Thanh and Yapwattanaphun, 2015)**. Citrus farmers in the study area prefer to use organic fertilizers because they are lower in price compared to inorganic fertilizers since the region is famous for raising livestock.

Also, about 86.1% of citrus farmers implement the conservation tillage especially reduced tillage system; where the number of tillage processes is reduced by either elimination of one or more tillage processes from the conventional tillage program **(Khursheed et al., 2019)**. The narrow distance between the trees is one of the major motivations for the implementation the conservation tillage.

Biological control is an effective and environmentally friendly way to dominant insect pests and diseases through the work of natural control agents **(Sanda and Sunusi, 2016)**. However, no one of the farmers in the study area used such a technique. On the contrary, we find that around 81.7% of farmers depend on using chemical pesticides. As is known, the use of pesticides leaves amounts of residue on fruits and vegetables **(Roy, Chan and Rainis, 2014; Nakano et al., 2016)**.

Regarding the mulches for weed control, no citrus farmers applied this technique. Mulches are fundamental to alleviate soil erosion and improve the water-holding capacity,

maintain moisture in the soil facilitating infiltration, and reduce weed growth **(Patil et al., 2013)**. Besides, no citrus farmers applied the cover crop technique for pest control. Farmers have often used these crops to conserve the soil from erosion and prevent loss of nutrients in deep layers out of leaching and surface runoff, which means they reduce the need for chemical inputs such as fertilizers herbicides, and insecticides **(Sharma et al., 2018; Shakiru et al., 2018)**.

To expand the consumption market and improve product prices, it is necessary to quality check products every year, where this technique plays a significant role in improving consumer's perception and belief about products and improving product prices at the same time **(Baldwin et al., 2014)**. None the less, no farmers applied this technique. Even register product labels that have the same role did not obtain the attention of farmers.

Finally, the popular trend in agricultural production is presently collaboration between farmers and enterprises in product consumption through contracts that help farmers to achieve more gains and profits through value-adding and decrease price fluctuation in the market (**Thanh and Yapwattanaphun, 2015)**. However, the result displayed that no contracts were carried out in selling products between farmers and enterprises. To prevent contamination and spoilage of the produce, fresh equipment and materials should be used to harvest the fruits **(Cantwell and Suslow, 2002; Rahman, 2007)**. Nevertheless, the rate of farmers adopted this technique was not high.

**Factors influencing citrus farmers SAPsadoption**

To determine the factors influencing citrus farmer's SAPs adoption, a regression analysis (the Multiple Regression Model) with entre method was conducted. The regression model integrated all of the explanatory variables which had significant correlations with the SAPs adoption.

The citrus farmer's adoption towards selected SAPs index, which was specified according to their scores gained from the statements associated with 23 selected practices was considered as a dependent variable.

There were 23 explanatory variables, described in the research methodology section entered in the model, of which only 4 variables had a statistically significant influence at the 5% level on citrus farmer's SAPs adoption. As revealed in Table 3, the partial regression coefficient of the age of the farmer was found to have a positive influence, while farm experience, primary education, and tertiary education were found to have a negative influence on citrus farmer's SAPs adoption. The R2 value for the model equals 0.232 indicating that 23.2% of the variation in the farmer's adoption could be explained by these 4 variables.

Although there is agreement about the influence of age on the adoption and use of sustainable practices, there are disagreements as to which age group is most likely to adopt new agricultural practices. Empirical evidence from studies by **(Bese, Zwane, and Cheteni, 2020; Cheteni, Mushunje, and Taruving, 2014)** shows that the elderly were more open to adopting new agricultural technologies and practices than the youth. On the contrary, **(Filho, 2018; Kassie et al. 2012)** noted that the elderly were hesitant to change.

Surprisingly, experience has a negative influence on the adoption of SAPs. Where β of experience was -.363. Results indicate that farmers who have long experience are generally less likely to adopt SAPs. It makes sense that the long experience would increase adoption of SAPs since it should enable farmers to improve their stock of farming knowledge, through leveraging on years of "learning by doing"; where more experienced farmers are possessed better knowledge of farming practices that could help improve yield and income earnings. However, though contrary to the findings of other studies, such as the study of **Adeola (2010)**, the result in this study may be significant and bear other explanations. The plausible explanation is that farmers who spent a long time in agricultural work are accustomed to traditional agricultural practices. This finding corroborates with the results of a study by **Oladimeji et al. (2020)**.

Besides, primary education and tertiary education were other important factors negatively influencing farmers' SAPs adoption with $\beta = -0.266$ and $\beta = -0.316$, respectively. For every standard deviation change in primary education and tertiary education farmers, SAPs adoption decreases by 0.266 and 0.316 standard deviation, respectively. In other meaning, farmers who had attended primary education and tertiary education were less likely to SAPs adoption and the variable was statically significant at 5%, p= 0.019, and $p = 0.021$ respectively.

Concerning the educational attainment variable as clear discrimination in the adoption of SAPs. It is found that the present study results conflict with studies of **(D'Souza and Gebremedhin 2019; Thanha and Yapwattanaphuna, 2015; Ngombe et al., 2014; Teklewold, Kassie, Shiferaw, 2013; Omoare and Oyediran, 2020; Purba et al., 2021)** which suggested that a farmer with higher education is more likely to adopt SAPs. According to these studies, with increased knowledge, a farmer becomes less risk-averse when evaluating SAPs, and more willing to accept innovation that requires a change in the farm operation. It is also in conflict with a study by **Clay et al. (1998)** and **D'Emden et al. (2006)** which did not find an influence of education levels on the adoption of SAPs.

## CONCLUSION

The study pointed out that the largest proportion 44.4% of the citrus farmers was of fairly high adoption group of SAPs while 13.0% of ones belonged to the high adoption group of SAPs. In addition, the result of the regression analysis showed that 4 variables affecting farmers' SAPs adoption. These variables were the age of the farmer, farm experience, primary education, and tertiary education.

The study recommends that efforts to consolidate citrus farmers' SAPs adoption in Northern Ghor should be focus on environmental and economic benefits and give special attention to older farmers to exploit their skills and receptive to implementing SAPs. In addition, the government can encourage and guide farmers in implementing sustainable agriculture techniques and suitable inputs by providing premium and incentive payments to them. Moreover, the government should take deterrent penalties against farmers who using inappropriate and harmful applications such as using excessive chemicals pesticides and fertilizers, or who do not use appropriate applications such as not properly ravage of defective fruit.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This article does not contain any studies that would require an ethical statement.

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# DEVELOPMENT OF NUTRITIONAL MEALS AND GRUELS FROM BLENDS OF PRO-VITAMIN A CASSAVA GRITS AND AFRICAN YAM

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## ABSTRACT

The potentials of underutilized African yam bean (AYB) and pro-vitamin A cassava in the development of nutritious food products with acceptable sensory properties were studied. Grits were produced from freshly harvested yellow root pro-vitamin A cassava by peeling, washing, cutting, soaking, dewatering, roasting, sieving, and milling to obtain yellow root cassava grits while AYB flour was obtained by cleaning, roasting, dehulling, milling, and sieving (425 µm). A simple lattice design was used to obtain formulations of blends (100:0; 90:10; 80:20; 70:30; 60:40 and 0:100) of yellow root cassava grits and AYB flour. Gruels were prepared from these formulations using 4:5 w/v in boiling water while meals were prepared using 1:1 w/v of blend in boiling water for 5 min. Moisture, fat, ash, protein, crude fibre, carbohydrate, β-carotene and calorific content of the blends were in the ranges of 4.66 – 7.92%, 2.20 – 2.82%, 2.16 – 2.66%, 2.72 – 20.43%, 1.15 – 1.40%, 68.65 – 83.23%, 1.33 to 3.97 µg/g and 348.37 – 358.96 kcal/100 g, respectively. Saponin, tannin, trypsin inhibitor, hemagglutinin, starchyose, raffinose, phytate and Hydrogen Cyanide ranged from 0.039 – 0.087%, 0.11 – 0.15%, 1.24 – 3.15 mg/g, 1.47 – 3.49 mg/100 g, 1.51 – 1.81%, 0.38 – 0.45%, 0.82 – 2.69 mg/g, 0.07 – 4.47 mg/kg, respectively. The sensory evaluation revealed that the meal and the gruel samples had acceptable sensory attributes. The developed products have the potentials in alleviating the problem of protein malnutrition in developing countries.

**Keywords:** Cassava; grits; legume; malnutrition; Gruel

## INTRODUCTION

Cassava (*Manihot esculenta Crantz*), commonly referred to as manioc, tapioca, or yuca, is a tropical food crop primarily grown for its starchy tuberous roots and consumed as staples for more than 800 million people **(Ikegwu et al., 2009; Vincenza et al., 2016)**. It is mostly consumed in some parts of Africa, Asia and America **(Burns et al., 2010)**. Cassava thrives in low-nutrient and drought-prone environments making it a food security crop especially in developing countries **(Nyerhovwo, 2004; Vinduranga, 2018; Shackelford et al., 2018)**. Common cassava food products include flour, confectionaries, starch, and gari **(Osunde and Fadeyibi, 2011; Aniedu, Aniedu and Nwakor, 2012)**.

African Yam Bean (*Sphenostylis stenocarpa*) is a legume, hard to cook, and underutilized **(George, Obilana and Oyeyinka, 2020)** with a lot of nutritional advantages **(Adegboyega et al., 2020)**. It is mostly cultivated for its tubers in the Central African Republic, Zaire, East Africa, and Ethiopia while the seeds are mostly cultivated in Southeastern Nigeria **(Ndidi et al., 2014)**. A large variation and different accessions of African yam bean have been reported recently **(Aremu and Ibirinde, 2012; Abioye, Olanipekun and Omotosho, 2015; Ajibola and Olapade, 2016; Baiyeri, Uguru and Ogbonna, 2018; Aina et al., 2020)**. The AYB seed is rich in protein and previous reports indicated values ranging between 19 and 30 percent **(George, Obilana and Oyeyinka, 2020)** and the amino acid profile is similar or even better than that of soybeans in terms of lysine and methionine **(Obiakor, 2008; Atinuke, 2015)**. AYB seeds contain high dietary fiber, carbohydrate, and essential minerals such as phosphorus, calcium, iron, zinc, magnesium, potassium **(Abioye, Olanipekun and Omotosho, 2015; Ajibola and Olapade, 2016; Ojuederie and Balogun, 2017; Anya and Ozung, 2019)**. However, it is underutilized due to the characteristic hardness of its seed coat which increases the processing cost and cooking time **(Ene-obong and Okoye, 2007; Iwuchukwu et al., 2017)**, anti-nutritional factors in high concentration **(Oboh et al., 1998; Ajibade et al., 2006; Fasoyiro et al., 2006; Adegboyega et al., 2020)**, beany flavor and the tendency to cause flatulence in humans **(Machuka and Okeola, 2000; Ngwu, Aburime and Ani, 2014)**.

Yellow root Cassava bio-fortification has been developed primarily to help in reducing the prevalence of vitamin A deficiency where cassava is consumed as a staple food. It can be transformed into retinol within a human body, and this bioconversion can contribute to the reduction of vitamin

A deficiency diseases (VAD) among the vulnerable groups. Vitamin A deficiency (VAD) is common in impoverished tropical nations, and it is especially prevalent in Sub-Saharan Africa. **(Maziya-Dixon et al., 2004; Getu et al., 2017)**. Dependence on cassava diets has also been implicated in protein deficit challenges. Consumers who depend solely on cassava and cassava products for their protein sources with few or no high-protein food sources as supplements are at risk of protein malnutrition. More than a few studies have been published in fortification of cassava flour and cassava food products with protein sources such as cowpea **(Oyarekua, 2009; Agbon, Ngozi and Onabanjo, 2010; Pedhuru, Tuarira and Mutetwa, 2017; Maziya-dixon et al., 2017)**, cassava leaf **(Pedhuru, Tuarira and Mutetwa, 2017)**, soybean **(Rokeya et al., 2011; Ajani et al., 2016; Maziya-Dixon et al., 2017)**, groundnut flour **(Ajani et al., 2016)**, African yam bean **(Ogbonnaya, Onumadu and Nwogu, 2018; Ajibola and Olapade, 2019)**.

Hence, there is scanty information on the development of nutritious food products from yellow root cassava grits and AYB flour blends. Therefore, this study aimed at developing nutritious food products with acceptable sensory attributes from yellow root cassava grits and African yam bean seeds flour which could increase the potentials of these crops and also improve the protein intake of the populace where cassava is consumed as a major staple food.

**Scientific Hypothesis**

This project was carried out to determine the effects of processing and combination of African yam bean and yellow root cassava on the chemical and antinutritional properties of the blends. It also determined the effects of the combination on the sensory properties of the food products.

## MATERIAL AND METHODOLOGY

**Samples**

Yellow root cassava tuber (UMUCASS 37 variety) used for this research was procured from the Teaching and Research Farm of Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria. Nigeria. The AYB seeds were bought at an indigenous market and identified at the LAUTECH teaching and research farm.

Cassava grits were produced from yellow root cassava using the method of **Sanni and Jaji (2003)** Grits were produced from freshly harvested yellow root pro-vitamin A cassava by peeling, washing, cutting, soaking (72 h, 28 ±2 °C), dewatering, roasting (120 °C), sieving and milling to obtain yellow root cassava grits while AYB flour was obtained as described by Aniedu and Aniedu (2014) by cleaning, roasting (190 °C, 10 min), dehulling, milling and sieving (425 μm). Simplex lattice design was used to obtain different formulations from blends of yellow root cassava grit and AYB flour. The formulations were done with (60 – 100%) cassava grit and (0 – 40%) AYB flour, while 100% AYB flour and 100% cassava grits were used as controls. The blends were thoroughly mixed for about 20 minutes using a Kenwood mixer (Model: Chef XL KVL4100S, made in China) to achieve uniform blending.

Gruel samples were prepared from different blends of yellow root cassava grit fortified with AYB flour as described by **Ngwu, Aburime and, Ani (2014)**. Four hundred grams (400 g) of each blend was reconstituted with 500 milliliters of water, and 2 liters of water was used to bring it to boil reconstituted flour was progressively added to the hot water while continually whisking to avoid lumps. The mix was left to simmer for around five (5) minutes while being constantly stirred until it was done.

The meal was prepared from different blends of yellow root cassava grit and AYB flour as described by **Aniedu and Aniedu (2014)**. The blends were reconstituted into meals by cooking 100 grams of each of the blends in hundred (100) mL of boiling water while stirring vigorously for 5 minutes.

**Laboratory Methods**

The proximate composition of the blends was determined as described by the Association of Official Analytical Chemists **(AOAC, 2010)**. Carbohydrate content was determined by difference **AOAC (2010)**. Calorific content was calculated using the values obtained for carbohydrate, fat, and protein values. The β-carotene of the flour mixes was estimated using **Rodriguez-Amaya and Kimura (2004)** method that uses acetone extraction for carotenoid analysis. Tannin was determined with the method based on the fact that tannin-like substances in an alkaline solution diminish phosphotungstomolybdic acid, resulting in a strongly colored blue solution as devised by Adegunwa, **Alamu and Omitogun (2011)**, Spectrophotometer was used to measure the absorbance at 760 (AA Analyse Perkin Nerma)/ Phytate was determined according to AOAC (2010). **Kakade *et al.* (1974)** technique was employed in the determination of trypsin inhibitors. Absorbance reading was taken at 410 nm with a spectrophotometer (AA Analyse Perkin Nerma). The hydrogen cyanide concentration was determined using the method of Alkaline picrate calorimetric while a spectrophotometer was then used to measure the absorbance of elutes from the standard and the sample at 510 nm. **(Mburu, Swaleh and Njue, 2013)**. Raffinose and Stachyose (oligosaccharides) were both determined with HPLC techniques **(František *et al.*, 1995)**. The mobile phase used was demineralized water in a reverse phase HPLC (RP-HPLC) and on a Silica C18 column.

**Sensory evaluation of the food products**

Sensory attributes of the cooked gruel and meal samples were carried out using a 9-point hedonic scale preference test. A total number of 50 panelists were used and to effectively compare the sensory ratings between the panelists, the panelists were semi-trained. Panelists were given coded samples of meal and gruel and asked to assess the items on a 9-point hedonic scale. (where one represents dislike significantly, and (nine) represents like significantly) for sensory attributes such as flavor, color, consistency, mouthfeel, texture, appearance, taste, and overall acceptability. On the panel were staff and students from the Ladoke Akintola University of Technology's Department of Food Science and Engineering in Ogbomoso, Oyo State, Nigeria.

**Description of the Experiment**

**Number of samples analyzed:** 1
**Number of repeated analyses:** 1
**Number of experiment replication:** 1

**Statistical analysis**

Data obtained were analyzed using Analysis of variance (ANOVA) while the means were separated using Duncan's multiple range test (DMRT), a significance level of 5% was considered.

## RESULTS AND DISCUSSION

### Chemical composition

The proximate components of yellow root cassava grits and AYB seed flour blends are indicated in Table 1. The values obtained for the moisture varied between 4.66 to 7.92 percent and a significant ($p$ <0.05) difference was observed in each of the blends except in the blends that had 10-30% of AYB flour substitution. The roasted AYB flour had the least moisture content which was close to 4.88% reported by **Ndidi *et al.* (2014)** for roasted AYB flour. All of the formulations had low moisture content which could be enough to have long storage when packaged in moisture-proof materials. The fat content of the samples varied significantly 2.20 – 2.82% with the highest fat content in the 100% yellow root cassava grits (2.82%). The values obtained were close to the value reported by **Eleazu and Eleazu (2012)** for yellow root cassava. The total ash (minerals) ranged from 2.16 to 2.66% and the highest value was recorded with roasted AYB flour which is close to the report of **Ndidi *et al.* (2014)**. The protein level of the blended samples ranged between 2.72 and 20.43%, with the highest inclusion of roasted AYB flour having the highest protein content. Significant differences ($p$ <0.05) were recorded among the blends, validating the fact that AYB contributed significantly to the protein composition of the blends. This backs up prior research that shows leguminous plants are good sources of nutrients, including a diverse spectrum of minerals and amino acids **(Fagbemi, Oshodi and Ipinmoroti, 2004; Maphosa and Jideani, 2017)**. The crude protein content of the yellow root cassava grits was consistent with previous observations that cassava root food products are low in protein and that its consumption in meals necessitates the addition of adequate amino acids supplement **(Burrell, 2003; Esonu, 2006; Olugbemi, Mutayoba and Lekule, 2010; Ngiki, Igwebuike and Moruppa, 2014)**. The crude fibre of the samples ranged from 1.15 to 1.40%. Higher fibre content was recorded in flour blends with AYB flour substitution and was significant though the values were lower than the values reported. This could be as a result of the processing method used to process African yam beans into flour. The carbohydrate contents of the blended samples were in the range of 68.65 and 83.23%. These samples' high carbohydrate readings are attributable to the carbohydrate content of their basic raw materials, especially the yellow root cassava tubers **(El-sahy and Siliha, 2008; Awoyale et al., 2015)**.

The beta–carotene of the yellow root cassava grits and AYB flour blends is as shown in Fig 3. It ranged between 1.33 and 3.97 µg/g, the hundred percent yellow root cassava grit had the highest amount of beta–carotene due to the variety of the cassava used, which was enriched with beta–carotene. The value falls within the reported range by **Eyinla et al. (2019)** for food products from yellow root cassava. The blended samples also recorded some amounts of beta-carotene, which is a vitamin A precursor. As the substitution of AYB flour increased, the value of beta–carotene of the blended sample decreased. Consumption of these food products can contribute to the source of vitamin A in the diets of the people where cassava is their major staples. There were significant ($p$ <0.05) differences among the blends.

### Antinutritional factors

Saponin levels of the blends ranged between 0.039 to 0.087%. The results are consistent with those obtained by previous researchers for processed African yam beans. **(Ajibola and Olapade, 2016: Anya and Ozung, 2019)**. The least value was observed in 100% yellow cassava grits while the sample with 100% AYB flour had the highest. This is a pointer to the fact that African yam bean contains some anti-nutrients. Saponins bind to iron, zinc, calcium, and vitamins to create insoluble mineral complexes, rendering them inaccessible **(Samtiya, Aluko and Dhewa, 2020)**. There have been reports that processing such as roasting reduced the saponin content in African yam beans **(Onyeike and Omubo-Dede, 2002; Ojuederie, Ajiboye and Babalola, 2020)**. Indicating that the low levels of saponin recorded in the roasted African yam bean were traceable to the effect of heat on the anti-nutrient. It has been found that low quantities of saponins in beans are not harmful to health, but larger concentrations in the diet can be poisonous at about ≥150 mg/kg body weight **(Samtiya, Aluko and Dhewa, 2020)**.

Tannin content was in the range of 0.14 to 0.15%, and in all of the blends, there were no significant changes in the tannin level. For AYB flour, the results were lower than those reported by **Anya and Ozung (2019)** for AYB (0.31 – 0.34%). Tannins are high-molecular-weight (>500), water-soluble phenolic chemicals that can precipitate protein, particularly pepsin **(Adamczyk et al., 2017)**. Studies have shown that tannins are concentrated in the seed coat of legume seeds and are heat resistant. Indicating that the dehulling of the seed coat in the processing method reduced the tannin content in African yam bean flour. Tannin consumption of 1.5 – 2.5 g per day in the diet is considered safe **(Sharma et al., 2019)**.

Therefore, the tannin level found in this study should not have any detrimental effects associated with tannins, such as antagonistic competition reducing accessible protein. Trypsin inhibitor content was in the range of 1.24 and 3.15 mg/g. The values are within the range of 2.22 – 3.05 reported by **Ajibola and Olapade (2016)** for processed AYB seeds.

Phytate concentrations of the flour blends were in the ranges of 0.82 and 2.69 mg/g, and all the blended samples differed significantly ($p$ <0.05). In both animal and human nutrition, phytic acid has been recognized as the most potent antinutritional factor in foods and a source of mineral ion deficit **(Grases, Prieto and Costa-Bauza, 2017)**. The low phytate contents suggest that minerals otherwise chelated by phytate can be much more available. Hydrogen cyanide levels are as shown in Fig 3; they ranged between 0.07 and 4.47 mg/kg. Roasting and fermentation processes involved in the production of cassava grits reduced the hydrogen cyanide to safe levels. As indicated by the Joint FAO/WHO Expert Committee on Food Additives **(JECFA)**, a codex level of 10 mg HCN/kg body weight (10 ppm) in cassava flour does not cause severe toxicity. **(WHO, 2004)**.



**Figure 1** Yellow root cassava and African yam bean seeds.

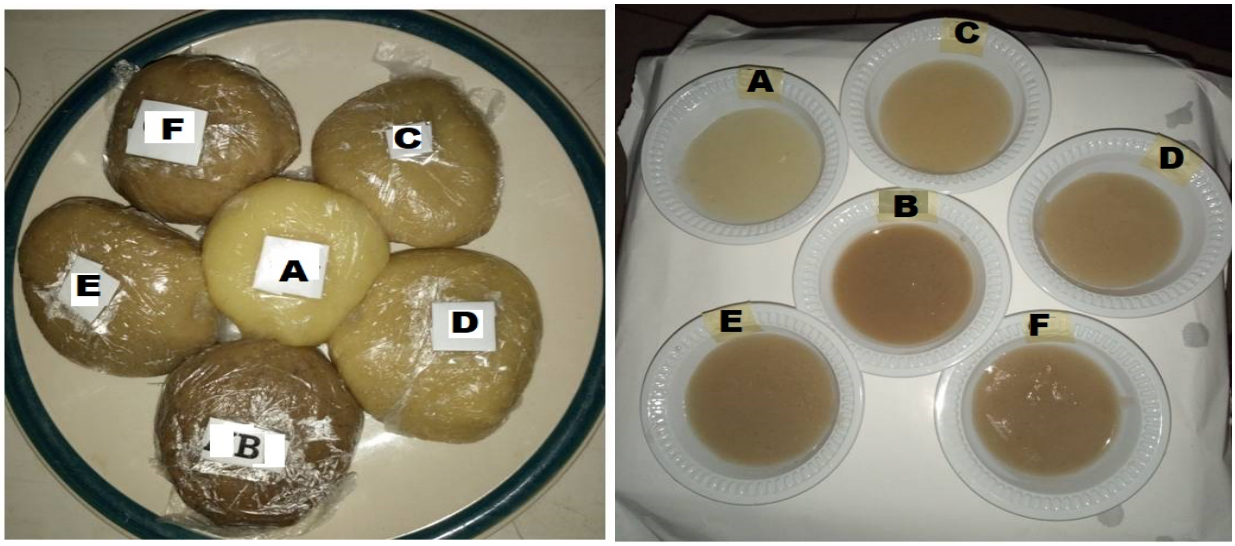


**Figure 2** Meal and gruel samples prepared from blends of yellow root cassava grits and AYB flour.
Note: A =100% Yellow root cassava grit, B = 100% AYB flour, C= 90% Yellow root cassava grit and 10% AYB, D = 80% Yellow root cassava grit and 20% AYB, E =70% Yellow root cassava grit and 30% AYB, F = 60% Yellow root cassava grit and 40% AYB.

Indicating that the food products are within the safe level and will pose no hazard to the consumers.

Haemagglutin contents varied from 1.47 to 3.49 mg/100 g. In this investigation, the flour blends containing 100 percent AYB flour had the highest hemagglutin level (3.49 mg/100 g), which is lesser than the 5.30 mg/100 mg reported by **Ngwu, Aburime and Ani (2014)** for roasted AYB.

Stachyose levels ranged from 1.51 to 1.81%, while raffinose ranged from 0.38 to 0.45%. The reduction of stachyose and raffinose in all the blended samples is of interest. This is because oligosaccharides (raffinose and stachyose) are implicated in flatulence and diarrhea.

**Sensory Evaluation**

The result of the sensory evaluation of gruel samples made with cassava grit and African yam bean flour mixes is as shown in Table 3. All of the samples received good sensory ratings for all of the qualities, as well as overall acceptability. The panelists' ratings for the gruels' taste and flavor were not different significantly ($p$ >0.05) across all samples. In appearance, samples manufactured from 100% AYB flour and 20 – 30% AYB flour substitute did not differ substantially ($p$ <0.05). The sample created with 100 percent AYB flour and the samples manufactured with 10 – 30 percent AYB flour had no discernible variation in general acceptability. but they varied significantly with the samples made from 100% cassava grit and 40% AYB flour substitution. The least score for color, consistency, mouth feels texture, and appearance was observed in the sample with 40% Substitution of AYB flour. The high mean scores observed for flavor, color, consistency, mouthfeel texture, appearance, taste, and overall acceptability indicated that all the gruel samples had acceptable sensory properties. as the percentage of substitution increased up to 30%, the degree of similarity in all of the samples increased for practically all of the criteria. The high mean scores observed for appearance, color, hand feel, mouthfeel, mouldability, and overall acceptability indicated that all the meal samples were of good sensory attributes except the meal prepared from 100% AYB. The highest score for overall acceptability was observed in the sample with 70% yellow root cassava grits and 30% AYB flour.

**Table 1** Proximate and calorific content of yellow root cassava grits and AYB flour blends.

| Sample (%) | MC (%) | Fat (%) | Ash (%) | Protein (%) | Crude fibre (%) | Carbohydrate (%) | Calorific Content kcal/100 g |
|---|---|---|---|---|---|---|---|
| **A** | 7.92$^{d}$ | 2.82$^{c}$ | 2.16$^{a}$ | 2.72$^{a}$ | 1.15$^{a}$ | 83.23$^{c}$ | 348.37 |
| **B** | 4.66$^{a}$ | 2.20$^{a}$ | 2.66$^{f}$ | 20.43$^{f}$ | 1.40$^{f}$ | 68.65$^{a}$ | 358.96 |
| **C** | 7.28$^{c}$ | 2.66$^{b}$ | 2.20$^{b}$ | 5.58$^{b}$ | 1.22$^{b}$ | 81.06$^{c}$ | 350.22 |
| **D** | 7.26$^{c}$ | 2.64$^{b}$ | 2.36$^{c}$ | 7.36$^{c}$ | 1.29$^{c}$ | 79.09$^{c}$ | 348.20 |
| **E** | 7.26$^{c}$ | 2.67$^{b}$ | 2.37$^{c}$ | 8.63$^{d}$ | 1.33$^{d}$ | 77.74$^{b}$ | 350.08 |
| **F** | 7.10$^{b}$ | 2.71$^{b}$ | 2.46$^{d}$ | 10.60$^{e}$ | 1.39$^{e}$ | 75.74$^{b}$ | 350.82 |

Note: The mean values in the same column with varied superscripts are significantly different ($p$<0.05). A =100% Yellow root cassava grit, B = 100% AYB flour, C= 90% Yellow root cassava grit and 10% AYB, D = 80% Yellow root cassava grit and 20% AYB, E =70% Yellow root cassava grit and 30% AYB, F = 60% Yellow root cassava grit and 40% AYB.

**Table 2** Anti-nutritional composition of the yellow root cassava grits and AYB flour blends.

| Sample | Saponin % | Tannin % | Trypsin Inhibitor (mg/g) | Hemaglutin (mg/100 g) | Stachylose % | Raffinose % | Phytate (mg/g) |
|---|---|---|---|---|---|---|---|
| **A** | 0.039$^{a}$ | 0.14 | 1.24$^{a}$ | 1.47$^{a}$ | 1.79$^{d}$ | 0.45$^{d}$ | 0.82$^{a}$ |
| **B** | 0.087$^{e}$ | 0.15 | 3.15$^{f}$ | 3.49$^{f}$ | 1.81$^{e}$ | 0.45$^{d}$ | 2.69$^{f}$ |
| **C** | 0.050$^{b}$ | 0.11 | 1.28$^{b}$ | 2.14$^{b}$ | 1.51$^{a}$ | 0.38$^{a}$ | 1.07$^{b}$ |
| **D** | 0.060$^{c}$ | 0.12 | 1.35$^{c}$ | 2.42$^{c}$ | 1.55$^{b}$ | 0.39$^{b}$ | 1.19$^{c}$ |
| **E** | 0.062$^{c}$ | 0.13 | 1.91$^{d}$ | 2.69$^{d}$ | 1.75$^{c}$ | 0.44$^{c}$ | 1.40$^{d}$ |
| **F** | 0.065$^{d}$ | 0.14 | 2.14$^{e}$ | 3.03$^{e}$ | 1.79$^{d}$ | 0.45$^{d}$ | 1.62$^{e}$ |

Note: The mean values of the same column with different superscripts differ by a significant amount ($p$ <0.05). A =100% Yellow root cassava grit, B = 100% AYB flour, C= 90% Yellow root cassava grit and 10% AYB, D = 80% Yellow root cassava grit and 20% AYB, E =70% Yellow root cassava grit and 30% AYB, F = 60% Yellow root cassava grit and 40% AYB.

**Table 3** Sensory evaluation of gruel samples produced from yellow root cassava grits and AYB flour blends.

| Sample | Flavour | Colour | Consistency | Mouthfeel Texture | Appearance | Taste | Overall Acceptability |
|---|---|---|---|---|---|---|---|
| **A** | 6.32$^{a}$ | 7.66$^{d}$ | 7.18$^{d}$ | 7.22$^{c}$ | 7.34$^{c}$ | 6.14$^{a}$ | 7.38$^{c}$ |
| **B** | 6.58$^{a}$ | 6.52$^{bc}$ | 5.76$^{ab}$ | 6.16$^{ab}$ | 6.56$^{bc}$ | 5.86$^{a}$ | 6.32$^{b}$ |
| **C** | 6.18$^{a}$ | 6.72$^{bc}$ | 6.70$^{cd}$ | 6.78$^{bc}$ | 6.34$^{ab}$ | 6.22$^{a}$ | 5.94$^{b}$ |
| **D** | 6.72$^{a}$ | 7.20$^{cd}$ | 6.24$^{abc}$ | 6.14$^{ab}$ | 6.62$^{bc}$ | 6.20$^{a}$ | 6.42$^{b}$ |
| **E** | 6.68$^{a}$ | 6.28$^{cd}$ | 6.36$^{bc}$ | 6.10$^{ab}$ | 6.60$^{bc}$ | 5.98$^{a}$ | 5.84$^{b}$ |
| **F** | 6.42$^{a}$ | 5.78$^{a}$ | 5.50$^{a}$ | 5.86$^{a}$ | 5.58$^{a}$ | 5.56$^{a}$ | 5.06$^{a}$ |

Note: Mean values in the same column with different superscripts are considerably different from each other ($p$ <0.05). A =100% Yellow root cassava grit, B = 100% AYB flour, C= 90% Yellow root cassava grit and 10% AYB, D = 80% Yellow root cassava grit and 20% AYB, E =70% Yellow root cassava grit and 30% AYB, F = 60% Yellow root cassava grit and 40% AYB.

**Table 4** Sensory evaluation of meal samples produced from yellow root cassava grits and AYB flour blends.

| Sample | Appearance | Colour | Hand feel | Mouthfeel | Mouldability | Overall Acceptability |
|---|---|---|---|---|---|---|
| A | 7.60$^{b}$ | 7.52$^{c}$ | 7.04$^{b}$ | 6.62$^{b}$ | 6.14$^{b}$ | 6.84$^{c}$ |
| B | 5.48$^{a}$ | 6.36$^{b}$ | 4.22$^{a}$ | 3.86$^{a}$ | 3.56$^{a}$ | 3.42$^{a}$ |
| C | 7.08$^{b}$ | 6.68$^{b}$ | 6.54$^{b}$ | 6.52$^{b}$ | 6.62$^{b}$ | 6.60$^{c}$ |
| D | 7.04$^{b}$ | 6.64$^{b}$ | 6.76$^{b}$ | 6.70$^{b}$ | 6.52$^{b}$ | 6.34$^{c}$ |
| E | 8.26$^{c}$ | 7.80$^{c}$ | 8.02$^{c}$ | 7.80$^{c}$ | 7.64$^{c}$ | 7.80$^{d}$ |
| F | 5.84$^{a}$ | 5.70$^{a}$ | 6.28$^{b}$ | 6.36$^{b}$ | 6.20$^{b}$ | 5.50$^{b}$ |

Note: The mean values in the same column with different superscripts vary significantly ($p$<0.05). A = 100% Yellow root cassava grit, B = 100% AYB flour, C= 90% Yellow root cassava grit and 10% AYB, D = 80% Yellow root cassava grit and 20% AYB, E =70% Yellow root cassava grit and 30% AYB, F = 60% Yellow root cassava grit and 40% AYB.

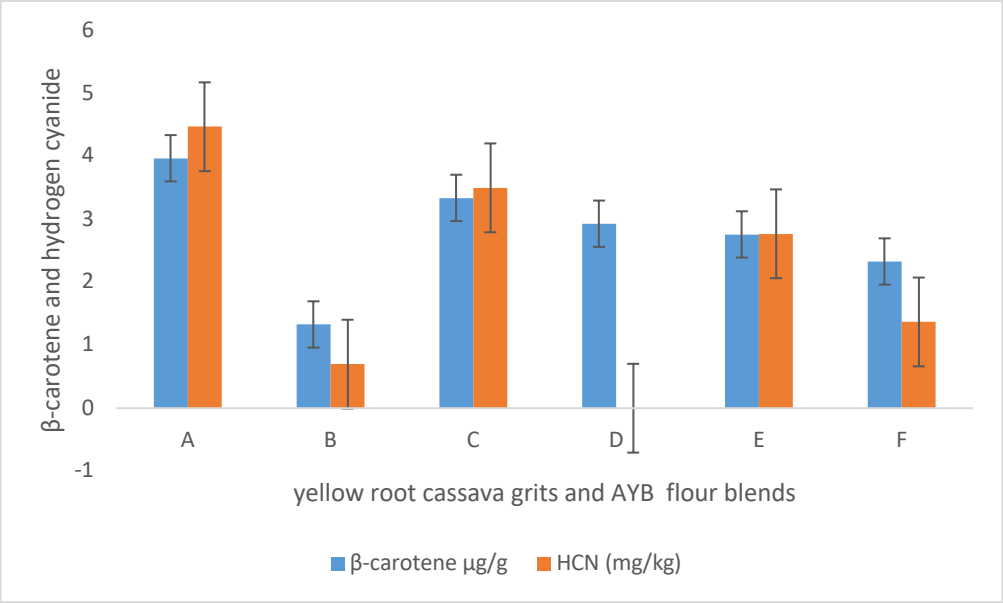


**Figure 3** The β-carotene and the hydrogen cyanide contents of the yellow root cassava and AYB flour blends.
Note: A =100% Yellow root cassava grit, B = 100% AYB flour, C= 90% Yellow root cassava grit and 10% AYB, D = 80% Yellow root cassava grit and 20% AYB, E =70% Yellow root cassava grit and 30% AYB, F = 60% Yellow root cassava grit and 40% AYB.

## CONCLUSION

This study has shown that nutritious gruel and meals with acceptable sensory properties can be obtained from blends of 80% cassava grits with 20% roasted AYB flour and 70% yellow cassava grit with 30% roasted AYB flour, respectively. The protein content and overall sensory characteristics of prepared meals and gruels were positively affected with AYB flour substitution. Hence, the potential of African yam bean in alleviating the problems of protein malnutrition in the developing world among the most vulnerable groups was established.

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# PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF *ECHINOCYSTIS LOBATA* (MICH.) TORR. ET GRAY (*CUCURBITACEAE*)

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## ABSTRACT

*Echinocystis lobata* (Mich.) Torr. et Gray extracts were investigated for their bioactive compounds and their potential for natural antioxidant properties. In vitro antioxidant activity of methanol, ethanol, and water extracts were evaluated by 1,1-diphenyl-picrylhydrazyl (DPPH) radical scavenging activity. Total phenolic content (TPC) and total flavonoid content (TFC) of fruits and seeds were determined. TPC is a fairly constant value and varies weakly both among the different organs of *Echinocystis lobata* and among the geographical locations of the populations studied. Like other invasive species, *Echinocystis lobata* is superior to many other plants in terms of the total amount of flavonoids contained in its fruits and seeds. The dried fruits contain more flavonoids than the seeds. Seeds from Slovak populations have higher flavonoid content than those from Russian populations. The results demonstrated that *Echinocystis lobata* can be a good source of natural antioxidants and have a great potential for commercialization, especially in the pharmaceutical industry.

**Keywords:** *Echinocystis lobata*; leaves; flowers; fruits; seed; biological activity

## INTRODUCTION

The secondary plant metabolites, polyphenols, have a wide spectrum of actions such as modulation of lipid peroxidation, free radical scavenging, inhibition of hydrolytic and oxidative enzymes (phospholipase A2, cyclooxygenase), and anti-inflammatory activity **(Lu et al. 2016)**. The current focus is on the antioxidant activity of polyphenols. It is this activity that determines the pharmacological effects. Antioxidants play an important role in protecting human cells against free radicals, which cause several physiological and pathological abnormalities, such as cardiovascular disease, rheumatoid arthritis, cancer, and aging **(Atanassova et al., 2011; Ghafar et al., 2017)**.

Scientists are constantly searching for plants whose organs contain substances with high antioxidant activity, namely *Cydonia* Mill. and *Pseudocydonia* C.K. Schneid. **(Monka et al., 2014; Grygorieva et al., 2020)**, *Cornus mas* L. **(Klymenko, Grygorieva and Brindza, 2017)**, *Diospyros virginiana* L. **(Grygorieva et al., 2018)**, *Sambucus nigra* L. **(Horčinová Sedláčková et al., 2018, 2019)**, *Ziziphus jujuba* Mill. **(Ivanišová et al., 2017)**, *Hippophae rhamnoides* L. **(Ivanišová et al., 2020)**, *Lycium* spp. **(Szot, Zhurba and Klymenko, 2020)**. However, a whole group of invasive plants is little studied in this respect. Meanwhile, the secondary distribution range of invasive species expands year by year and their resource reserves in the new homeland are very large. Our previous studies have already demonstrated very high antioxidant activity in several invasive species: *Aronia mitschurinii* A.K.Skvortsov & Maitul. **(Vinogradova et al., 2017, 2020)** and *Solidago canadensis* L. **(Shelepova et al., 2020)**.

In this context, we began to study a North American *Echinocystis lobata* (Mich.) Torr. et Gray, which have been unintentionally introduced in Europe at the beginning of the XX century, no later than 1904, but had only isolated localities until World War II. From 1946 onwards, it gradually expanded its range and moved north-east. *This species* became established in Central and South-Eastern Europe, and the Far East, forming a secondary distribution range. The northern limit of the range in Russia runs along the line St. Petersburg – Vologda – Perm – Krasnoyarsk – Irkutsk – Tynda – Komsomolsk-on-Amur **(Vinogradova et al., 2010)**. Over the past 30 years, its secondary range has expanded considerably and the species has invaded natural riverine plant communities, becoming a "transformer" **(Richardson and Pyšek, 2006)**. Invasive populations have been very stable: *Echinocystis lobata* actively displaces native riparian species from their habitats, producing a lot of seeds.

*Echinocystis lobata* belongs to the family Cucurbitaceae, and closely related species in the same family contain cucurbitacins that are used as antiproliferative, anti-inflammatory, and antioxidant agents. Several species of this family, widely used in folk medicine, are now

attracting the increasing attention of biochemists as valuable raw materials for new medicinal preparations. For example, the oil isolated from the seeds of *Ecballium elaterium* (L.) A. Rich. has antitumor activity and is promising for use in oncology **(Touihri et al., 2019)**. The presence of cucurbitacins in seeds of the Cucurbitaceae family (and *Echinocystis* in particular) is known from the literature **(Sokolov, 1986)**. All these compounds have a bitter, unpleasant taste, but can induce cytotoxicity against malignant cells in mammals **(Panosyan, 1985)** and are used to treat benign tumors and to kill worms (pumpkin seeds are known to have this pharmacological effect).

North American Indians use *Echinocystis* roots as a remedy: the bitterness extracted from the roots contains analgesics **(Burton et al., 2015)**. Traditional medicine refers to its use for headaches, menstrual disorders, rheumatism, fever, kidney, or stomach problems **(Melymuka et al., 2005)**. However, there is little scientific information on the composition of bioactive compounds of *Echinocystis*. There are indications of the presence of quercetin and isorhamnetin derivatives **(Krauze-Baranowska and Cisowski, 1996)**. Various enzymes **(Gillard and Walton, 1976)** and trypsin inhibitors **(Stachowiak et al., 1996)** have been reported. The fruit contains carbohydrates: galactose, xylose, rhamnose, arabinose, and mucus **(Budantsev and Lesiovskaya, 2014)**.

The presence in *Echinocystis lobata* of substances with a wide range of pharmacological effects, in particular, antitumor and antioxidant activity, increases interest in detailing the chemical composition of this species. Such studies may be promising and relevant, as they will allow evaluating the phytotherapeutic prospects of the plant and its resource potential.

Phenolic content and antioxidant activity have been studied in detail only in Romania. However, only the leaves, flowers, and above-ground parts of the plants have been studied; the seeds have not been examined. Ethanolic extracts from leaves and aerial parts of *Echinocystis lobata* contain mainly p-coumaric acid, isoquercitrin, rutin, quercitrin, and kaempferol **(Ielciu et al., 2017)**. The flowers contained the same substances and in addition ferulic acid. Biological assays showed a significant antioxidant effect and no cytotoxic and anti-plasmodial activity **(Ielciu et al., 2018)**.

When analyzing seeds of *Echinocystis* from geographically different populations of Central Russia, it was found that climatic and soil conditions of growth influence the accumulation of flavonoids – the total content of these compounds in the seeds of *Echinocystis* from the Moscow population was 0.085 ±0.005%, and in seeds from the Tver population was 0.105 ±0.009%. The flavonoid composition of seeds of this plant is practically not studied. Thus, only one literary source **(Krauze-Baranowska and Cisowski, 1996)** knows that C-glycosides are distinguished in flavonoid complex. Considering the significant content of flavonoids in the seeds of *Echinocystis lobata* and their undoubted importance in the biological activity of the raw material, furthermore, detailed study of the flavonoid composition of *Echinocystis* seeds seems to be advisable.

This work aims to compare the total content of polyphenols and flavonoids in different organs of *Echinocystis* in Central Russia and Slovakia.

### Scientific Hypothesis

First, we assumed (based on our previous research on invasive species) that the *Echinocystis lobata* will have the same high levels of antiradical activity. Second, we believe that the content of beneficial substances will be higher in Slovak invasive populations, as the weather conditions here are more favorable for the growth of *Echinocystis lobata*.

## MATERIAL AND METHODOLOGY

### Samples

The material was collected from five sites in Russia and two sites in Slovakia (Table 1). The plants were collected in September 2019 during the fruiting season. Five to seven plants were selected from each population. Leaves, flowers, fruits, and seeds were studied (Figure 1, 2).

### Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

**Table 1** Studied samples of *Echinocystis lobata* (Mich.) Torr. et Gray.

| Harvesting place | Geographical coordinates | Organ |
|---|---|---|
| **Russia** | | |
| **Zvenigorod, Moscow district, Russia** | N 55.44 E 36.51 | Leaves (ZL)<br>Flowers (ZFl)<br>Dry ripe fruit without seeds (ZF)<br>Dry unripe fruit with milk seeds (ZFS)<br>Seeds (ZS) |
| **Redkino, Tver district, Russia** | N 56.38 E 36.17 | Seeds (TS) |
| **Kaluga, Russia** | N 54.31 E 36.15 | Seeds (KS) |
| **Uglich, Yaroslavl district, Russia** | N 57.32 E 38.20 | Seeds (YS) |
| **Saransk, Republic of Mordovia, Russia** | N 54.18 E 45.17 | Seeds (MS) |
| **Slovakia** | | |
| **Nitra, Slovakia** | N 48.18 E 18.05 | Seeds (NiS) |
| **Nova Bana, Slovakia** | N 48.25 E 18.38 | Seeds (NoS) |



**Figure 1** Invasive populations of *Echinocystis lobata* (Mich.) Torr. et Gray.



**Figure 2** Fruit and seeds of *Echinocystis lobata* (Mich.) Torr. et Gray (each seed has been collected from single individual).

**Description of the Experiment**

**Sample preparation:**

a) **for the antioxidant activity measurements**: 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.

b) **for the polyphenols and flavonoids measurements:** the air-dried plant material was mechanically ground to a homogeneous powder in a laboratory mill. All Echinocystis samples weighing approximately 0.7 g (weighed to the nearest 0.0001 g) were extracted in 15 ml of a mixture of 90% ethanol and 1% chloric acid in a reflux condenser water bath at 60 °C for 30 min. The obtained extract was passed through a 0.25 μm filter. The extraction was repeated three times. The total volume of the extract is 45 ml, stored in a dark place at 4 °C until analysis.

**Number of samples analyzed:** A total of 55 samples were analyzed.

**Determination of antiradical activity**

The antiradical activity was measured by the 2.2-diphenyl-1-picrylhydrazyl (DPPH·) method according to **Brand-Williams, et al. (1995)**. This test is based on the reaction of radical discoloration (the color of the radical solution is purple). The procedure of determination of optical density was measured with a spectrophotometer (Genesys 20 UV-VIS, USA) at wavelength 515 nm. Investigated plants were dried at room temperature and

powdered. Dry mass (1 g) of plant raw material was mixed with 25 ml of solvent. Extraction was carried out with methanol, ethanol, and water for 12 hours with constant stirring on a shaker. Obtained extracts were filtrated (Whatman No. 1) and 0.1 ml of antioxidant solution was added to 3.9 ml of methanol DPPH·solution (25 mg of radical per 100 ml of methanol with further dilution). The optical density of the solution was measured immediately and after adding a sample. The incubation for 10 minutes was in the dark. Obtained results were calculated in percentage by using the following equation (1):

$$\% \, Inh = \frac{A_0 - A_1}{A_0} x \, 100 \qquad (1)$$

where:

$A_0$ – absorbance of control reaction;

$A_1$ – absorbance in presence of the sample.

**Determination of Total Phenolic Content (TPC)**

The total phenolic content was determined using **the** Folin-Ciocalteu procedure by using gallic acid as standard. The crude extract (40 µg) was mixed with Folin – Ciocalteu reagent (200 µL) and distilled water (3.16 mL). The mixture was left between 30 sec to 8 min before **being** added with 20% sodium carbonate (600 µL). The mixture was kept at 20 °C for 2 hours and the absorbance was read at 765 nm using a Spectrophotometer Spekol 1300 (Analytik Jena). The TPC values were calculated using a gallic acid calibration curve within the range 0 – 2000 mg/L (R2 = 0.9982). The results were expressed as gallic acid equivalents (GAE) mg/g dw vegetal product. All samples were analyzed in triplicate.

**Determination of Total Flavonoid Content (TFC)**

The total flavonoid content of the methanol extract was determined using the aluminium chloride colorimetric method with quercetin [2] and rutin [3] as standard. The crude extracts (1 mg) were diluted with water (4 mL) in a 10 mL volumetric flask. Initially, 5% sodium nitrate solution (0.3mL) was added to each volumetric flask then 10% aluminium chloride (0.3 mL) was added into the flask and followed by 1.0M NaOH (2ml). Water (2.4mL) was then added to the flask and mixed well. The absorbance of the mixture was kept at 22 °C for 20 minutes and the absorbance was read at 430 nm (quercetin) and kept at 22 °C for 40 minutes and the absorbance was read at 415 (rutin). TFC values were determined as quercetin equivalents (QE) mg/g and rutin equivalents (RE) mg/g dw vegetal product. All samples were analyzed in triplicate.

**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

*Echinocystis lobata* is an annual vine with stems that can be as long as 6 m **(Vinogradova, 2006)** and which climb, with the help of coiling, branched tendrils, over shrubs and fences or trail across the ground. The leaves are alternate with long petioles and five palmate lobes. The flowers are monoecious, with separate male and female blooms on the same plant. The male flowers are in long-stemmed, upright panicles. Each flower has a white, or greenish-yellow, corolla with six slender lobes and a single central stamen with a yellow anther. The female flower has a single stigma with the spiky globular inferior ovary. The fruit is a prickly, inflated capsule up to 5 cm long with two pores and four seeds. It persists all winter and then opens at the bottom, liberating the seeds.

The seed of *Echinocystis lobata*, collected from a single individual, has its shape, color, and seed coat pattern (like a person's fingerprints). Only seed weight and size vary. Seeds of 17 discrete variations (phenotypes) have been identified, occurring both in the natural and in secondary habitat. The spermoderm texture can be smooth or wrinkled, with or without a pattern. The shape of the semen varies from rounded to oval or, less frequently, lanceolate. Spermoderm coloring may be black, brown, or grey, with or without a stripe along the lateral seam. Spermoderm pattern may be marbled or spotted, in the latter case, the spots may be rounded, triangular, or shapeless**.** In all populations, oval-shaped seeds with wrinkled patterned spermoderm prevailed **(Vinogradova, et al., 2010)**. High variability in seed size and coloration has also been noted by other researchers **(Golivets, 2014)**.

The biochemical composition of *Echinocystis* seed oil has been best studied. Linoleic, oleic, palmitic, stearic, and linolenic acids, as well as phytosteroids (spinasterol, sigmastadiene) and glycoside of echinocystic acid, were isolated in seed oil **(Sokolov, 1986)**. Our previous studies **(Shelepova, et al., 2016)** have also shown that the component composition of *Echinocystis* seed oil from populations of the secondary distribution range of Central Russia is almost similar to that of plants from North American populations. The seeds of *Echinocystis lobata* are of interest for their possible use in phytotherapy. They can be used for the prevention of some diseases and, in particular, as an anti-inflammatory component, as they contain up to 1% of triterpene saponins, up to 0.1% of flavonoids, and up to 40% of oil. Fatty acid oil composition is represented by almost 60% linoleic acid **(Shelepova and Vinogradova, 2016, 2017)**.

As a result, numerous reports are dedicated to the study of useful properties of invasive plants, among which different biological activities. The antioxidant activity of these plants demonstrated valuable results comparing with widely used species **(Guzzetti, et al., 2017; Sari, et al., 2018; Maema, Potgieter and Samie, 2019)**. Several studies showed that the content of polyphenol compounds of invasive plants had higher values than native **(Kim and Lee, 2011)**.

**Determination of antiradical activity**

As we expected, samples of *Echinocystis* contain the same high levels of bioactive substances as other invasive species. The antiradical activity of alcohol extracts for all specimens was quite high. The free radical scavenging activity in the leaf extracts had 60.93 ±2.66% (methanol extracts), 37.30 ±0.60% (ethanol extracts), and 26.47 ±1.60% (water extracts) (Figure 3). The same characteristic in the inflorescences was higher in alcohol extracts: 76.93 ±0.92% (methanol extracts), 49.48 ±2.18% (ethanol extracts) but was lower in water extracts 13.46 ±1.22%.

**Rodge and Biradar (2017)** found in ethanol extracts of other Cucurbitaceae the antiradical activity for the following species: *Coccinia grandis* L. fruits (72.14%) and leaves (18.40%), *Lagenaria siceraria* (Molina) Standl. fruits (23.20%) and leaves (14.85%), *Thrichosanthes tricuspidata* Lour. fruits (65.0%) and leaves (54.0%), *Diplocyclos palmatus* (L.) Jeffrey fruits (60.33%), and leaves (22.33%), *Cucumis setosus* Cogn. fruits (59.66%) and leaves (29.26%). Similar to our data, closely related above species from the Cucurbitaceae family accumulate more phenol in the fruit than in the leaves. *Echinocystis* fruits accumulate fewer substances with antiradical activity than *Coccinia grandis, Thrichosanthes tricuspidata, Diplocyclos palmatus,* and *Cucumis setosus*, but more than *Lagenaria siceraria* (49% versus 23%). However, it should not be forgotten that all these species, already actively used for medicinal purposes, grow mainly in subtropical or tropical climates, whereas *Echinocystis* is a temperate plant.

**Determination of Total Phenolic Content (TPC)**

There are about 8000 plant phenolics in nature. They are one of the main groups of compounds working as primary antioxidants or free radical scavengers. TPC is almost independent of the climatic conditions of the plant habitats and varies from 12.9 to 21.2 mg GAE/100g in Russian invasive populations and from 17.5 to 19.9 in invasive populations in Slovakia (Table 2).

Moreover, mature fruit, immature fruit, and seed also do not differ in this indicator, a comparison for which has been made for the Zvenigorod population. The highest TPC was found in seeds of Ys (Uglich, Yaroslavl district,

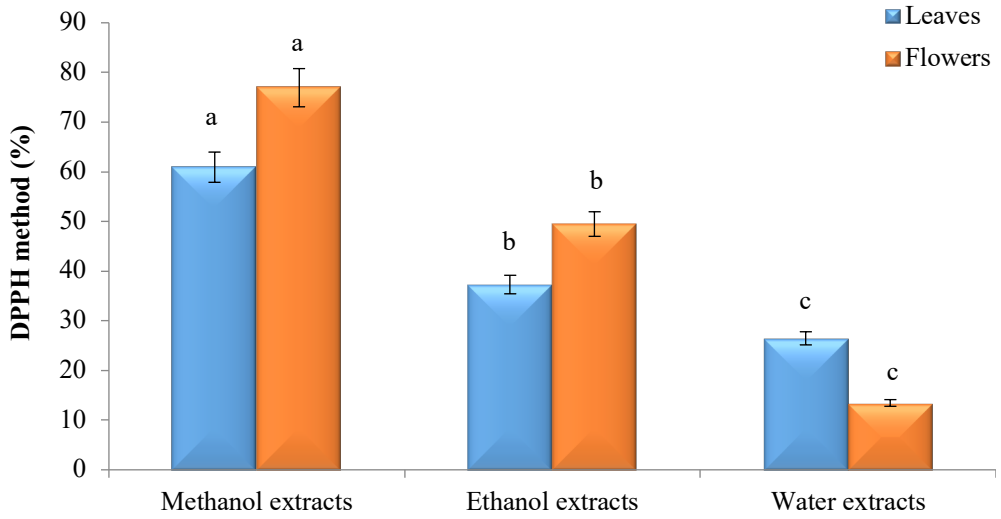


**Figure 3** Antioxidant activity of leaves and flowers of *Echinocystis lobata* (Mich.) Torr. et Gray.
Note: (DPPH method, %). Means in columns followed by different letters are different at $p$ <0.05.

**Table 2** Total Phenolic Content and Total Flavonoid Content in studied samples of *Echinocystis lobata* (Mich.) Torr. et Gray.

| Sample (according Table 1) | Total Phenolic Content (TPC), mg GAE/100g | Total Flavonoid Content (TFC) | |
|---|---|---|---|
| | | Quercetin, mg QE/g | Rutin, mg RE/g |
| **Russia** | | | |
| **ZF** | 15.33 ±1.21c | 266.52 ±2.11a | 1264.85 ±3.17a |
| **ZFS** | 12.93 ±0.87d | 10.64 ±0.51e | 242.78 ±1.12d |
| **ZS** | 15.32 ±0.91c | 80.14 ±0.77d | 297.14 ±1.20c |
| **TS** | 15.62 ±0.93c | 10.92 ±0.45e | 74.75 ±0.83e |
| **MS** | 17.08 ±1.05b | 128.79 ±0.91c | 440.77 ±1.57b |
| **KS** | 19.21 ±0.95a | 84.38 ±0.71d | 312.86 ±1.21c |
| **YS** | 21.19 ±1.17a | 164.86 ±0.93b | 470.20 ±1.62b |
| **Slovakia** | | | |
| **NiS** | 17.46 ±1.01b | 227.17 ±1.63a | 779.54 ±1.54a |
| **NoS** | 19.87 ±0.93a | 173.23 ±0.85b | 538.67 ±1.69b |

Note: Values are expressed as the mean ±SD (n = 3). Means in columns followed by different letters are different at $p$ <0.05.

Russia), whereas the lowest was found in seeds of Zs (Zvenigorod, Moscow district, Russia).

The TPC levels obtained are significantly different from those found in dry unripe fruit with milk seeds of *Echinocystis lobata*. These results highlight the significant differences in total phenolic content among different organs of *Echinocystis lobata*. It seems to suggest that seeds from *Echinocystis lobata* may be the best source to obtain extracts with high TPC.

**Determination of Total Flavonoid Content (TFC)**

In contrast, the TFC indicator demonstrates a difference in flavonoid content between mature fruit, immature fruit, and seeds. Unripe fruit contains the least flavonoids (10.6 mg QE/g and 242.8 mg RE/g), seeds contain somewhat more flavonoids (80.1 mg QE/g and 297.1 mg RE/g). Most flavonoids are accumulated in the dried fruits. As for quercetin, this index (266.5 mg QE/g) is 26 times higher than in unripe fruits and 3 times higher than in seeds. For rutin, this index (1264.8 mg RE/g) is 5 times higher than in unripe fruits and 4 times higher than in seeds.

There is also a difference in flavonoid accumulation depending on where the seeds are harvested. Seeds collected in Slovakia contain significantly more flavonoids than seeds collected in Central Russia. In seeds collected in Russia, the TFC for quercetin ranges from 10.9 to 164.9 mg QE/g, while in Slovak populations it ranges from 173.2 to 227.2 mg QE/g. The TFC value for rutin ranges from 74.7 to 470.2 mg RE/g for Russian populations, while in Slovak populations it is much higher and ranges from 538.7 to 779.5 mg RE/g.

As we suggested, the flavonoid content of invasive *Echinocystis* is higher than that of many other plants. For example, the TFC of *Manilkara zapota* (L.) P Royen seeds are 97.2- 116.1 mg RE/g **(Shafii, et al., 2017)** compared to 538.7-779.5 for the seed of *Echinocystis lobata* in Slovakia. The TFC values in *Moringa oleifera* seeds **(Ghafar, et al., 2017)** were 99.72 (mg QE/g of extract weight), which is also significantly lower than that of *Echinocystis*. **Abifarin, et al. (2019)** found TFC of methanol extracts for wild vegetable *Cucumis africanus* in fruits and leaves 20.63 and 228.96 mg QE/g, respectively, and TPC 17.46 and 43.31 mg GAE/g.

## CONCLUSION

The fruits and seeds of *Echinocystis lobata* are of interest for further investigation into their possible use in phytotherapy. They can be used for the prevention of several diseases and, in particular, as an anti-inflammatory component as they contain up to 1% triterpene saponins, up to 40% oil, whose fatty acid composition is represented by almost 60% linoleic acid, and up to 0.1% flavonoids. *Echinocystis* is superior to many other plants in terms of the total amount of flavonoids contained in its fruits and seeds. The dried fruits contain more flavonoids than the seeds. Seeds from Slovak populations of *Echinocystis* have a higher flavonoid content than those from Russian populations. The results demonstrated that *Echinocystis lobata* can be a valuable raw material resource for the bioeconomy and there is the possibility of its wider application in the future.

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# DETERMINING THE EFFECT OF REPLACING COMPOSITE FLOUR MIXTURE WITH CASSAVA STARCH ON NUTRITIONAL AND PHYSICAL PROPERTIES OF CHICKEN SAUSAGES

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## ABSTRACT

Cassava starch extracted from cassava roots (*Manihot esculanta*) is a highly used ingredient for most commercial food products and has a high viscosity, water holding capacity, and binding abilities. The objective of this study was to determine the effect of replacing composite flour mixture with cassava starch on the nutritional and physical properties of chicken sausages. A scientific experiment was designed for the sausage formulation to incorporate 50% to 100% with cassava modified and native starch compared to the control sample. These samples were evaluated for sensory and textural properties. Nonparametric data obtained through sensory evaluation were analyzed using the Friedman ranking test. According to the test results, the best sample was analyzed for the nutritional toxicological composition and storage stability. According to the statistical analysis, the sample which was 50% replaced with native cassava starch showed the best sensory profile. The best sample showed average moisture, crude fat, crude protein, total solids, ash, and acid-insoluble ash content of 65.98, 7.19, 8.79, 34.02, 2.36, and 0.32 percent respectively. The starch content of the best-ranked sample was 3.12 and the cyanide content of that sample was 1.54 ppm on a dry basis. According to the microbial analysis results, the sausage sample was coliform negative and zero initial plate count was obtained. Besides, the best-ranked fresh chicken sausage was nutritionally acceptable and could keep for 14 days without adding preservatives.

**Keywords:** cassava starch; sausage; nutritional property; toxicological analysis; microbial analysis

## INTRODUCTION

Cassava (*Manihot esculanta*) is one of the most important food crops in tropical countries. Cassava has higher carbohydrate content and its tapioca starch and modified tapioca starch are highly used as an ingredient of many food products **(Zhu et al., 2012)**. The Sri Lankan annual cassava production in 2015 was 324,097 million Tons and the area of land under the cultivation of cassava is 23,970 Hectares **(Statistics, 2016)**. Over the last decades, cassava production in Sri Lanka grew by 45% between 2005 and 2015. According to the National Statistics of Household Income and Expenditure Survey, 2012/13 the average monthly consumption of cassava was 171.53g per person in Sri Lanka. Sausage is the most popular product among the oldest meat products in history about 1500 B.C. Sausage can be simply defined as a product manufactured from ground meat; mixed with salt, spices, and composite flour mixture; and shaped in some manner, using various sizes and types of casings. Raw sausages are produced without curing and are made from selected cuts of fresh meat. Fresh sausages must always be kept under refrigeration and also must be cooked thoroughly before serving.

The composite flour mixture is included with rice flour, maize powder, milk powder, and bread crumbs. Composite flour mixture has been used as binders in comminuted meat products due to their economic benefits. The amount of composite flour mixture used in processed meat varies depending upon the type of product, manufacturing procedure, and government regulations **(Hedrick et al., 1994)**.

Nowadays, starches are used as binders in processed meat products to absorb moisture, which is released from meat protein during heating. A satisfactory binder should have the ability to hold more moisture throughout the processing, cooking, chilling, and storage of meat products. Today scientific principles are employed to improve production procedures, product quality, and product safety.

To maintain the water-holding ability the binder should also assist in binding the fat and maintaining its dispersion throughout the mix. Starches are commonly added to emulsion-type meat products and are popular not only for their functional properties but also to extend the more expensive lean meat portion of products **(Hedrick et al., 1994)**. The effect is based on the ability of starch to

**Table 1** Ingredients content in sausage formulae.

| Ingredient | $a_0b_0$ CFM 50% Starch 50% | $a_0b_1$ CFM 0% Starch 100% | C CFM 100% Starch 0% | $a_1b_0$ CFM 50% Starch 50% | $a_1b_1$ CFM 0% Starch 100% |
|---|---|---|---|---|---|
| Meat % | 57.6 | 57.6 | 57.6 | 57.6 | 57.6 |
| Emulsion % | 33.3 | 33.3 | 33.3 | 33.3 | 33.3 |
| Composite Flour Mixture (CFM) % | 3.05 | 0 | 6.1 | 3.05 | 0 |
| Cassava Native starch % | 3.05 | 6.1 | 0 | 0 | 0 |
| Cassava Modified starch % | 0 | 0 | 0 | 3.05 | 3.05 |
| Sugar & Salts % | 2 | 2 | 2 | 2 | 2 |
| Spices Mixture % | 1 | 1 | 1 | 1 | 1 |

gelatinize when heated in a water-containing medium, thereby binding a relatively large amount of water **(Hodge, Osman and Fennema, 1976)**.

Extracted cassava native starch and modified starch were used for the replacement of composite flour mixture as a source of dietary fiber content **(Ruhee et al., 2020)** and due to its specific physical properties as high viscosity, water holding capacity, and binding ability. Even though Sri Lanka has higher production of cassava the industrial utilization is limited **(Somendrika et al., 2016)**. Also, cassava has lower postharvest life since it is a perishable food. Increasing the application of cassava starch will directly be beneficial to cassava farmers and for the Sri Lankan economy as it reduces the wastage of cassava. Therefore the objective of the study was to determine the effect of replacing composite flour mixture with cassava starch on the nutritional and physical properties of chicken sausages. Cassava MU51 cultivar was used for the extraction of cassava starch due to the higher availability and higher starch extraction yield (25% – 30%). Textural properties of cassava starch are also important to improve the texture of sausage. Also, the use of cassava starch as a composite flour mixture reduces the cost of sausage raw material.

## Scientific hypothesis

The replacement of composite flour mixture with cassava starch decreases the cost of preparation, improves the organoleptic properties and nutritional composition of fresh chicken sausage.

## MATERIAL AND METHODOLOGY

### Samples

MU51 variety is one of the most cultivated and recommended cassava varieties in Sri Lanka. Thus, the pre-determined quantity of cassava MU51 variety was purchased from a cassava cultivator in Bandaragama, Sri Lanka.

### Chemicals

Kjeldahl Tablets (Kjeldahl Tablets, Suphelco, Sigma-Aldrich Chemie GmbH).

Conc. $H_2SO_4$ (Concentrated Sulphuric acid, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 99.999%).

Conc. NaOH (Sodium Hydroxide, Sigald, Sigma-Aldrich Chemie GmbH, ≥98%)

95% Ethyl Alcohol (Etanol, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 95%).

6N HCl (Hydrochloric Acid, Sigma, Sigma-Aldrich Chemie GmbH, 37%).

Diethyl Ether (Diethyl Ether, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 99.7%).

Petroleom Ether ( Petroleom Ether, SIGALD, Sigma-Aldrich Chemie GmbH, 99%).

Phenol (Phenol, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 99%).

Potassium cyanide (Potassium cyanide, Sigald, Sigma-Aldrich Chemie GmbH, 99%).

Pictric Acid (2,4,6-Trinitrophenol, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 99%).

Methyl Red (Methyl Red, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 99.5%).

Methyl Blue (Mthyl blue, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, 70%).

Peptone (Peptone, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH).

Nutrient agar (Nutrient Agar, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH).

Potato Dextrose Agar (Potato Dextrose Agar, Sigma-Aldrich, Sigma-Aldrich Chemie GmbH).

### Instruments

The Brookfield texture analysis (Model: CT3-4500, Brookfield Engineering, USA).

UV–Vis spectrophotometer (Model: UV mini-1240, SHIMADZU CORPORATION, Japan).

Muffle furnace (Model: SHIMADEN SR1, SHIMADEN CO., LTD, Tokyo).

Polarized Zeeman atomic absorption spectrophotometer (Model ZA3000, HITACHI, Japan).

### Laboratory Methods

Analysis of storage stability – **(SLS 516, 2013)**.

Pre-gelatinized modification (PG) – **Rajapaksha et al. (2017)**.

Nutrition Analysis of sausage – **AOAC (2012)**.

Toxicological Analysis – Spectrophotometric Determination of Cyanide (Picric acid method) by **Wood (1965)**.

### Description of the Experiment

### Sample preparation:

***Extraction of cassava starch***

Cassava starch was extracted according to the method described by **Rajapaksha et al. (2017)**. Roots of the best quality were washed and peeled manually and grated into small pieces. They were ground with normal tap water in a 1:4 ratio. The slurry was filtrated through a muslin cloth and the filtrate was allowed to sediment for 4 – 5 hours. The sediment starch was separated by decanting and starch was dried by keeping in the mechanical dryer at 60 ºC for 5 hours until the moisture content reduced up to around

10%. The dried starch was ground and passed through a 300-micron sieve.

Finally, ground starch was packed in a polyethylene package and was stored (-5 °C) in an airtight container.

***Pre-gelatinization modification of cassava starch***

Pre-gelatinized modification (PG) was done with slight modifications of the method described by **Rajapaksha et al. (2017)**. As 1:10 starch solution (100 g of starch in 1000 mL deionized water) was incubated at 65 °C for 10 minutes. The starch slurry was allowed to sediment for 4 – 5 hours. Then starch sediment was dried at 45 °C till the moisture level dropped to 10% – 13%.

The pre-gelatinized cassava starch was packed in a polyethylene package and stored under cold room conditions at -5 °C.

***Formulation of sausage***

The basic ingredients used for the chicken sausage were lean meat (minced chicken) fat, ice, isolated soy protein, composite flour mixture, rice flour, maize powder, bread crumbs, milk powder, cassava starch (native and modified), sugar, salt, garlic, black pepper, and chili powder. The amount of ingredients added to sausage preparation is summarized in Table 1.

**Number of samples analyzed:** Sensory and color analysis – five samples, Nutritional composition, and microbial quality analysis – One sample with three replications for the final sample.

**Number of repeated analyses:** Three times.

## Design of the experiment

Two cassava starch types (i.e., native cassava starch ($a_0$) and modified cassava starch ($a_1$)) were used in the experimental design. In addition, two replacement levels (i.e., 50% ($b_0$) and 100% ($b_1$)) were included.

Four different sausage batters were prepared by replacing composite flour mixture with cassava native starch in 50% ($a_0b_0$) and 100% ($a_0b_1$) levels and with cassava modified starch in 50% ($a_1b_0$) and 100% ($a_1b_1$) levels. A sausage was prepared without replacing the composite flour mixture as a control. Other ingredients and additives were incorporated in the formulated batters (Table 1).

The batter was filled into cellulose casings and cooked in steam for 15 minutes until the core temperature becomes 72 °C. Then the prepared sausage was stored at -5 °C in a freezer for further analysis.

## Analyzing the effect of sensory and physical properties of replacements (developed formulae)

***Analysis of sensory attributes of sausages***

Six sensory attributes (i.e., Appearance, Colour, Aroma, Flavour, Hardness, Overall acceptability) were analyzed by using the five-point hedonic scale (5 = Like extremely, 1= Extremely dislike) and 30 untrained panelists.

***Texture profile analysis of sausages***

The texture profile (TPA) was analyzed according to the method described by **Bourne (2002)** with slight modifications by using the Brookfield texture analysis (Model: CT3-4500, Brookfield engineering, USA) at a constant crosshead velocity of 1 $mm.s^{-1}$, Trigger point of 10.0 g, and cylindrical sausage samples with a diameter of about 17 mm and a height of about 10 mm were used for TPA evaluation. Each test was performed with four replications. Hardness, Springiness, Gumminess, and Chewiness factors were evaluated in the test.

***The Color analysis of sausages***

Values for the color dimensions L*, a*, b*, C*, and h* were measured by using the Colorimeter (Lovibond).

## Analyzing the nutritional and toxicological composition of the developed formulae

***Nutritional composition analysis of sausage***

Moisture content, crude protein content, crude fat content ash content, and acid insoluble ash contents were analyzed for the top-ranked sausage samples ($a_0b_0$ - 50% replacement with Cassava Native starch) following the **AOAC (2012)** standard methods.

The total solids content was analyzed according to the arithmetic calculation.

The top-ranked sausage sample was subjected to dry ashing (using muffle furnace at 500 °C) and analyzed for the presence/quantity of Na, K, Ca and Mg minerals using Atomic Absorption Spectrophotometric (AAS) method.

Starch content was measured by the method of Dubois **(Dubois and Cotter, 1955)** with slight modifications.

***Toxicological composition analysis (Cyanide content)***

The cyanide content of the top-ranked sausage sample ($a_0b_0$ – 50% replacement with Cassava Native starch) was analyzed according to Spectrophotometric Determination of Cyanide (Picric acid method) by **Wood (1965)**.

About 10 – 20g of the grounded top-ranked sausage sample was weighed and the weight was recorded. Then the starch sample was introduced into a triple-necked round bottom flask containing 100 mL of deionized water. About 50 mL of 2N $H_2SO_4$ was added to the round bottom flask. The flask was immediately connected to the steam generator and the distillate was collected in 50 mL of $Na_2CO_3$ solution till it becomes 200 ml. The solution was transferred into a 250 mL volumetric flask and the volume was made up to 250 mL using deionized water.

Then 10.00 mL of the distillate (final solution should be contained 3 mL of 5% $Na_2CO_3$), and 4 mL of 1% Pictric acid were added into a boiling tube and the boiling tube was immersed in a boiling water bath for 12 – 15 minutes. Then the solution was left to cool to room temperature and volume was made up to 25 mL.

Finally, the absorbance was measured at 530 nm in UV-Vis spectrophotometer (VU mini – 1240) for the developed color of CN- with Picric acid. Reading of absorbance was noted down and CN- concentration was calculated based on the equation (Cyanide Content = (absorbance+0.03)/0.0048) which was been first developed using standard concentration series of KCN solution.

***Analysis of storage stability***

The total plate count and coliforms of the prepared sausage samples were determined during the storage using the methods described by **SLS 516 (2013)**.

***Total plate count-(SLS 516: part 1 Section 2:2013)***

The total plate count of the sausage sample was performed using a Colony count at 30 °C by the surface plating technique.

***Detection and enumeration of Coliform (SLS 516: Part 3 Section 1: 2013)***

Coliform detection and enumeration of frozen fries samples were carried by the Most Probable Number (MPN) method.

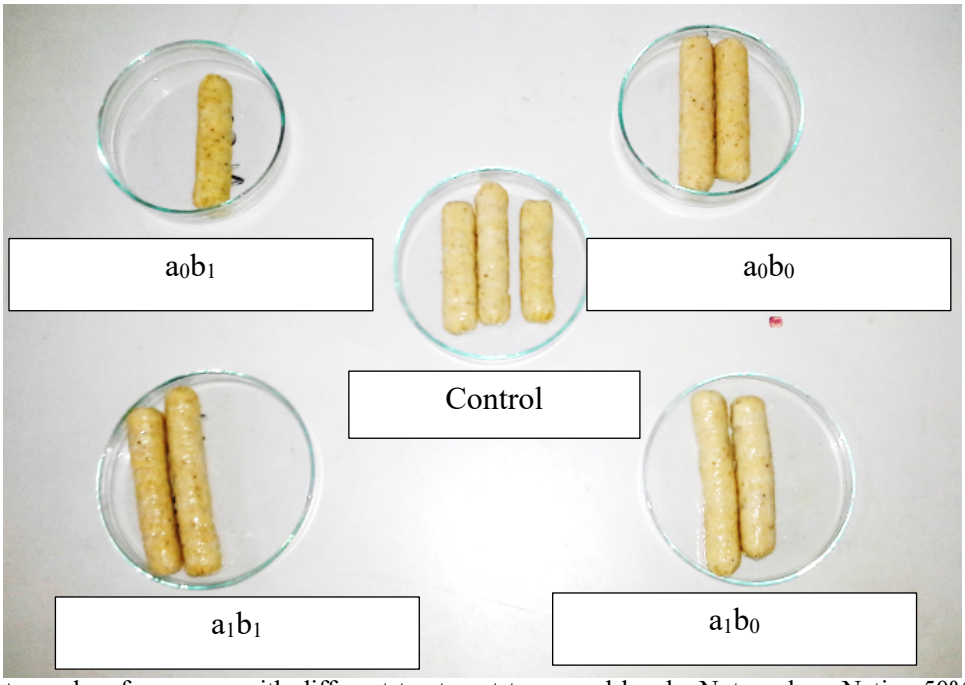


**Figure 1** Photographs of sausages with different treatment types and levels. Note: $a_0b_0$ – Native 50%, $a_0b_1$– Native 100%, $a_1b_0$ – Modified 50%, $a_1b_1$ – Modified 100%, C- 0% Replacement.

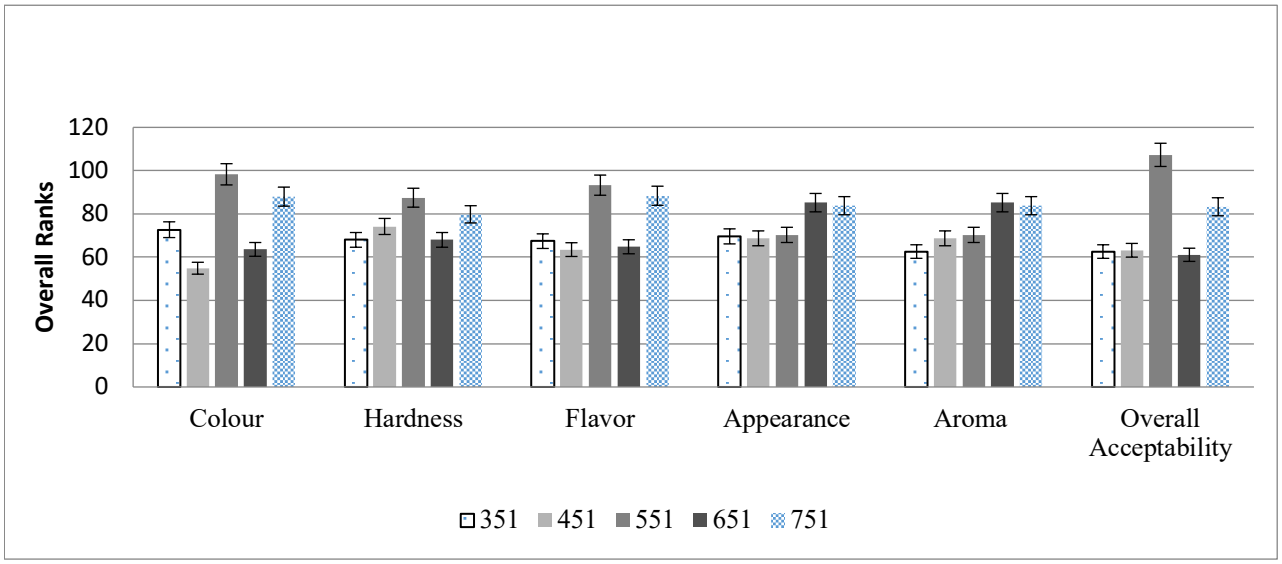


**Figure 2** Bar Chart of Friedman overall of Ranks 751 – 50% replacement with Cassava Native starch, 351 – 100% replacement with Cassava Native starch, 451 – 50% replacement with Cassava Modified starch, 651 – 100% replacement with Cassava Modified starch, 551 – Control.

### Statistical Analysis

All the parametric and non-parametric data were analyzed by using MINITAB 17 statistical tool. Non-parametric data which are results obtained from the sensory analysis were analyzed using the Friedman ranking test. One-way ANOVA was used to analyze the significant differences of mean values of each sample, followed by Tukey Pairwise comparison test to analyze the samples that had a significant difference in color and texture. Two sample t-test was used to analyze the significant difference in nutritional composition by referring to the reference values obtained from SLS and USDA standards.

## RESULTS AND DISCUSSION

### Sensory analysis

As the sensory profile is the most critical factor results of the sensory analysis were used for the selection of the best sample. The top-ranked sample was 551(Control sausage-0% replacement) according to the average ranking of Friedman analysis. The second best-ranked sample was 751 ($a_0b_0$ – 50% replacement with cassava native starch) according to the results of the Friedman ranking analysis. Results further suggest that the color, appearance, and overall acceptability of all the treatment levels are significantly different from the control sausage sample, ($p$ <0.05) caused by the treatment on that parameters. Since the p-value for hardness, flavor, and aroma of all the

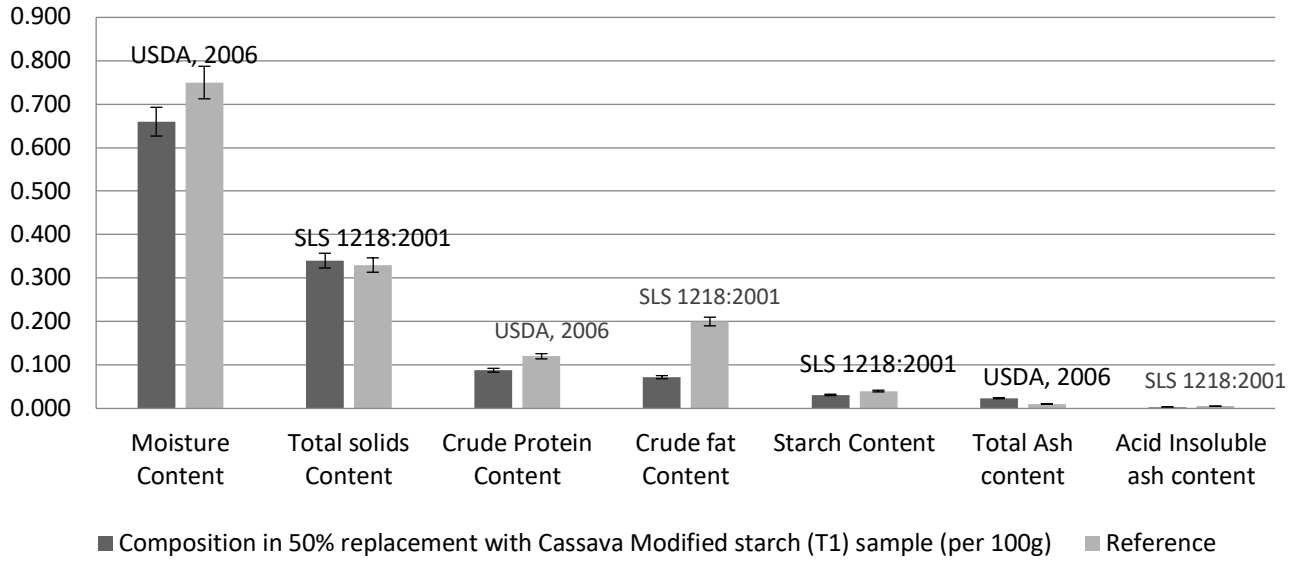


**Figure 3** Bar chart of compares nutritional composition of sausage with referred SLS and USDA standards.

treatment levels was 0.05, there was no significant effect caused by the treatment on those parameters.

**Garcia-Santos et al. (2019)** showed that no effect on the sensory parameters including appearance, color, flavor, texture, aroma, and overall acceptance attributes of the Beef and Chicken mix sausages with the treatment of cassava starch. Also, **Grizotto et al. (2012)** carried out a study with the addition of two types of okara flour in Frankfurter type sausages with partial (1.5%) and total (4.0%) replacement of texturized soy protein and concluded that the addition of okara flour did not influence sensory acceptance.

## Texture profile analysis

According to the texture profile analysis data $a_0b_0$ sample has been given 1475 ±278 (g) for gumminess, 99.5 ±19.7 (mJ) for Chewiness, 0.24 ±0.01 for cohesiveness, and 6.88 ±0.06 (mm) for springiness values.

Since the *p* values were higher than 0.01 (at 1% significant level) there was no relationship between the treatment level of each treatment type with gumminess, chewiness, cohesiveness, and springiness. However, the addition of wheat fiber had a significant effect on the hardness of sausages **(Choe et al., 2013)**.

According to **Chang et al. (2012)**, the textural characteristic of sausages with modified starch were better than sausage with native starch. But in their research, the starch modifications used were starch acetate, cross-linked esterified starch, and phosphate starch, wherein current research heat moisture treated starch was used for the experiment **(Chang et al., 2012)**.

## Colour analysis

In the colorimetric values analysis L*, a*, b*, c*, $h_o$ values refer to lightness, redness, yellowness, saturation, and hue, and those dimensions were considered. According to the color values analysis results, there was no significant effect (at 0.05 significant level) by the 50% replacement by cassava native starch ($a_0b_0$) on lightness and hue. However, that treatment ($a_0b_0$) affected the redness, yellowness, and saturation of sausage.

The 100% replacement by the cassava native starch ($a_0b_1$) significantly affected (at 0.05 significant level) lightness, redness, and hue. However, it was not significantly affected by yellowness and saturation. A similar result was observed by **Hughes, Mullen and Troy (1998)**. According to **Hughes, Mullen and Troy (1998)**, incorporation of tapioca starch decreased the redness and lightness of the frankfurters, whereas yellowness was not significantly altered.

The 50% replacement with Cassava Modified starch ($a_1b_0$) was significantly influenced (at 0.05 significant level) on the lightness and redness. Also, it was not significantly affected by yellowness and saturation.

But the treatment of 100% replacement with Cassava Modified starch ($a_1b_1$) was significantly affected (at 0.05 significant level) on the lightness, redness, yellowness, and saturation and significantly not affected on hue according to Tukey's pairwise comparison results.

Since the p values are less than 0.01 (at 1% significant level) for the correlation of $a_0$ vs. L*, a1 vs. a*, $a_1$ vs. L*, $a_1$ vs. a*, $a_1$ vs. b*, $a_1$ vs. C* and $a_1$ vs. $h_o$ ($p$ <0.01) the treatment with cassava starch was significantly affected on color.

According to the Pearson correlation analysis results, there was a strong negative relationship between $a_0$ vs. L*, $a_1$ vs. L*, and $a_0$ vs. $h_0$. Also, there was a strong positive relationship between $a_1$ vs. a*, $a_1$ vs. b*, and $a_1$ vs. C*.

## Nutritional Composition analysis

The top-ranked sample showed average moisture, crude fat, crude protein, total solids, ash, and acid-insoluble ash content of 65.98%, 7.19%, 8.79%, 34.02%, 2.36%, and 0.32% respectively. The starch content of the top-ranked sample was 3.12%. According to **Bozhko et al. (2020)**, the protein content in meat containing semi-smoked sausages

with different ratios of duck and fish meat varied within 17.90%-21.34%.

According to the SLS and USDA standards moisture, crude fat, crude protein, total solids, ash, and acid-insoluble ash contents were 74.6%, 33%, 12.1%, 20%, 4 %, 1%, and 0.5% respectively **(SLS 1218:2001; USDA, 2006)**. According to the two-sample t-test results, the p-value for total solids content, there was not a significant effect on the total solids content by the 50% replacement with cassava native starch. Also since the p values for other nutritional properties are less than 0.05 the replacement was significantly affected on that nutritional property.

Previous research on the effect of substitution of fat with Sorgam and finger-millet showed protein, fat, ash, crude fiber, and carbohydrates contents as 10.4%, 3.1%, 1.6%, 2.0%, and 70.7% respectively **(Das et al., 2013)**.

High fat consumption has been associated with cardiovascular diseases, obesity, cancer, and hypertension, among other illnesses **(Mapiye et al., 2012; Hygreeva, Pandey and Radhakrishna, 2014)**.

According to the AAS analysis results, there was a high Na content in the sausage sample which was 1348.39 mg.100g$^{-1}$. Also, the K and Mg contents were 232.15 mg.100g$^{-1}$ and 73.21 mg.100g$^{-1}$ respectively. According to the analysis the absorbance value for the Fe content was not observed in the standard range. Calculation suggests that the Fe content of the sausage sample should be less than 0.012 mg.100g$^{-1}$.

### Toxicological composition

Cassava starch has a cyanide content of 7.05 mg.kg$^{-1}$ on a dry basis **(Rajapaksha et. al, 2017).** The cyanide content in the sausage sample was around 1.54 ±0.02 mg.kg$^{-1}$ on a dry basis. Around 0.0528 mg amount of cyanide contained in added 7.5g of starch sample. The cyanide content of the product has been further reduced due to the processing steps in sausage processing such as steaming. **(Akingbala, Oguntimein and Abass (1991)**.

### pH analysis

The detected average pH value of the sausage was 5.64 ±0.11. The standard level for pH 6.6 has been shown by **Venturini et al., (2011).** According to **Garcia-Santos et al. (2019)** addition of resistant starch in sausages increased pH values where the average varied from 6.10 to 6.21.

### Analysis of storage stability

Microbial analysis was conducted by TPC and coliform test. Total plate count was analyzed with the purpose of shelf life analysis of the best-ranked sausage and coliform analysis was conducted to determine whether the coliform bacteria are present or absent in the sausage sample.

Sausage is usually exposed to high temperatures by heating till the middle temperature becomes greater than 74 °C by using the steaming process. But this temperature might not be enough to inactivate all the microorganisms.

Sausages are also re-contaminated with spoilage bacteria during the processing stages followed after cooking. Since that the initial plate count was around 10 C.F.U.g$^{-1}$.

The excessive proliferation of present microflora in the sausage content or on the surface during storage causes economic losses because of spoilage and deterioration. Especially, lactic acid bacteria are considered to be a major component of the microbial population found on vacuum-packaged sausages **(Özdemir, 1997)**. Lactic acid bacterial growth on the surface of the sausages produces undesirable sensory attributes **(Korkeala and Björkroth, 1997)**.

Findings from research by **Onishi. 1957** has shown that the growth of osmophilic yeast in the presence of 18% NaCl was limited to the range pH 4.0 – 5.0 and according to the findings in current research the pH was higher than this limited range and because of that, the analysis was not done for yeasts and molds.

## CONCLUSION

The best composite flour mixture replacement formula was a 50% composite flour mixture with cassava native starch. There was no significant effect on the total solids content by the 50% replacement with cassava native starch and it was significantly affected on moisture content, protein content, fat content, starch content, ash content, acid insoluble ash content, and pH value of the final product. Shelf life analysis of developed sausage suggests that the product was safer to consume up to 14 days of manufacturing under frozen conditions (-5 °C).

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**Conflict of Interest:**
No conflicts of interest to declare regarding the present study.

**Ethical Statement:**
Not applicable.

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# *SATUREJA MONTANA* L. ESSENTIAL OIL VARIOUS DOSAGES EFFECT ON THE MAIN RATS' BIOLOGICAL FEATURES

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## ABSTRACT

An application of natural antioxidants remains the focus of research groups. The effect of *Satureja montana* L. essential oil in various doses on the main biological characteristics of Wistar rats was the main aim of the study. The intensification of protein metabolism in the blood plasma of rats on the background of the use of *Satureja montana* L. essential oil was noted. Total protein increases by 17.9 – 19.7%, and albumin by 27.6% in rats of the experimental group received the essential oil at a dose of 0.6 ml per kg of feed. A significant increase in the AST level in control group rats to 207.3 $U.L^{-1}$ was revealed. Its one to a certain extent indicates the hepatoprotective effect of mountain savory oil and a decrease in inflammatory processes in the organs of the gastrointestinal tract in the conditions of cell maintenance of rats of the experimental groups. Gastric epithelium thickness of rats of both experimental groups was lower than the control animals. But it did not bear any signs of atrophy. The difference of this indicator in comparison with control was 12.75 μm ($p \leq 0.05$) in the second group and it was reliable. The number of chief stomach cells increases in animals of the experimental groups, which may indicate a greater enzymatic activity. An increased dose of mountain savory oil contributes to the formation of more damage to hepatocytes on the periphery of the liver lobule. Thus, the relationship between liver enzymes and the state of peripheral hepatocytes was noted.

**Keywords:** *Satureja montana* L.; essential oil; rats; live weight; plasma; stomach; liver

## INTRODUCTION

The interest in natural-origin antioxidants due to the significant content of biologically active substances increased in recent years all over the world. The study of the effect of natural antioxidants on rats is widely practiced because of a simple and understandable model for research **(Nurmawati et al., 2021; Widyastuti et al., 2020; Fauza et al., 2019)**.

Essential oils (EO) are one of these antioxidants. EO has antiseptic, antibacterial, antiparasitic, antifungal, insecticidal, anti-inflammatory, and regenerating properties. It exerts a defined effect on the body, regardless of the different ways of application. The effect is usually intensified also with prolonged use **(Krishan and Narang, 2014)**.

*Piper guineense* EO effect investigated in mice by intraperitoneal application on the central nervous system (CNS). Sedative, anticonvulsant, and hypothermic effects were detected **(Oyemitan et al., 2015)**. A pronounced anxiolytic, antidepressant, and antioxidant effect in the study of *Ferulago angulata* essential oil in rats was recorded with inhalation use **(Bagci et al., 2016)**. The antidepressant EO effect of the *Asarum heterotropoides* was studied **(Park et al., 2015)**.

The anxiolytic effect of vetiveria essential oil, when inhaled for 7 minutes to reduce the symptom of anxiety on Wistar line male rats, was studied. 2.5% is the most effective concentration was found **(Saiyudthong et al., 2015)**. The anxiolytic and antidepressant effects of inhaled basil (*Ocimum sanctum* L.) and holy basil (*Ocimum basilicum* L.) EOs on the background of experimental Alzheimer's disease in rats have been proven **(Gradinariu et al., 2015)**. Elsholtzia ciliate (*Elsholtzia ciliate*), angelica (*Angelicae gigantis*), and clove (*Eugenia caryophyllata*) essential oils in the complex therapy of withdrawal syndrome can be used **(Choi et al., 2013)**.

Cypress obtuse (*Chamaecyparis obtusa*) essential oil at a concentration of 7.0 mg/l of air on male mice of the ICR line was studied. An anxiolytic effect was recorded **(Bae et al., 2012)**. *Toona ciliata* M. Roem essential oil antidepressant effect on mice in doses of 10, 20, 40, and 80 $mg.kg^{-1}$ was administered creating. Clove tree

(*Syzygium aromaticum*) EO with the intragastric influence of 200 mg.kg$^{-1}$ dosage was established **(Castro et al., 2015)**.

The neuroprotective effect of *Lavandula angustifolia* EO on the background of an experimental stroke in Swiss line male mice at a dose of 0.3 – 2 mg.kg$^{-1}$ intraperitoneally was proved **(Vakili et al., 2014)**.

*Lavandula angustifolia* essential oil at the background of insomnia reduces anxiety **(Chioca et al., 2013)**. Similar results were obtained with intraperitoneal administration at a dose of 0.7 mg.kg$^{-1}$ of male Wistar rats in the study of *Lavandula angustifolia ssp.* and *Lavandula hybrida Rev. (Lamiaceae)* essential oils **(Hritcu et al., 2012)**. The use of these essential oils significantly reduced the level of anxiety and inhibition, showed an antidepressant effect, reduced the level of nitric oxide and malondialdehyde in brain tissues. Lavender officinalis (*Lavandula officinalis*) essential oil in the conditions of insomnia to rats a sedative effect was rendered **(Takahashi et al., 2014)**.

The effect of perilla essential oil (*Perilla frutescens*) by intragastric administration in rats was studied **(Yi et al., 2013)**. The psychophysiological effect occurs only with prolonged use at least 3 weeks. The effect of lemongrass (*Cymbopogon citratus*) essential oil on Swiss line male mice per intragastric introduction in dosage 0.5 – 1.0 g.kg$^{-1}$: sedative, anxiolytic, anticonvulsant, anticonvulsive effects were recorded **(Campélo et al., 2011)**.

The effect of chamomile essential oil (*Matricaria chamomilla* L.) in mice that received chamomile EO in 5000, 2500, and 1250 ppm concentrations was studied **(Fabian et al., 2011)**. Chamomile essential oil can improve some parameters of inflammatory models, depending on the concentration used. The essential oil of clove (*Calamintha officinalis*) has antifungal and antimicrobial activity against gram-positive bacteria was founded **(Monforte et al., 2011)**.

The effectiveness of the use of mountain savory has already been studied by us in poultry farming **(Pashtetsky et al., 2020)**. Mountain savory (*Satureja Montana* L.) is a perennial semi-shrub with lignified shoots at the base of the root, belonging to the family of Clear-cut flowers (*Lamiaceae* L), has a sharp, spicy aroma, with a slight burning pepper taste. The biological value of S. montana is due to the complex of biologically active substances, among which the leading position is occupied by volatile compounds. Of the proven therapeutic properties, mountain savory essential oil has anti fungicidal, antibacterial, antiviral, immuno-strengthening, hepatoprotective, analgesic, tonic effects **(Mihajilov-Krstev et al., 2014)**. Mountain savory essential oil on nine phytopathogenic fungi of the genus Fusarium has an effect that suppresses their growth **(Rahimian and Eisvand, 2016)**.

Carvacrol and thymol are the main components of the essential oil. Their mechanism of action is the destruction of the cytoplasmic membrane, which increases its permeability and depolarizes its potential. **(Allaoua et al., 2018)**. The effect of thymol on the smooth muscles of the trachea, the ciliary apparatus of the respiratory tract and the ileum in rats and found that thymol has a dose-dependent antispasmodic property and increases the rate of contraction of the mucous membrane due to the movement of cilia was founded **(Orchardand and van Vuuren, 2017)**.

Essential oils in the Republic of Crimea occupy a significant part in the agrarian-and-industrial complex of the region. In addition to the perfumery, cosmetics, and pharmaceutical areas, the use of essential oils in animal husbandry is little studied, so the construction of an antioxidant model for the use of essential oils is very necessary **(Ostapchuk et al., 2020)**. It is also reported about its effective use in complex therapy in the treatment of combined bacterial and fungal infections, including COVID-19 **(Postnikova et al., 2021)**.

Based on the analyzed sources, the use of essential oils as natural antioxidants continues to be the focus of scientists ' attention. Since essential oils provide a wide range of effects on a living organism, the purpose of these studies is the effect of mountain savory (*Satureja montana* L.) essential oil in various dosages on the growth and basic biological features of rats.

## Scientific hypothesis

Research work hypothesis hinged on the assumptions that the phenolic substances contained in mountain savory oil have an antioxidant effect on the body of rats. As an object of the study has been selected *Satureja montana* L. essential oil due to the high content of valuable phenolic substances. According to obtained results the improvement of indicators of the main rats' biological features under the influence of plant phenols, has been vindicated.

## MATERIAL AND METHODS

The experimental study was conducted jointly by the staff of the Research Institute of Agriculture of Crimea and the Medical Academy named after S. I. Georgievsky of V.I. Vernadsky Crimean Federal University.

**Animals and Biological Material**

Wistar line adult male rats aged 5 – 6 months with 180 g average weight. The animals were weighed daily. After removing the animals from the experiment, samples of the liver and stomach were taken.

**Chemical**

Mountain savory essential oil is obtained by steam distillation. In the laboratory of selection of essential oil crops on the chromatograph. The composition of mountain savory essential oil includes the following plant phenols: a-pinene, camphene, b-pinene, β-myrcene, a-terpinene, limonene, eucalyptol, g-terpinene, p-cymene, cis-linalool oxide, sabinene hydrate, camphor, linalool, linalyl acetate, caryophyllene, caryophyllene oxide, thymol, and carvacrol. The highest content was of the following components: carvacrol (49.88%), p-cymene (15.76%), y-terpinene (15.28%), a-pinene (2.52%), a-terpinene (2.07%) and thymol (0.23%).

The biochemical composition of animal blood plasma was determined according to the following indicators: total protein (TP, g.L$^{-1}$), albumin (ALB, g.L$^{-1}$), glucose (GLUC, mmol.L$^{-1}$), alanine aminotransferase (ALT, U.L$^{-1}$), aspartate transaminase (AST, U.L$^{-1}$), alkaline phosphatase (ALP, U.L$^{-1}$), creatinine (CREA, μmol.L$^{-1}$), urea (UREA, mmol.L$^{-1}$), bilirubin (BILT, μmol.L$^{-1}$). Whole blood was also separated to study the shaped elements of the blood.

**Laboratory Methods**

After three weeks, the animals were removed from the experiment under ether anesthesia, the stomach and liver were separated, fixed in 10% buffered formalin, and subjected to dehydration and paraffin impregnation on a histology processor. Thin sections with a thickness of 4 microns were made from the obtained paraffin blocks, stained with Hematoxylin and Eosin, and viewed in a light field under a microscope. The images obtained in the *ImajeG* program, after appropriate calibration on the TS-M1 scale of 0.01 mm/100 div stage micrometer, measured the height of the gastric epithelium, the depth of the own glands, the thickness of the gastric mucosa, the number of epithelial cells in the epithelium (per 1 villus), the relative number of the chief and parietal epithelium cells in glands of the stomach. In the photo of liver preparations, the number of binucleated cells, the relative areas of sinuses and hepatocytes in the central and peripheral parts of the hepatic lobule were counted, and the nuclear-cytoplasmic ratio of hepatocytes was calculated.

**Instruments**

Chromatek-Crystal 5000.2 chromatography device on a capillary quartz column with a fixed phase CR-WAXms (polyethylene glycol salt-and-gel matrix). Capillary quartz column with a length of 60 mm with an internal diameter of 0.32 mm, fixed phase CR-WAXms (polyethylene glycol salt-and-gel matrix). The thermostat temperature is 80 °C, lasting 6 minutes. The next step is programming with a speed of 2.1 °C per min. up to 220 °C. Evaporator temperature: 260 °C. Detector temperature: 240 °C. Column inlet pressure: 100 kPa flow division: 1/50. Sample volume: $0.5\times10^{-3}$ $cm^3$.

Biochemical Vitalab Flexor E automatic analyzer.

Hematology Biobase analyzer.

LOGOS microwave histology processor.

DM 2000 Leica microscope with Plan 10× and Plan 40× lenses and a DFC295 camera.

**Description of the Experiment**

The number of heads in each group was 5. The animals were kept in standard vivarium conditions. The rats have divided into 3 groups: the control group received the main diet (MD) (1st group). The 2nd group was experimental: animals received mountain savory (*Satureja montana* L.) essential oil at the rate of 0.3 mg per group daily on the background of MD. The 3rd group was experimental too: animals received essential oil at the rate of 0.6 mg per group daily on the background of MD. The experiment duration was 20 days. The animals obtained the main diet in *ad libitum* conditions and 12-hour daylight hours were. The MD included dry food and fresh vegetables and herbs.

**Sample preparation:** The blood was collected from the sublingual plexus under weak ether anesthesia into EDTA tubes. The blood was centrifuged at 3000 rpm for 5 minutes to separate the plasma. The stomach and liver were sent for pathomorphological studies.

**Number of samples analyzed:** 15 heads

**Number of repeated analyses:** 15

**Number of experiment replication:** There is no experiment replication in our study

**Statistical Analysis.**

The obtained data were processed by methods of descriptive statistics with the determination of the normality of the distribution by the Shapiro-Wilk method, calculation of the arithmetic mean, confidence interval, and mean statistic error. Nonparametric statistics are the determination of differences between groups by multiple comparisons of several groups by the Kruskal-Wallis method was used. All data were analyzed statistically using Excel for Windows. Significant differences were considered at $p \leq 0.05$.

## RESULTS AND DISCUSSION

The body weight increasing in both experimental groups of rats mountain savory essential oil using contributed. Live weight increasing in the middle and at the end of the experiment was most expressed. The results in Table 1 were stated.

Rats' live weight of the second group on the 10th day of the experiment has no significant advantages in comparison with the control group ones. A significant difference on the 20th day of the experiment was 3.4% ($p \leq 0.05$).

The 3rd group rats have a significant tendency of the live weight during the entire period of the experiment over the control ones. A significant difference was on the 10th day by 6.8% ($p \leq 0.05$), and on the 20th day by 10.5% ($p \leq 0.05$).

Live weight dynamics of all individuals in the experiment are stated in Figure 1.

Thus, the conducted studies have established that the tested essential oil has a growth-stimulating effect on rats. However, live weight data is not enough to fully characterize the development of animals at the background of essential oil use.

Blood plasma biochemistry data on the 21st day of the experiment were obtained. These results are stated in Table 2.

AST increment of the control group animals to 207.3 $U.L^{-1}$ ($p < 0.001$) was significant. One of the main reasons for transaminase enzyme activity level increasing in the animals of the control group probably can be assumed to be the presence of non-critical destructive processes in hepatocytes. This one, probably, is they tend to develop at the standard content of rats in cells. Bilirubin increasing tendency in animals of the control group was noted. The amount of bilirubin decreasing in the 3rd group may be associated with a decrease of this enzyme in the liver. Thus, a mountain savory essential oil hepatoprotective effect on the rats' body to a certain extent.

AST dynamics concentration during the experiment to the studied groups of rats was studied and shown in Figure 2. Unstable variation of this indicator in the animals of the control group was noted. In the animals of the second group, the tendency to decrease from 154.9 $U.L^{-1}$ on the 10th day of the experiment to 135.3 $U.L^{-1}$ on the 20th day was noted. This indicator fluctuation in the animals of the 3rd group during the experiment was practically absent.

The urease level of the experimental groups increased by 2.0 – 1.9 times ($p < 0.001$) on the 10th day. It indicates an increased urease role in the process of amino acid synthesis by bacteria, which are intestinal microbiota nitrogen source forming. That effect in the animals of the experimental groups from the 10th day at the level of 12.5 – 13.2 to 12.6 – 12.2 $mmol.L^{-1}$ was observed (Figure 3).

**Table 1** Rats' live weight dynamics in the experiment, g.

| Statistical indicator | Day of experiment | | |
|---|---|---|---|
| | 1st | 10th | 20th |
| | | 1st group | |
| X±mx | 179.0 ±1.2 | 190.5 ±2.5 | 208.3 ±3.9 |
| σ | 2.7 | 5.5 | 8.8 |
| Cv, % | 1.5 | 2.9 | 4.2 |
| | | 2nd group | |
| X±mx | 180.0 ±1.1 | 197.0 ±2.7 | 223.2 ±6.2* |
| σ | 2.6 | 6.1 | 13.8 |
| Cv, % | 1.4 | 3.1 | 6.2 |
| | | 3rd group | |
| X±mx | 178.0 ±1.2 | 203.5 ±3.8* | 230.2 ±9.6* |
| σ | 2.7 | 8.4 | 21.5 |
| Cv, % | 1.5 | 4.1 | 9.4 |

Note: Here in table level of statistical significance: * – $p \leq 0.05$.

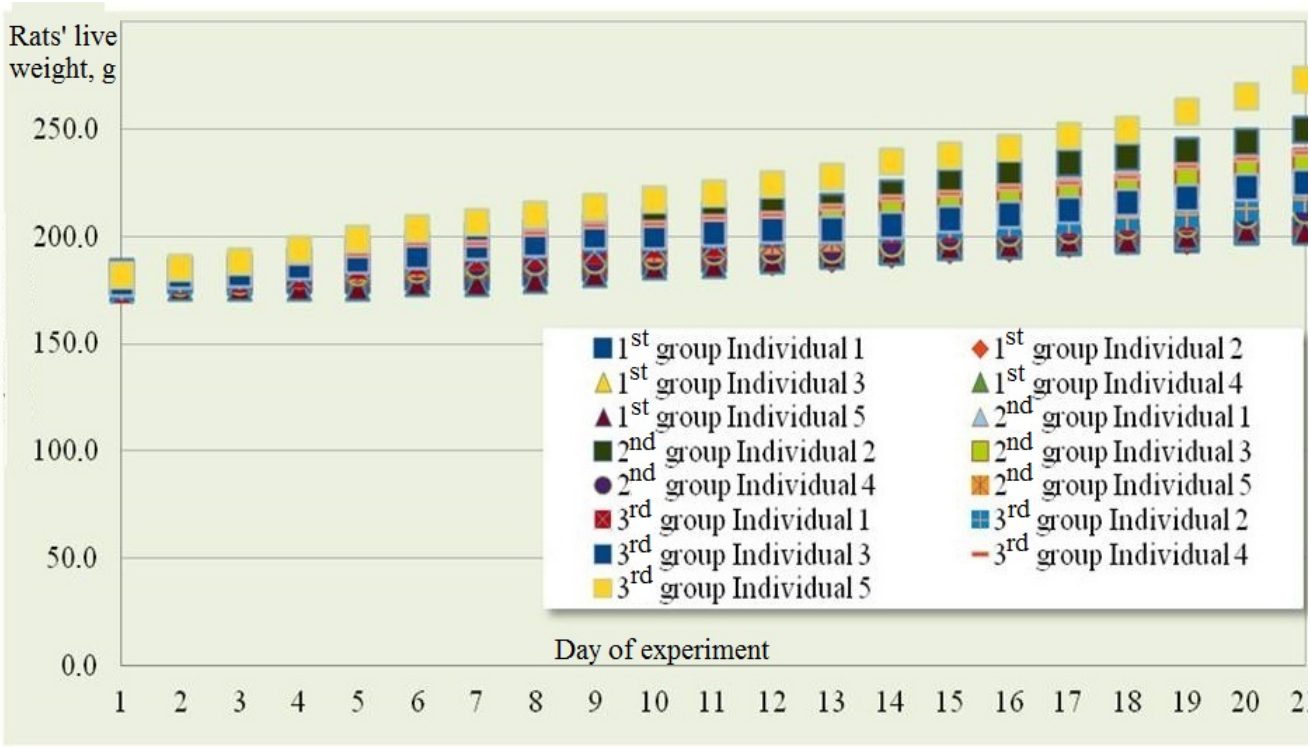


**Figure 1** Rats' live weight dynamics of all individuals in the experiment, g.

**Table 2** Rats' blood plasma biochemical features on the 21st day of experiment (n = 3).

| Biochemistry indicator | 1st group | | 2nd group | | 3rd group | |
|---|---|---|---|---|---|---|
| | X±mx | Cv, % | X±mx | Cv, % | X±mx | Cv, % |
| ALT | 59.3 ±1.3 | 3.7 | 56.1 ±2.4 | 7.5 | 64.5 ±4.3 | 11.4 |
| AST | 207.3 ±7.8*** | 6.5 | 135.3 ±4.0 | 5.1 | 134.2 ±2.7 | 3.5 |
| BILT | 2.4 ±0.3 | 20.7 | 2.5 ±0.2 | 11.0 | 2.0 ±0.2 | 14.0 |
| UREA | 6.3 ±0.3 | 8.5 | 12.6 ±0.4*** | 5.5 | 12.2 ±0.3*** | 3.9 |
| ALP | 323.7 ±3.3 | 1.8 | 326.7 ±10.9 | 5.8 | 322.7 ±18.0 | 9.6 |
| CREA | 46.7 ±2.1 | 7.9 | 41.3 ±3.0 | 12.5 | 45.2 ±2.1 | 7.9 |
| GLUC | 8.9 ±0.2 | 4.5 | 9.4 ±0.3 | 5.5 | 9.5 ±0.3 | 5.3 |
| TP | 67.0 ±1.2 | 3.0 | 80.2 ±1.7*** | 3.7 | 79.0 ±1.1*** | 2.5 |
| ALB | 29.3 ±0.2 | 1.3 | 37.4 ±1.5*** | 6.8 | 31.3 ±1.4 | 8.0 |

Note: Here in table level of statistical significance: * – $p \leq 0.05$; ** – $p \leq 0.01$; *** – $p \leq 0.001$.

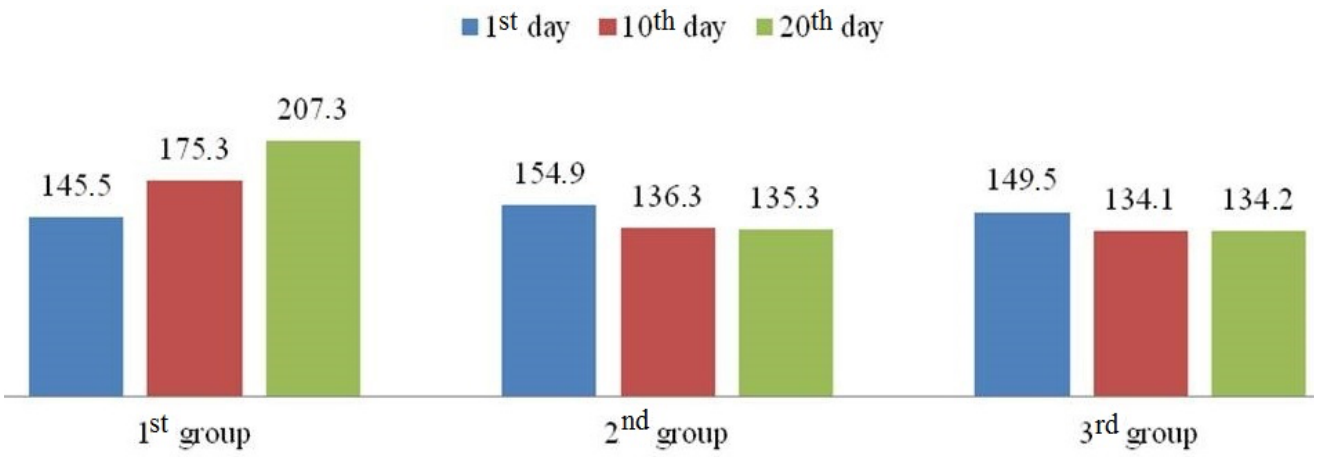


**Figure 2** AST dynamics concentration during the experiment, $U.L^{-1}$.

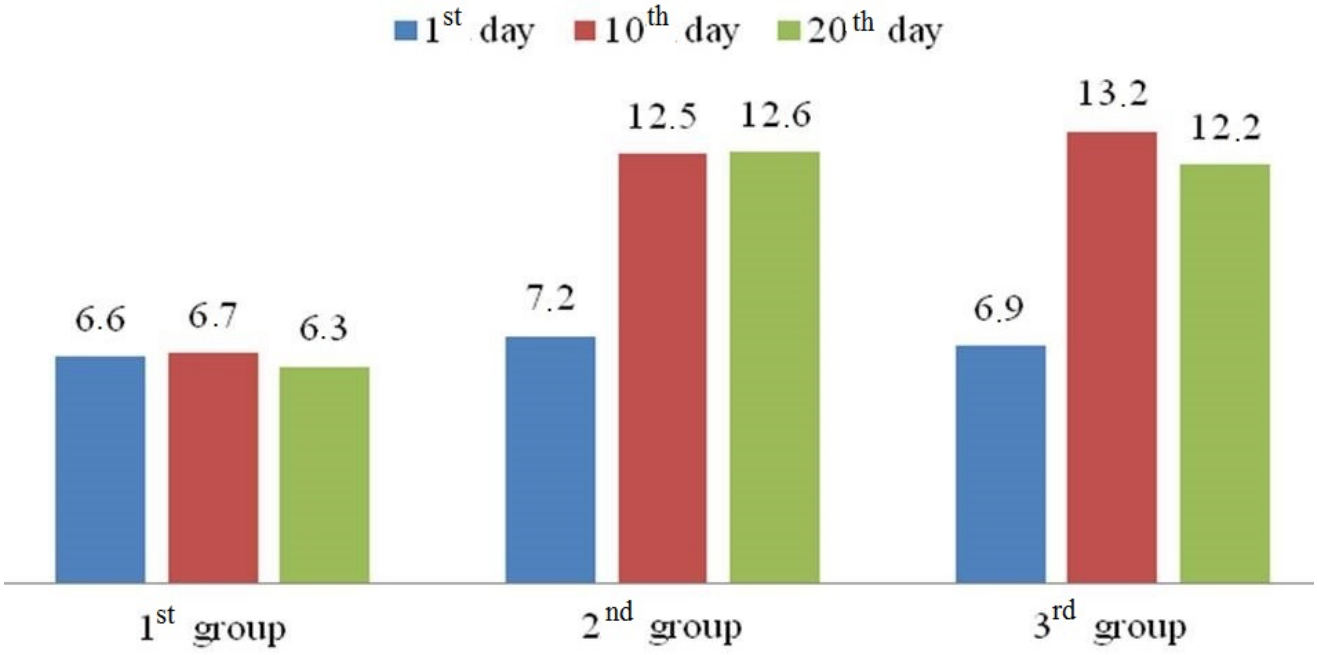


**Figure 3** Urease dynamics concentration during the experiment, $mmol.L^{-1}$.

Total protein content in blood plasma in the control group slightly fluctuates. A decrease in the protein content in the blood plasma also indicates a downgrade of kidney function. In our studies, there was an increase in total protein by 19.7% in the 2nd group and by 17.9% in the 3rd group. Albumin increasing observed in animals only in second group 27.6%; in 3rd group, this increase is tendentious, which indicates the feasibility suppression of inflammatory processes in the gastrointestinal tract in rats. Figure 4 shows the total protein dynamics concentration in the blood plasma of rats during the experiment, and the albumin dynamic is in Figure 5.

The positive total protein dynamics concentration in the rats' blood plasma of both experimental groups was noted. This indicator varies in the range of 80.2 – 80.3 $g.L^{-1}$ on the 10th day of the experiment. Including in animals of the second group, this concentration occurs due to albumin, the content of which in the plasma increases during the experiment from 30.0 to 37.4 $g.L^{-1}$. In the animals of the 3rd group, the albumin content varies slightly during the experiment, ranging from 31.0 to 31.7 $g.L^{-1}$. In the control group rats, this indicator takes values from 30.0 $g.L^{-1}$ at the beginning of the experiment, with a tendency to decrease at the end of the experiment to 29.3 $g.L^{-1}$.

Glucose concentration is the most important nutrient base for the vast majority of nervous cells, especially brain tissue. Half of the energy consumed by the body is released from glucose. Hypoglycemia is often associated with a decrease in insulin blood level **(Salman et al., 2017)**. Thus, the observed decreased glucose concentration in rats of the control group is a consequence of the presence of possible inflammatory processes in the internal organs but is not critical because of the unreliable difference in indicators between the study groups (Figure 6).

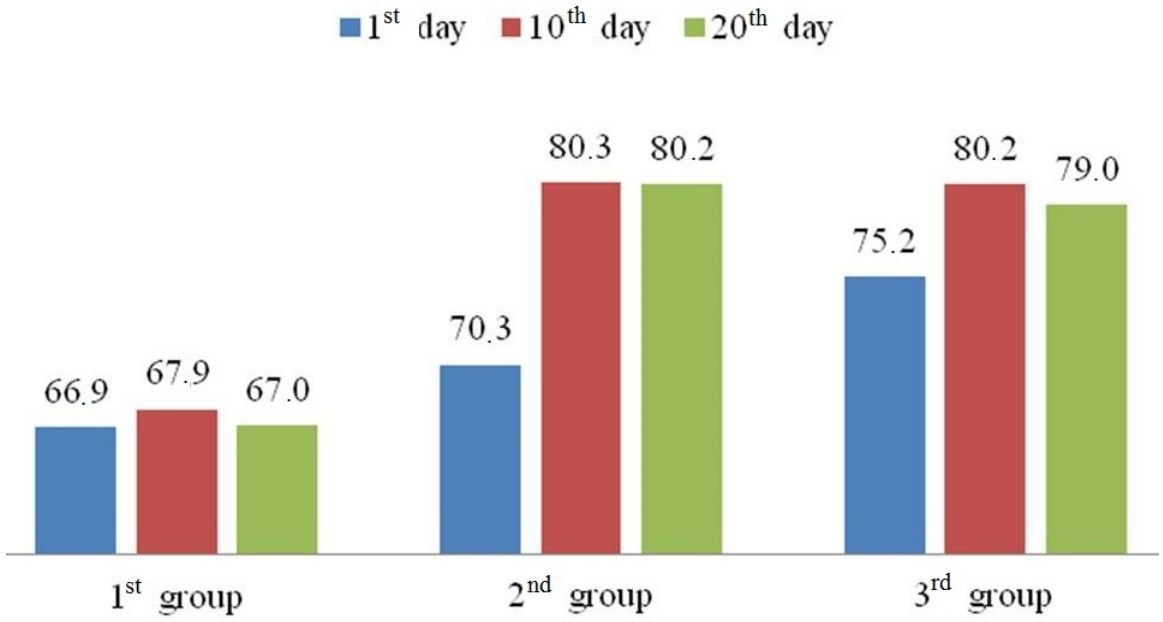


**Figure 4** Total Protein dynamics concentration, g.L$^{-1}$.

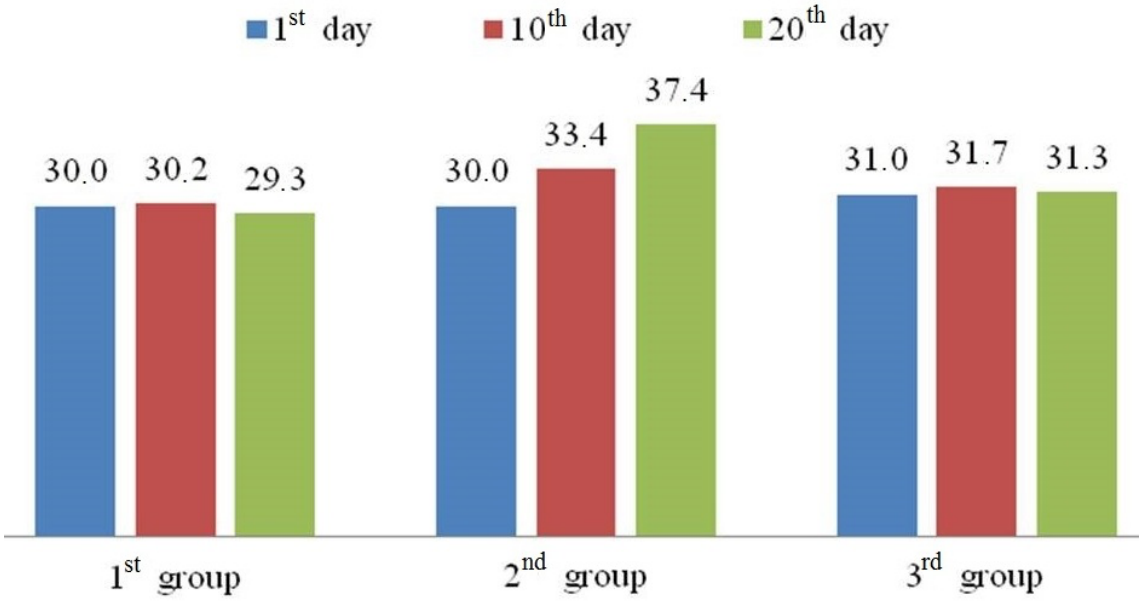


**Figure 5** Albumin dynamics concentration, g.L$^{-1}$.

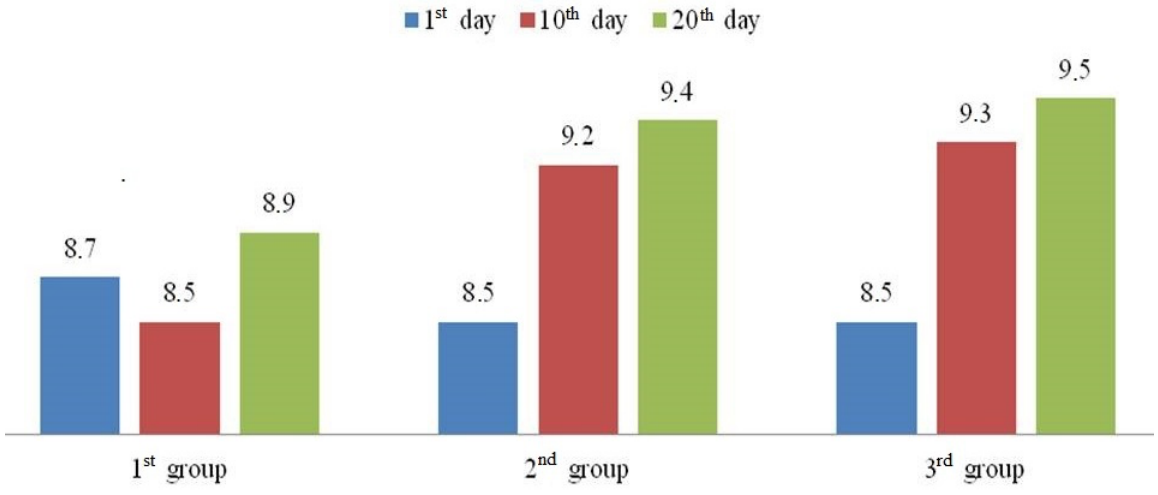


**Figure 6** Glucose dynamics concentration, mmol.L$^{-1}$.

RBCs are an important component of the blood, which is involved in the saturation of tissues with oxygen, and WBCs are an indicator of the physiological norm, and their dynamics indicate the development of inflammatory and other pathological processes. An increase in blood cells was observed: RBCs by 0.44 cells × $10^{12}$ per liter in animals of the 2nd group, and the content of WBCs practically did not change in all the studied groups (Table 3).

However, the WBCs' composition in the experimental groups was mainly due to neutrophils responsible for the phagocytic activity, and monocytes in the second group were formed (Table 4).

Non-specific immune response provides by neutrophils and monocytes. Such a leukocyte formula contributes to an increased level of protection against pathogenic environmental influences and provides a high level of protection in lung diseases of a coldlike nature **(Luo et al., 2014)**. Regular consumption of small doses (about 0.3 mcg per day) of essential oil with food or drinking water increased the life expectancy of AKR high-cancer line mice by 30% and reduced the incidence of leukemia **(Oyemitan et al., 2015)**.

The systematic intake of small doses of oregano essential oil by healthy BALB/c line mice increased their average life expectancy by 120 days or 17%. At the same time, it was found that the intake of oil throughout life did not cause toxic effects, did not affect the body weight, the size of the immune-competent organs, and the blood formula **(Saiyudthong et al., 2015)**.

The gastric mucosa of rats has a covering epithelium, between the folds of which the mouths of simple tubular glands open. The mucosal layer is underlain by its own muscle plate of the mucosa, formed by smooth myocytes organized parallel to the longitudinal axis of the organ, as well as the connective tissue base.

The main function of the gastric epithelium is protective. Its thickness is normally about 150 μm in rats and begins to increase when exposed to pathogenic factors, both physical and chemical. It is worth noting that in all groups of animals that consumed oil, the thickness of the integumentary epithelium was slightly lower than the control values, while it did not bear any signs of atrophy (Figure 7).

The depth of the glands also became slightly smaller, which generally affected the overall thickness of the mucous layer (Table 5). These changes are most pronounced in the 2nd group. In general, the thickness of the mucous layer remained within the normal range for this age group of animals.

The number of epithelial cells in the integumentary epithelium, main and lining cells in the glands of the fundal part of the stomach in the control group also agree with the literature data **(Yuldashev et al., 2014)**.

At the same time, in the groups that received mountain savory essential oil, the number of cells decreased slightly, which is more pronounced in the group with ginger (Table 5). However, these differences are not reliable. The number of chief cells, on the contrary, increases, which may indicate a greater enzymatic activity and, consequently, an improvement in the functional characteristics of the stomach is approximately the same in all groups that received oil. Changes in the number of parietal cells are reversed and more pronounced in groups with a single dose of any of the oils.

**Table 3** Rats' blood hematology (n = 3).

| Biometrics indicator | 1st group | 2nd group | 3rd group |
|---|---|---|---|
| | RBCs count (×$10^6$ Cells per $mm^3$ ) | | |
| **X±$m_X$** | 6.67 ±0.06 | 7.24 ±0.21 | 7.11 ±0.28 |
| **$C_v$, %** | 1.69 | 5.00 | 6.90 |
| | WBCs count (× $10^9$ Cells per $mm^3$) | | |
| **X±$m_X$** | 10.18 ±0.6 | 9.62 ±0.51 | 10.13 ±0.46 |
| **$C_v$, %** | 11.80 | 9.13 | 7.79 |

**Table 4** Rats' blood leukocyte formula, %.

| Rats' group | Neutrophils | Lymphocytes | Eosinophils | Monocytes | Basophils |
|---|---|---|---|---|---|
| 1st | 40.7 ±1.4 | 51.7 ±1.7 | 2.3 ±0.3 | 5.0 ±0.4 | 0.3 ±0.3 |
| 2nd | 42.0 ±1.2 | 49.7 ±0.6 | 2.7 ±0.5 | 5.7 ±0.3 | 0.0 ±0.0 |
| 3rd | 42.7 ±1.4 | 49.3 ±1.3 | 3.0 ±0.4 | 4.7 ±0.6 | 0.3 ±0.3 |

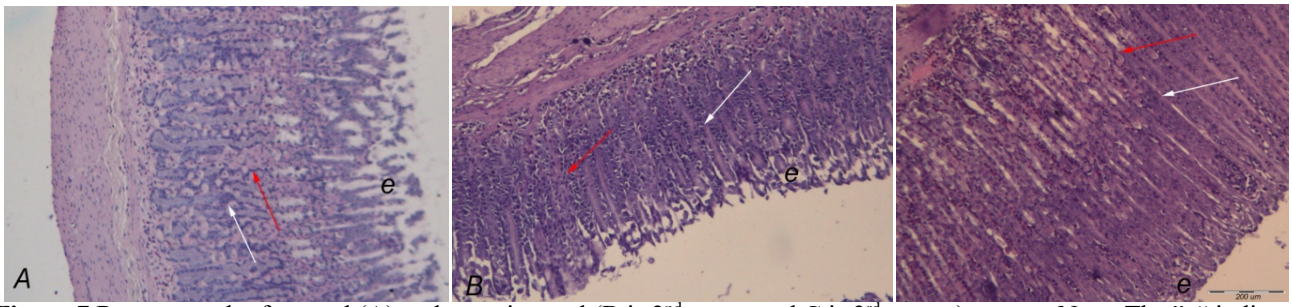


**Figure 7** Rats stomach of control (A) and experimental (B is 2nd group and C is 3rd group) groups. Note: The "e" indicates the integumentary epithelium. Red arrows show the lining cells of the gastric glands, and white arrow is the main ones. Hematoxylin and Eosin stained. The lens is 40×.

**Table 5** Rats' stomach morphometric characteristics, μm.

| Rats' group | Epithelial height | Glands thickness | Stomach wall mucous layer thickness | Superficial epithelial cells number | Chief exocrine cells number | Parietal exocrine cells number |
|---|---|---|---|---|---|---|
| 1st | 152.64 ±5.54 | 479.70 ±10.92 | 632.34 ±12.03 | 21.2 ±7.6 | 34.2 ±8.014 | 51.4 ±10.14 |
| 2nd | 139.89 ±3.18* | 290.33 ±24.24* | 380.23 ±25.96* | 21.00 ±0.52 | 53.0 ±1.0 | 29.00 ±1.0* |
| 3rd | 124.50 ±3.66 | 470.78 ±11.36 | 595.28 ±13.92 | 20.00 ±0.84 | 54.4 ±5.54 | 36.3 ±6.64 |

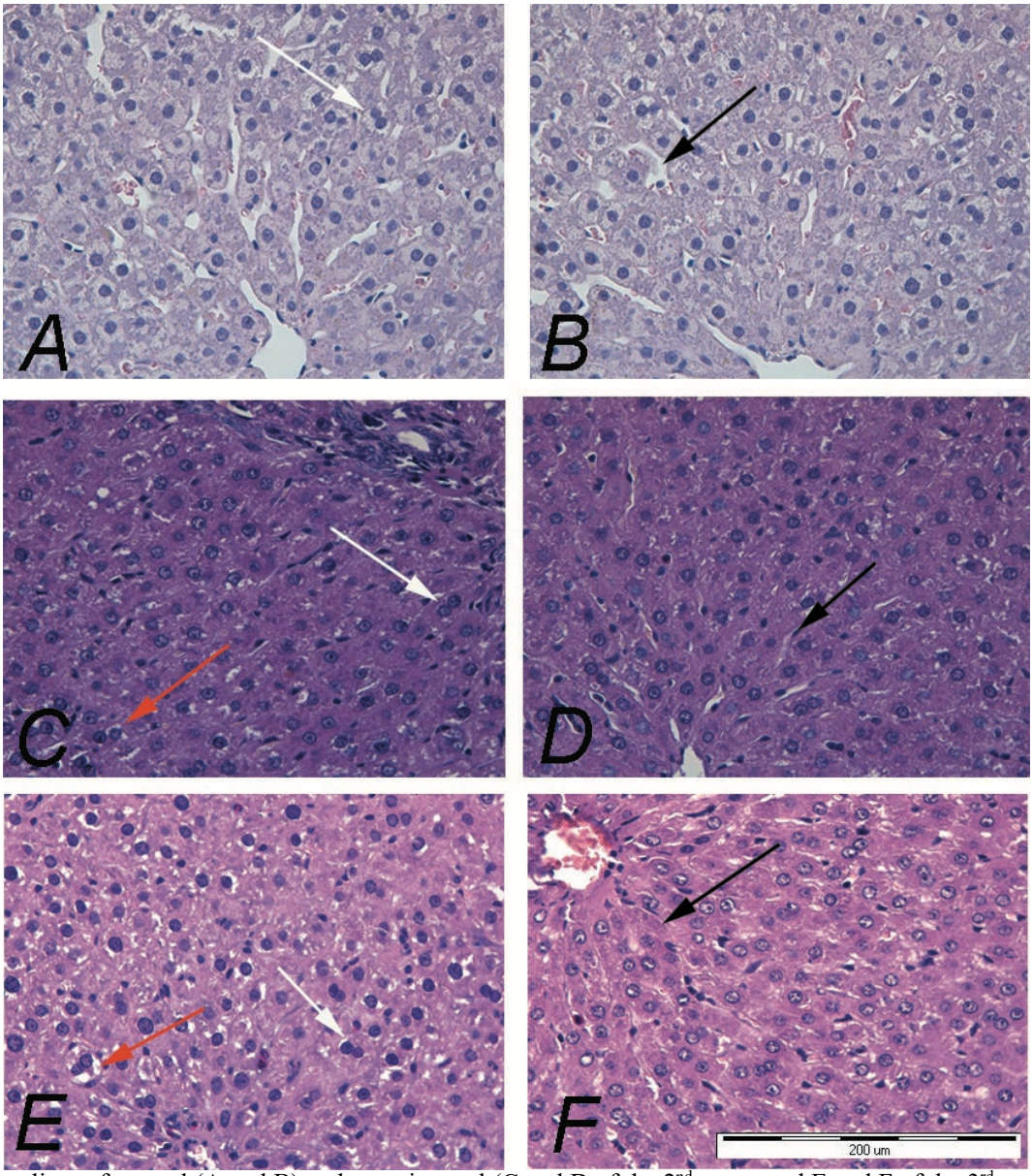


**Figure 8** Rats liver of control (A and B) and experimental (C and D of the 2nd group and E and F of the 3rd group) groups. Note: In the left column, the peripheral part of the hepatic lobule, in the right – the central one. The black arrows indicate the sinuses of the hepatic lobule. The white arrows represent binucleated hepatocytes. Fatty cell dystrophy was marked by red arrows. Hematoxylin and Eosin stained. The lens is 40×.

**Table 6** Rats' liver morphometric characteristics in the central (c) and peripheral (p) parts of the classical liver lobes, μm.

| Rats' group | | Binuclear cells number | Parenchyma percentage | Stroma percentage | Cell area | Core area | Nuclear and Cytoplasmic Ratio |
|---|---|---|---|---|---|---|---|
| 1st | c | 6.60 ±0.98 | 81.20 ±1.32 | 18.80 ±1.32 | 300.35 ±9.98 | 54.19 ±2.30 | 0.23 ±0.01 |
| | p | 7.80 ±0.97 | 79.80 ±2.48 | 20.20 ±2.48 | 338.15 ±11.04 | 54.35 ±2.54 | 0.17 ±0.01 |
| 2nd | c | 8.20 ±2.31 | 87.60 ±1.16 | 12.40 ±1.16 | 360.47 ±13.82 | 55.72 ±2.91 | 0.19 ±0.01 |
| | p | 5.40 ±1.70 | 85.80 ±1.69 | 14.20 ±1.69 | 355.98 ±11.52 | 59.19 ±2.89 | 0.20 ±0.01 |
| 3rd | c | 5.60 ±0.93 | 82.80 ±1.93 | 17.20 ±1.93 | 283.07 ±10.18** | 49.57 ±2.74 | 0.22 ±0.01 |
| | p | 8.40 ±1.50 | 89.40 ±0.60 | 10.60 ±0.60 | 320.34 ±12.14 | 51.90 ±2.36 | 0.20 ±0.01 |

The liver of the rats of all groups retained a typical structure. The lobules of the liver consisted of beams of hepatocytes, between which sinusoids and blood capillaries were located. The use of oils as a top dressing led to a narrowing of the sinus-capillary network, they began to look less transparent on histopreparations, which may indicate the saturation of the sinuses with fats. Hepatocytes of the liver of rats of the experimental groups differed from the control group by the presence of fat inclusions in the cytoplasm in the form of rounded drops (Figure 2, Table 6).

There is some regularity in the morphometric indicators, depending on the site of the hepatic lobule. Thus, the number of binuclear cells in the 3rd experimental group is greater on the periphery of the lobule, which corresponds to a greater degree of damage to hepatocytes in this area, the first to meet the blood entering the lobule. The same region showed a greater narrowing of the capillary network compared to the central regions of the lobules.

## CONCLUSIONS

The intensification of protein metabolism in the rats' blood plasma on the background of the use of mountain savory essential oil was noted. Total protein increases by 17.9 – 19.7%, and albumin by 27.6% in rats treated with essential oil at a dose of 0.6 mL per kg of feed. A significant increase in the level of aspartate aminotransferase in animals of the control group to 207.3 $U.L^{-1}$ was revealed, which to a certain extent indicates the hepatoprotective effect of mountain savory oil and a decrease in inflammatory processes in the organs of the gastrointestinal tract in the conditions of cell maintenance of rats of the experimental groups. Gastric epithelium thickness of experimental groups rats was lower than the control animals, while it did not bear any signs of atrophy. The difference of this indicator in comparison with control was 12.75 μm ($p \leq 0.05$) in the second group and it was reliable. The number of chief stomach cells increases in animals of the experimental groups, which may indicate a greater enzymatic activity. An increased dose of mountain savory oil contributes to the formation of more damage to hepatocytes on the periphery of the liver lobule.

Thus, the relationship between liver enzymes and the state of peripheral hepatocytes was noted.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

Wistar line adult male rats in the experiment were used. The experimental work in accordance with the Declaration on the Humane Treatment of Animals was carried out. Animals of all groups from the experiment by decapitation under ether anesthesia were removed.

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# FORMATION OF HEAT AND MASS TRANSFER BONDS WHEN MIXING COMPONENTS IN A SUSPENDED STATE

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## ABSTRACT

The method of preparation of wheat paste based on discrete and impulse influence on components is revealed. The basis of the research of the vapors preparation method is the formation of a liquid mixture of interacting dosing components in a suspended state. The interaction occurs due to the humidification of the pulverulent suspended state of the layer of flour particles by a scattered jet of liquid under pressure. The development of this technological process and equipment is considered. A thorough analysis of the mixing of components in a suspended state. The influence of a set of experimental and theoretical researches is noted, where the results of the theoretical direction are aimed at the creation of mathematical models, simulation of the process with the use of the possibility of computer engineering. The characteristics of thermal processes for the transition region from the surface of the flour to the massive formed medium are given, taking into account the effective thermophysical characteristics of the medium in the form of dependences obtained theoretically and experimentally. Dependencies are taken into account as well as heat and mass transfer processes when the liquid phase interacts with flour: external heat and mass transfer processes when interacting in a suspended state on the flour surface and change of working environment with the formation of bonds, and internal mass transfer processes when moving moisture and heat inside the flour particle.

**Keywords:** mathematical model; mixing; suspended state; steam; heat and mass transfer

## INTRODUCTION

The process of mixing the components is used in the food industry for the formation of homogeneous viscous media during the intensification of the technological process. Mixing of components is carried out in mixers in different ways using different designs of working chambers and working bodies in gas and liquid streams. The mixing mechanism in the mathematical description is represented in the form of dependences on the nature of the hydrodynamic motion of the particles of the components and the factors that cause the deformation movement. Under such conditions, the characteristics which cause a change of position of particles of components or their mix in space with the use of probable physical and chemical parameters are revealed.

The uniformity of the interaction of the components during mixing is estimated by the standard deviation of the concentrations of the components for all elementary volumes of the working chamber, i.e. the mixing index, the intensity of the process. Uniform intensive of components mixing is to obtain a homogeneous mixture affects the quantitative indicators of technological processes of production. Achieving equilibrium during mixing depends on the relative balance between the structural elements of the machine and the interaction of components.

Currently, the mixing of components is provided not only with practical knowledge and extensive experience of specialists but also with theoretical knowledge necessary for detailed study and modeling of the processes that occur during their interaction. Numerical simulation is particularly convenient when describing the processes of gravitational and bulk mixing when it occurs most actively in a thin layer of material in which there is a random transition of the particles of the components in the adjacent layer. An example of such a device is a mixer, the working cylindrical surface of which rotates together with the blades installed inside the working chamber. In this case, the main volume of the mixture filling the lower part of the chamber moves synchronously with the working surface without mixing, and only in the upper layer of material, rolled at an angle down the surface of the layer, there is a chaotic movement and redistribution of particles of different fractions. Among the two-phase methods of making wheat dough, liquid and thick pastes are most often used as the first phase. Schemes with liquid vapors due to several inherent shortcomings **(Hasatani et al., 1993; Dolomakin, 2015)** are not widely used in the baking industry of Ukraine. The main reason is that the units for the preparation of liquid mash are quite conservative, and high-quality using the energy of compressed air and water under

pressure **(Lisovenko, 2000)**, unbearable for bakery enterprises in Ukraine.

Sponge dough is a complex hydrophilic colloidal system **(Lisovenko**, **1982; Quail, 2019)**, the state of which depends on the properties of the raw material used for its preparation, design, and technological parameters of the process, changes that occur during the formation of the environment. The state of influence of the liquid phase on the flour is not only theoretical but also practical. Therefore, in our opinion, there is a need to develop similar domestic designs and justify rational modes of their work.

The basis of this development of the method of preparation of sponge dough **(Pat. No 134226, newsletter No 9, 2019; Pat. No 137278, newsletter No 19, 2019)** is the formation of a liquid mixture of interacting dosing components in a suspended state. The interaction occurs due to the humidification of the pulverulent suspended state of the layer of flour particles by a scattered jet of liquid under pressure **(Stadnyk et al., 2019)**.

The offered study presents for the first time the results of process modeling of the interaction of components in the suspended state, characteristic of the transition region of heat and mass transfer.

## Analysis of model approximations of medium types

Theoretical dependences and general provisions of the process of mixing and interaction of components with working bodies are given in the works of **Lisovenko (1982)**, **Stadnyk (2015)**, **Dolomakin (2015)**, and others. The mixing process has been covered in studies on the development of the technological scheme **(Danyliuk et al., 2017; Stadnyk, 2015; Kafarov, 1949)** the parameters of mixers **(Stadnyk et al., 2019; Krivoruchko, 2018)**, process modes **(Barabash et al., 2007)** and thermodynamic processes during mixing **(Kornienko, 2011)**. It is also offered to use different rotors for the production of water-flour mixture with the calculation of their technological and design characteristics. Heat engineering calculations and heat transfer processes, in general, are covered quite fully **(Kharlashinet, Beckett and Bendich, 2012; Stadnyk, 2015)** but there are no special works devoted to thermal processes when mixing steam by a new method in a suspended state.

The analysis of research on the development of technology for cooking stew allowed to establish the following:

- insufficiently studied issues of mass heat transfer;
- the use of the suspended state is constrained by the difference in the design of the mixer and the parameters of the technological process concerning the properties of the components;
- there is no thermodynamic model of the interaction of components in the formation of the environment.

The result of active mixing of the prescription components with the formation of the medium in a suspended state is due to the energy dissipation of the input stream. There is no information in the literature on the mutual influences of suspended state flows. Substantiation and improvement of the quality of components interaction in a suspended state are provided by us on the way of new constructive decisions use. This approach requires a review of the dosage of liquid and flour, which simultaneously leads to an increase in the flow of the resulting mixture of components.

It is clear that to increase the degree of mixing of the prepared components, it is necessary to provide an increase in the velocity of the liquid jets at the time of contact with the solid phase. It is obvious that with increasing distance between the nozzle and the line of separation of the jets, the flow rate decreases, which will reduce the degree of mixing.

In our opinion, the main reason why the interaction of the sprayed liquid during its dosing in the working chamber does not have time to reach equilibrium is that of the large droplet size, i.e insufficient degree of liquid dispersion by nozzles. During the stay (dosing) in the chamber, the large droplets do not have time to interact with the flour to equilibrium and, in addition, the falling time of large droplets in the chamber is significantly longer than small ones. This, in turn, leads to the fact that a significant amount of liquid during its dosing in the chamber does not have time to fully ensure the speed of interaction with the flour.

In addition, depending on the flow rate of the liquid, the width of the groove of the vibrating batcher of bulk components, the physical and mechanical properties of the components, can be formed areas in the form of dead zones. The trajectories of large droplets (of the order of 1 mm) are practically independent of the action of gravity due to their high inertia. For all droplets, regardless of the angle of departure, their trajectories are straight lines in the direction of the angle of departure from the nozzle. Due to the short residence time in the chamber, these droplets settle on the wall of the chamber with a noticeable formed medium, and it is the greater the bigger the angle of departure. The interaction of the components at the time of their collision is schematically shown in Figure 1.

These studies establish the fact that is revealed in the work of the author **(Stadnyk et al., 2019)**. The interface of the phases "solid raw material-liquid" depends on the degree of grinding and will be larger the smaller the size of its particles. At the same time **(Quail, 2019)** notes that the difference in concentration in the components is the driving

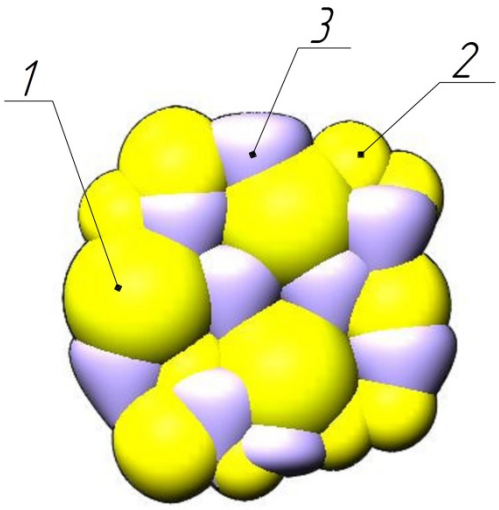


**Figure 1** The scheme of formation of the liquid phase-vapor: 1 – flour, 2 – the connection of components in the mixture, 3 – drops of liquid.

force of the mixing process. During mixing, it is necessary to strive for the maximum difference in concentrations, countercurrent process, etc .. Note that for the weighing state of mixing is important to ensure optimal contact time between phases. From the basic equation of mass transfer, it follows **(Aerov and Todes, 1968)** that the amount of the substance diffused through a layer of raw materials is directly proportional to the duration of the process.

The results of the numerical experiment show **(Kharlashin et al., 2012; Stadnyk**, **2015)** that small droplets (of the order of 100 μm) have time to interact to an equilibrium state in the upper part of the chamber even before the collision with the wall. This spray ensures that the conditions of the ideal mode in the chamber are met. Large drops (about 1 mm), regardless of the angle of departure, hit the wall of the chamber without interaction with the flour with a noticeable property. Note that the conditions for achieving equilibrium depend on the behavior of the liquid and flour in the chamber. Some of the drops return to the chamber volume after contacting with the flour and without contacting the wall. The rest of the droplets settle on the wall and flow down in a thin pellicle to the bottom of the chamber under the action of gravity. From the central part of the working chamber, the interacting components are fed to the rotating working bodies, ie intensive mechanical mixing. Therefore, the subsequent process of mixing the liquid phase with flour particles and drops of the mixture to equilibrium depends on the thickness and velocity of the film and its heat exchange with the working body and the chamber wall. In our opinion and the authors **(Kornienko, 2011)** increasing the energy efficiency of mixing is possible provided that the definition and establishment of rational parameters of the jet device and technological parameters of the mixing process. This result can be obtained by discrete-pulse dosing of components with the introduction of the design of the ejector nozzle system of the component mixer (jet device). The design of the device is based on the creation of the maximum difference of speeds of phases that allows reducing energy expenses for carrying out dispersion. The degree in the first minutes of binding a small amount of liquid phase of the active hydrophilic groups of flour particles promotes the formation of water shells. The interaction of the liquid phase with hydrophilic groups occurs not only on the surface of flour particles but also in volume. The process proceeds with the release and absorption of heat (exothermic). The amount of retained liquid phase is about 30% does not lead to a large increase in particle volume.

Therefore, the water absorption capacity of flour is affected by its dispersion, ie particle size. As the particle size decreases, the specific surface area per unit mass of flour increases, so more water may be adsorbed. Water absorption by small particles is much faster. Therefore experimental researches of the offered design of the mixer of discrete are impulse influence on uniformity of giving of the sprayed liquid components and weight of the dosing by a router of flour on their interaction in a suspended state are an actual direction of researches.

### Scientific hypothesis

The basis of the method of mixing under consideration is the moistening of dust particles of flour, which are in a suspended state, the flow of liquid under pressure, and the conditions of the thermodynamic model of interaction of components aimed at the intensity of heat and mass transfer in the model steam composition.

## MATERIAL AND METHODOLOGY

### Samples

Experimental and theoretical studies were performed based on research laboratories of the Department of Food Technology Equipment of Ternopil National Technical University. The dough was kneaded according to the recipe of shaped bread **(Drobot, 2010)**. The mixture was prepared from high-quality wheat flour, flour moisture was 13.9 ±0.2%, with a crude gluten content of 24% and a stretch of 14 cm. First-grade wheat flour was produced following **DSTU 46.004-99**. Manufacturer private enterprise "ZAKHID-KHLIB-ZBUT-2002", Ternopil, Ukraine. Yeast pressed bakery classic according to **TU U 10.8-00383320-001**. Manufacturer: Enzyme Company PJSC, Lviv, Ukraine. Granulated sugar – **GOST 21-94** Rivne, Ukraine, Factory of confectionery jewelry "Decoration" – **TU U 10.8-40570177-001 (2016)**.

### Chemicals

Water (chemical formula $H_2O$) was used to mix the components in the preparation of the paste. Water meets the national standard **TU U2.14-0051232-098 (2007)**.

### Instruments

The temperature was measured with a thermometer of Ukrainian TLS Figure 2. Devices for research manufacturer "Glassware". It is designed to accurately measure temperature. Dosing of dough components was performed on electronic scales with a measuring range up to 0.1g. Determination of temperature regimes in the process of preparation of the dough used the device Benetech GM533A; to determine the quality of scattering of liquid components and their interaction with flour in a suspended state used a digital camera CANON EOS Wi-Fi 4000D 18-55 DC III (3011C004AA; setting the quality of interaction of the components of the formed mixture was determined using a microscope My First Lab MFL-06 Duo-scope (Fig. 2).

Studies of the mixing process were carried out according to the Box-Benken plan. This is one of the types of statistical plans used in the planning of scientific experiments. This plan allows you to get the maximum amount of objective information about the influence of the studied factors on the mixing process with the least number of experiments.

Box-Benken plans belong to the second-order plans, ie they provide a regression model in the form of a complete quadratic polynomial:

$$Y = b_0 + \sum_{i=1}^{k} b_i \cdot x_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} b_{ij} \cdot x_i \cdot x_j + \sum_{i=1}^{k} b_{ii} \cdot x_i^2,$$

Where:

Y – target function; $b_i$, $b_{ij}$ $b_{ii}$ – calculated coefficients of the model; k – factor number.

Based on the constructed regression equation, the contribution of each independent variable to the variation of the studied dependent variable is determined, ie the influence of factors on the performance indicator.

The experimental data set was processed using the Statistica-12 software package for the computer. The coefficients of the regression or approximating function equation, under the condition of orthogonality and symmetry of the plan-matrix of the planned factorial experiment, were determined according to the standard method according to known dependences.

The obtained results were statistically processed using the standard Microsoft Office software package.

**Laboratory Methods**

Sampling was performed according to DSTU 4803:2013, Organoleptic evaluation "Descriptive (qualitative) method of profile analysis" GOST 5897-90. The quality of flour was determined according to GOST 9404-60 **(Drobot, 2010; Lisovska et al., 2020)**. Determination of the "strength" of the flour was performed by the vagueness of the dough ball **(Drobot, 2010)**. The moisture content of flour and semi-finished products was determined using an HF device according to GOST 21094-75 **(Lisovska et al., 2020)**.

***Method for determining the amount of raw materials***

The required amount of raw material was dosed by weight. The amount of water introduced during mixing Gv (in mL) was determined by the formula:

The Benetech GM533A device with a range of measurements to 0.1 °C.

The digital camera CANON EOS Wi-Fi 4000D 18-55 DC III (3011C004AA) specular/CMOS (KMOH)/depends on the lens/Wi-Fi/FullHD (1920x1080) Pix/HD (1280 x 720) Pix/VGA (640x480) Pix. Microphone – built-in (stereo)

The My First Lab MFL-06 Duo-scope training microscope is a biological monocular microscope with a full-fledged optical system with optical glass lenses. Country of origin of the USA.

$$G = G_c(W_0 - W_c) / (100 - W_c)$$

Where:

Gc is the total weight of flour, gr; $W_0$ is moisture content of pre-ferment, %; Wc is the average weighted moisture content of raw materials, %.

The water temperature ($t_0$, $^0$C) of water consumed for mixing the pre-ferment, provided its temperature is 280 °C, was calculated by the formula:

$$t_o = t_n + c_b G_b (t_n - t_b) / c_b G_b + K$$

Where:

To is set temperature, $^0$C; $c_b$ is heat capacity of flour, kJ.kg$^{-1}$ K, ($c_b$ =1.257); $G_b$ is heat capacity of water, 4.19; $t_b$ is flour temperature, °C; Gm– the amount of water, g; K is correction factor (in summer 0 – 1, spring and autumn – 2, winter – 3).

The ratio of the amount of flour and water for steam of different humidity is summarized in Table 1.

FlowVision software was used to determine the required design parameters and modes of operation and modeling of the corresponding process. FlowVision is based on a finite-volume method for solving hydrodynamic equations and uses a rectangular adaptive mesh with local grinding. To approximate curvilinear geometry with increased accuracy, FlowVision uses technology with a grid resolution of geometry. This technology allows you to import geometry from CAD systems and exchange information with finite element analysis systems.

Morphometric examination and microphotography were performed using a “Konus Biorex-3” microscope with a digital Sigeta UCMOS 5100 with Toup View software adapted for research data.

Description of the Experiment. According to the results obtained during experimental research on a new mixer (Figure 3) in determining the rational values of qualitative indicators of mixing components in a suspended state (homogeneity), the parameters were optimized based on thermodynamic.

The purpose of optimizing experimental studies was to determine the effect of the interaction of components in the suspended state on the process of heat and mass transfer. It should be noted that the technological process of the first stage of preparation of the paste is based on the fact that the accelerated jet of liquid components in the air meets with the sprayed particles of flour in free fall. The interaction time of flour and liquid components in the pre-mixing chamber and before they are in the lower part of the chamber is $\tau_1$ = 5 - 10 s.

We have already mentioned that the interaction of flour with the scattered jet of the liquid phase is mainly a chaotic process, ie their interaction with mixing. Thus, in our case under the action of vibration and stirring in the suspended state, the mixture tries to go into a quasi-equilibrium state. This takes into account the transformations of the forces of internal friction. However, the state of equilibrium is significantly affected by the gravitational forces arising in the process, which determine not only the size and density but also the shape and other parameters of the mixture (Figure 4).

Therefore, to study the temperature field and the homogeneity of the mixture, a layout of thermometers for measuring temperatures in the area of the suspended state of the interaction of the dosing components was developed. Using thermometers $T_1$, temperature measurements were performed in the coordinates Y = 0.15 m, Z = 0.2 m. $T_1$ was measured in the range of change X = 0.1 m, and $T_2$ in the range of change X = 0.1 - 0.3 m. The results are shown in Figure 5. The peculiarity of hydrodynamics is that in the range of values $0 \le X \le 0.1$ m there is a movement of components with a constant k = 0.8 (homogeneity). In the zone of axial interaction $0.1 \le X \le 0.3$, there is a 3D surface mixing of components at a temperature of 30.2 °C and k = 0.7. And at $0.3 \le X \le 0.4$ also became k = 0.8 and creates a downward movement along the wall of the chamber of the formed mixture.

Note that such hydrodynamics of the interaction of components due to the increase in the distribution zone of the liquid phase and flour provided a mechanically symmetrical geometric shape of the temperature zone from 32 to 32.2 °C, at a distance of 0.001 m from the working chamber wall.



**Figure 2** Devices for research.

**Table 1** Recipe for cooking sponge dough.

| Raw material | The amount of raw materials for sponge dough moisture | | |
|---|---|---|---|
| | 65% | 70% | 75% |
| Flour, gr | 2400 (40%) | 2100 (35%) | 1800 (30%) |
| Water, mg | 3600 (60%) | 3900 (65%) | 3900 (65%) |
| yeast, gr | 100 | 100 | 100 |
| Concentration, $kg.kg^{-1}$ | 0.666 | 0.538 | 0.428 |

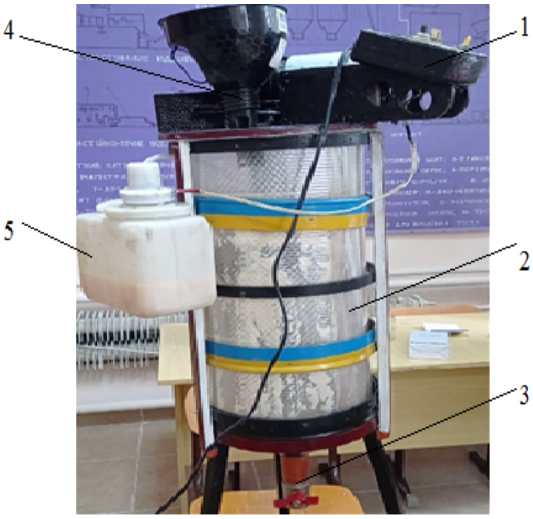


**Figure 3** General view of the mixer: 1 – the control panel, 2 – the cylindrical working chamber, 3 – a branch pipe for unloading of a paste, 4 – the flour vibrating batcher, 5 – the batcher of liquid components.

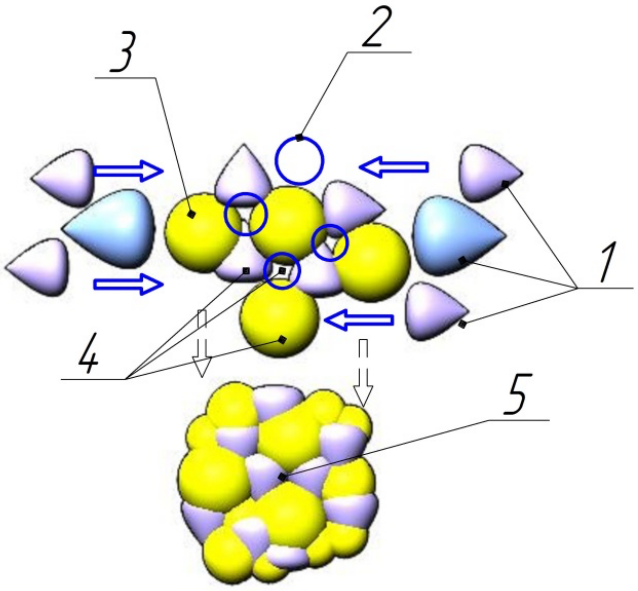


**Figure 4** The scheme of interaction of components in the formation of a mixture: 1 – liquid components, 2 – bubbles; 3 – part flour, 4 – mixing components, 5 – mixture.

**Number of repeated analyses**: All measurements of instrument readings were performed 5 times.
**Number of experiment replication**: The number of repetitions of each experiment to determine one value was also 5 times.

## Statistical analysis

To establish rational regime parameters of the component mixing process in the suspended state, a multifactor experiment was performed to determine the effect of mass flow rate Q, the oscillation amplitude of dosing flour A, height of their interaction h, flour mass m, filling factor K (homogeneity) on the quality of the mixture.

The target function Y is selected homogeneity-k. According to the program of experimental researches, 27 experiments were performed in five repetitions **(Response surface regression (Electronic resource)**.

The asymmetric Box-Benkin plan matrix for four factors and three levels of factor variation with the total number of experiments equal to 27 is given in table 2. The regression equation, which is written as a complete polynomial of the second degree has the form:

$$k(Q,A,h,m)=-1.83-207.94Q-15.01A-10.84h+662.19m+1246.72QA-501.53Qh+6239.84Qm+41.57Ah+409.76Am+18146.45Q^2+4.22A^2+16.53h^2-35053.24m^2$$

With the probability level $p$ = 0.95 and the value of the t-alpha criterion equal to 2.365, the following statistics were obtained (Figure 6): coefficient of multiple determination D = 0.926; multiple correlation coefficient R = 0.962; standard deviation of the estimate s = 0.084; Fisher's F-test is 10.776. The coefficient D is significant with the probability level $p$ = 0.99961.

All factors influencing the homogeneity of the mixture are important from the point of view of the interests of increasing the contact area of the components because the driving factor is the weighted state of interaction.

The importance of factors influencing the passage of the technological process is to create an objective control of the interaction of components. They direct the impact on the economy of material and energy resources. The state of the heterogeneous medium can be imagined in the diagram-illustration of the driving factors influencing the weighted state of mixing (Figure 6).

A graphical representation of the change in the homogeneity of the mixture according to experimental data, ie the response surface of the functional change in the homogeneity of the mixture as a functional:

$F_k = f(Q, h, m, A)$ is shown in Figure 7.

The simulations confirm the validity of the accepted assumptions of the origin and motion of the resulting mixture. The interaction of the phases in the liquid volume is high, there are pulsations of the solid phase in the liquid, which will additionally have a positive effect on the intensity of its dispersion. Therefore, the response surface shows that the factors influencing the homogeneity of the mixture are important for establishing and determining the area of interaction of the components in the weighing state. The interaction of the components in the suspended state quite clearly depends on the number of dosing components. It should be noted that the factors influencing (Q, kg.s$^{-1}$; A, mm; h, m; m, kg) on a more uniform interaction and increasing the contact area of the components, determined the change in the dosing method. Thus, the liquid components accelerated their interaction by installing two additional nozzles. Thus, without increasing the flow rate, we achieved an increase in the axial average influence of the liquid phase on the influence of mass transfer processes. The effect of the average scattered leakage also increased the area of contact with the flour in the working chamber of the machine. At the same time, a large part of the liquid mixture as a result of the formed inertial influence from the liquid side is in faster contact with the surface of the cylindrical working chamber. The action of gravitational forces contributes to the additional mixing of the formed mixture when it flows to the bottom of the chamber.

## RESULTS AND DISCUSSION.

Heat and mass transfer processes are widely used in the technology of preparation of liquid mash in the baking industry. Each process of dissolving flour is accompanied by a positive or negative thermal effect. Therefore, the physicochemical parameters of the medium, such as density, viscosity, thermal, and diffusion parameters, differ significantly from the center of the flow of the liquid phase. According to the data **(Dolomakin, 2016)**, there is a direct contact between the particles of dispersed systems and a connection occurs. The nature of the bond between water and flour refers to coagulation contacts through a layer of liquid. The phase separation between the particles is preserved.

From the above, we can see that the mode of mixing in the suspended state directly affects the heat and mass transfer in the mixture layer. Therefore, the design parameters of the mixer in the working chamber facilitate mixing. The formed homogeneous mode is characterized by the dosing of flour and liquid phase in the scattered state in the chamber space. The flour is on a uniformly distributed plane, ie the concentration of particles is scattered evenly in the volume of the chamber. Figure 8 shows the torch of the liquid spray during its flow from the device into the chamber and the interaction with the flour. You can see the particle size distribution zones of the sprayed liquid (A) and the formed droplets with flour (B).

Figure 8 quite clearly shows the pronounced zones of dense and dilute phases. The boundaries of their separation move gradually, mainly due to the interaction of the scattered liquid phase, which more quickly affects the interaction around the perimeter of the chamber. The formed liquid medium changes along the cross-section of the chamber as follows: in the central part the density changes sharply, near the sidewalls the flour slides with the liquid, closer to the working bodies their interaction decreases to the nature of a fixed layer and loose state. The central part of the chamber has a density of almost constant, the best property, which is explained by the input flows of flour on the axis of the velocity flow of the average liquid.

A detailed description of the formation of the liquid medium and the impact of the equipment is given in the paper **(Stadnyk, 2015)**.

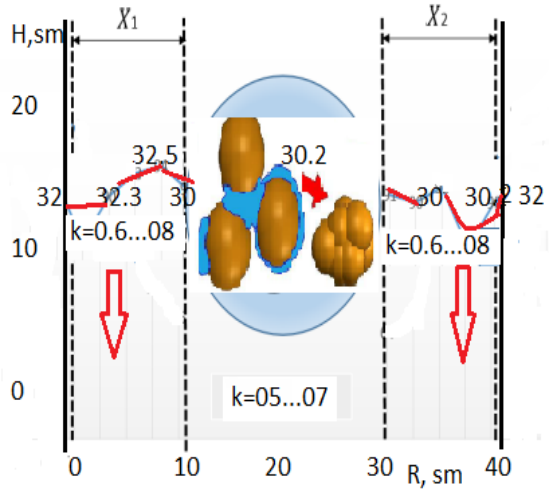


**Figure 5** The value of temperatures in the zone of suspended state.

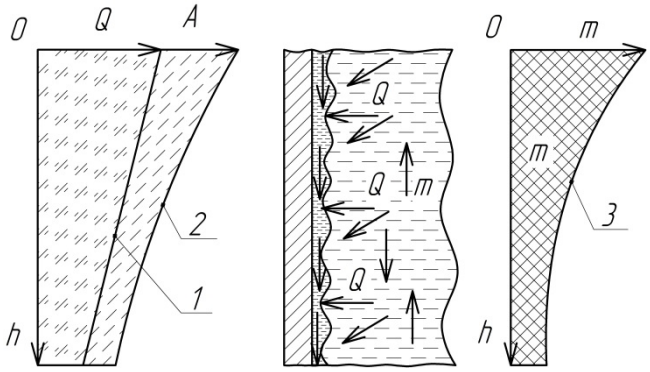


**Figure 6** Scheme-illustration of driving factors influencing the weighing state of mixing: 1 – dependence Q = Q (h); 2 – dependence A = A (h); 3 – dependence m = m (h).

**Table 2** Asymmetric Box-Benkin plan matrix for four factors and three levels of factor variation.

| No | Q.kg.s$^{-1}$ | A.m | h.m | m.kg | κ |
|---|---|---|---|---|---|
| 1 | (+) 0.007 | (+)0.11 | (0) 0.15 | (0) 0.011 | 1.05 |
| 2 | 0.007 | (-) 0.05 | 0.15 | 0.011 | 0.9 |
| 3 | (-) 0.003 | 0.11 | 0.15 | 0.011 | 0.65 |
| 4 | 0.003 | 0.05 | 0.15 | 0.011 | 0.8 |
| 5 | (0) 0.005 | (0) 0.08 | (+) 0.2 | (+) 0.013 | 1.05 |
| 6 | 0.005 | 0.08 | 0.2 | (-) 0.009 | 0.65 |
| 7 | 0.005 | 0.08 | (-) 0.1 | 0.013 | 0.55 |
| 8 | 0.005 | 0.08 | 0.1 | 0.009 | 0.45 |
| 9 | 0.005 | 0.08 | 0.15 | 0.011 | 0.75 |
| 10 | 0.007 | 0.08 | 0.15 | 0.013 | 1.0 |
| 11 | 0.007 | 0.08 | 0.15 | 0.009 | 0.65 |
| 12 | 0.003 | 0.08 | 0.15 | 0.013 | 0.6 |
| 13 | 0.003 | 0.08 | 0.15 | 0.009 | 0.35 |
| 14 | 0.005 | 0.11 | 0.2 | 0.011 | 1.15 |
| 15 | 0.005 | 0.11 | 0.1 | 0.011 | 0.7 |
| 16 | 0.005 | 0.05 | 0.2 | 0.011 | 0.7 |
| 17 | 0.005 | 0.05 | 0.1 | 0.011 | 0.5 |
| 18 | 0.005 | 0.08 | 0.15 | 0.011 | 0.75 |
| 19 | 0.007 | 0.08 | 0.2 | 0.011 | 1.05 |
| 20 | 0.007 | 0.08 | 0.1 | 0.011 | 0.9 |
| 21 | 0.003 | 0.08 | 0.2 | 0.011 | 0.95 |
| 22 | 0.003 | 0.08 | 0.1 | 0.011 | 0.6 |
| 23 | 0.005 | 0.11 | 0.15 | 0.013 | 0.85 |
| 24 | 0.005 | 0.11 | 0.15 | 0.009 | 0.55 |
| 25 | 0.005 | 0.05 | 0.15 | 0.013 | 0.65 |
| 26 | 0.005 | 0.05 | 0.15 | 0.009 | 0.45 |
| 27 | 0.005 | 0.08 | 0.15 | 0.011 | 0.75 |

The performed simulations confirm the validity of the accepted assumptions during the theoretical calculation of the origin and motion of the liquid medium and the dissipation of its energy in the working chamber of the machine.

At the same time in the work of the authors **(Dolomakin, et., al., 2017)** the formation of the semi-finished product depends on both the hydration of the flour components and the structural changes that occur as a result of mechanical mixing.

No less important is the fact that mixing leads to aeration of the mixture, during which the nuclei of bubbles are formed, which in turn form the final structure of the finished product.

We also observed that the homogeneity of the suspended state can be ensured only partially. At each point of the real layer of the suspended state, there are pulsating changes, pressure, velocity, temperature.

There are cases when due to poor distribution of dosing components (especially at the beginning and end) insufficient uniformity of weight distribution. This unevenness in the volume of the flow is compensated by the plate working bodies. Thanks to this design, dead zones are avoided and heat and mass transfer processes take place more intensively.

It is known **(Aerov amd Todes, 1968)** that the relatively small particle size of the bulk material and a small bulk density at low humidity of the material itself give them significant mobility. In our opinion, under the action of

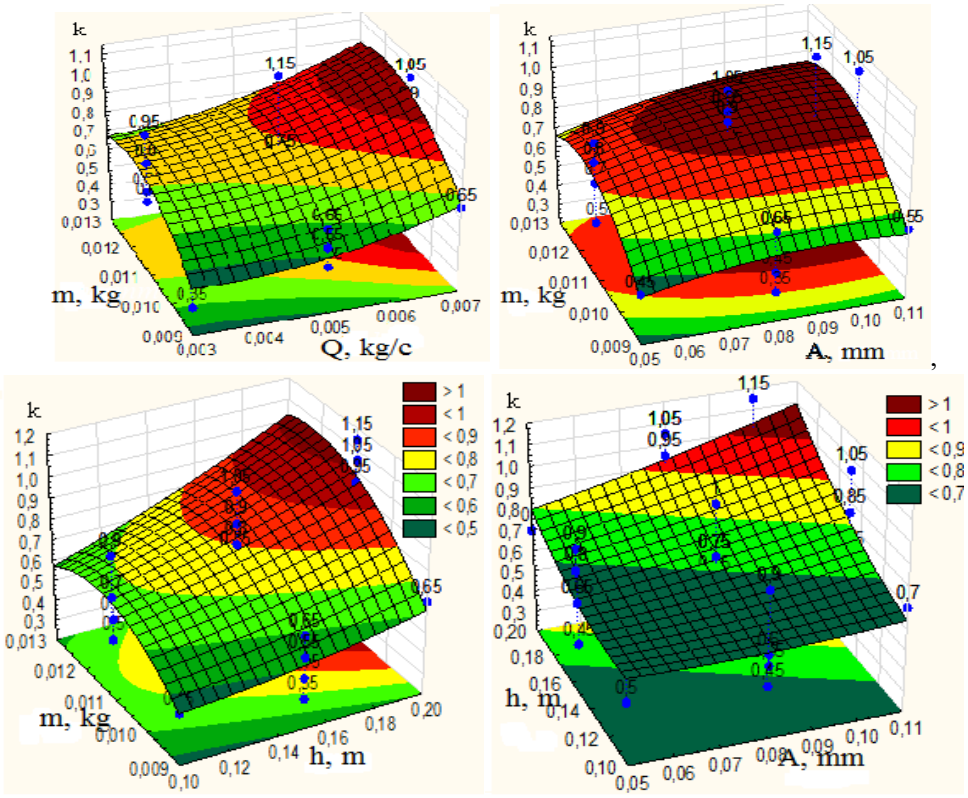


**Figure 7** The response surface changes the homogeneity of the mixture from the influencing factors: Q, kg / s; A, mm; h, m; m, kg.

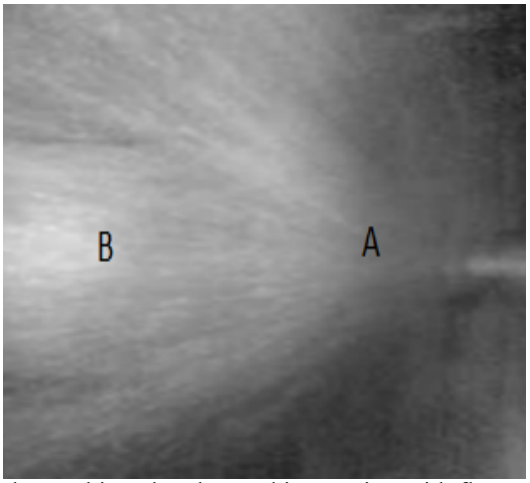


**Figure 8** Photo of spraying liquid into the working chamber and interaction with flour.

liquid flow - also volatility, where the rate of fall, the rotation of the particles of bulk material (flour) will significantly affect the intensity of mixing during convective heat supply by the liquid phase. Due to intensive mixing and direct contact of individual particles, the temperature in the volume of the layer is equalized.

Using the relationship between the liquid flow rate and the particle diameter d of flour at different values of the porosity of the layer ε, as studies have shown **(Baum and Bekmuradov, 1976)**, you can achieve the appropriate state of mixing.

According to the data **(Galle et al., 2011)**, the fragmentation of flour leads the system to a sharp increase in the phase interface and an excess of surface energy that causes various surface phenomena. The main ones are wetting, adsorption, and the formation of a double electric layer.

At the same time, using the concept of equivalent diameter when calculating the velocity of the onset of rarefaction (whirling), it should be borne in mind that the rarefaction of large particles is influenced by previously rarefied small fractions, which transmit part of their motion. This means that the rate of rarefaction depends on the total particle diameter distribution. In **(Gvozdev, 2008)**, based on the theory of similarity, a formula for calculating the velocity of a polydisperse material consisting of fractions of different densities is obtained.

The energy of fluidization **(Kornienko, 2011; Orishkevich and Sobchenko, 2017)** is transferred to the liquid mixture and causes its turbulent movements. This energy goes from large-scale turbulence to small-scale and is eventually dissipated in a given volume of solution without causing temperature changes.

From the analysis **(Symak et al., 2017; Grishkov, 2015)** and our research, we see that the mixing of flour is characteristic without considerable resistance to internal diffusion. In this case, it is necessary to solve the heat and mass transfer equations, which leads to difficulties in solving the corresponding differential equations. Therefore, when solving the differential equations of heat and mass transfer in the suspended layer, various approximate methods are used, as well as formulas obtained by mathematical processing of experimental data under different mixing conditions. However, these calculation formulas are usually presented in criterion form.

The authors **(Singh and MacRitchie, 2001)** revealed the effect of flour polymers to respond to three main stress processes: unraveling (a special form of bonding), reorientation of chains, and breaking the chains. Therefore, the authors of the work **(Piskunov et al., 2020)** showed that the rate of mixing and introduction of work must be above some critical value for the development of the gluten network to further a satisfactory process of manufacturing the final product.

Thus, to ensure optimal operation, it is necessary that the dosing liquid in the working chamber was as close as possible to the equilibrium state with the dosing flour. The solution of these conditions is based on compliance with the dosing regime, during which the particle size of the sprayed liquid will have the same speed and number of all the holes of the spray device. This contact contributes to the optimal flow of heat and mass transfer processes to ensure a high level of surface contact of the phases in the chamber of the machine in the first minutes of the process. Operation of batchers in the discrete mode close to ideal allows reducing expenses for all processes.

The nature of the spray takes place with an initial liquid velocity at the outlet of the nozzle of 50 $m.s^{-1}$ at an angle of opening of the torch up to 60°. When studying the trajectory of droplets in the volume of the chamber, the diameter of which is 0.4 m and the location of 6 nozzles in the upper part of the chamber at the same distance, it was found that for small droplets (about 0.1 m), the trajectories are determined mainly by gravity. high initial speed. When settling on the wall of the chamber, all the drops, regardless of their angle of departure have time to interact to equilibrium, which provides the conditions for the ideal mode.

The hydraulic spraying method is the most economical, simple, and reliable, given that the requirements for the average droplet size in the spray torch are not too strict (about 0.1 m). Therefore, a mathematical description of the heat transfer process when mixing components in a suspended state is not possible due to the large number of variables that determine the process. We decided on simplified theoretical models that will allow to some extent to approach the solution of the problem.

***Heat transfer model with heat transfer by liquid and flour particles.***

To ensure the most uniform distribution of the concentrations of the components in the mixing chamber, it is necessary to supply bulk components at a speed of 2 $m.s^{-1}$ and liquid at a speed of 50 $m.s^{-1}$. The rate of exchange processes and mixing are directly related to the frequency of dissipation of mechanical energy in the chamber. Due to the use of liquid under pressure, the surfaces of the interacting phases are continuously renewed, which causes a significant acceleration of the process of mixing the components. The conversion of energy into heat when kneading the dough should not lead to its overheating, because the optimal fermentation temperature of the dough and dough is 28-32 °C.

Jets of viscous liquid phase with a speed of 40 – 50 $m.s^{-1}$ hit the sprayed flour particles. At the same time, drops of the mix are formed, the size fluctuates in parameters of 0.3 – 0.8 mm depending on the viscosity of liquid and flour.

Based on the process of interaction in the suspended state and the shock-reflecting action of the spray, we can assume that the average diameter of the formed drop of viscous medium is a function of the following values:

$$d_{sr}=f(K_1,V_1,V_2,L,\sigma,\mu,\rho)$$

The value $d_{cp}$often have the view:

$$d_{sr}=K(V_1)^{\alpha 1},(V_2)^{\alpha 2},(L)^{\alpha 3},(\sigma)^{\alpha 4},(\mu)^{\alpha 5},(\rho)^{\alpha 6}$$

Where:

L – is the specific productivity of spraying, the output of the nozzle holes, $kg.s^{-1}$; ρ – is the density of the liquid mixture, $kg.m^{-3}$; σ– is the tension surface, $N.m^{-1}$; μ – is the viscosity of the formed mixture, PA s; $V_1$ – is the velocity of the viscous liquid phase to the interaction, $m.s^{-1}$; $V_2$– is the speed of flour movement to interaction, $m.s^{-1}$; n – is a parameter that characterizes the design of the mixer; $\alpha_1 – \alpha_6$

is a coefficient that takes into account the influence of quantities.

The formed drop of the medium as a result of the greater speed of the liquid, and the low speed of the flour, receives a rotational-translational motion. The two velocities of interaction create the rotation of the droplet. Given these conditions, to determine the average diameter of the droplet formed by the interaction, we use the proposed equation Chernyakova LM, Pashchenko VA, which considers the dependence of the average droplet on generalized number complexes.

$$d_{cp}=K(-\frac{\mu}{\sigma\rho})\ (\frac{n\mu}{\sigma})^{k1}\ (\frac{L\sigma\rho 2}{\mu 3})^{k2}$$

This equation has two dimensionless complexes and a dimensional coefficient A= $\frac{\mu}{\sigma\rho}$, which establishes the physical properties of the formed viscous drop of liquid.

An objective assessment of the droplet formation process is established by two main factors which are the intensity of interaction (mixing) and the time spent in a suspended state. In addition, the change in temperature during hydration is significant. The liquid phase arrives at a temperature of 320 °C, the particle size of the flour is 18 – 20 microns. The residence time of the formed drop is up to 5s.

Since the spraying of the liquid phase is discrete, the change in temperature during the interaction (dispersing phase) with the flour components can be determined by:

$$t=12+9.44\cos(\frac{\pi\tau}{12})$$

Where:
$\tau$ – is time.

At an ambient temperature of 250 °C, the heat loss of the liquid phase when fed into the working chamber is determined by the expression:

$$t_1=\alpha\ S(T_1-T_2)$$

Where:
α – is the heat transfer coefficient for the formed medium,

$$\alpha=132.3B\tau/mK.$$

$T_1$ – is the temperature of the liquid phase entering the chamber, $^0$C; $T_2$ – is the temperature of flour, °C.

The rate of heat loss when mixing the components (dispersed phase) is determined by the expression

$$v_1=\alpha_1 S\ (T_1 - t) \qquad (1)$$

Where:
S – is camera area, m$^2$;

The rate of heat accumulation by the mixture:

$$v_2=Vc\rho dT/d\tau \qquad (2)$$

Equating expressions 1 and 2,:

$Vc\rho dT/d\tau = \alpha_1 S\ (T_1 - t)$ and using the numerical data of these quantities, we obtain:

$$dT/d\tau=-\ \alpha_1 S/Vc\rho(T_1- t) \qquad (3)$$

Where:
V – is the volume of the mixer, m$^3$; ρ is phase density, 980 kg.m$^{-3}$; c – heat capacity of the environment 1.657 kcal.kg$^{-1}$ K.

Then $dT/d\tau+0.93T=6.7\ 0.93+\cos(\frac{\pi\tau}{12})\ 9.44\ 0.93= 6.23+8.5\cos(\frac{\pi\tau}{12})$ (4)

This expression is linear diffraction that can be solved using the integral factor $e^{0.93}$ as follows:

$$Te^{0.93}= 6.23\int e^{0.93}d\tau + 8.5\int e^{0.93}\cos\left(\frac{\tau\pi}{6.23}\right)d\tau$$

The second term of the right-hand side of the equation can be integrated in parts, we obtain:

$$T=6.7\frac{8.5\ 0.93}{0.93^2+0.262^2}cos0.263\tau + \frac{8.5\ 0.262}{0.93^2 0.262^2}\sin 0.262\tau+29.3e^{-0.93}$$

Due to the small values of the second and third terms of the equation, we can use the simplified equation $T = 6.7 + 29.3e^{-0.93\tau}$ and construct a graphical dependence of temperature change (Figure 9).

The theoretical and experimental analysis of heat transfer in the interaction of components in the suspended state allowed us in our case to establish its value when reaching the dosing rate of the liquid and solid phases at a small distance from the place of their entry into the working chamber. According to the process of dosing and structure of the mixer, the considered dynamics of the forming medium do not affect the temperature change, both theoretically and experimentally (Figure 11).

From Figure 9 and calculated data of temperature change

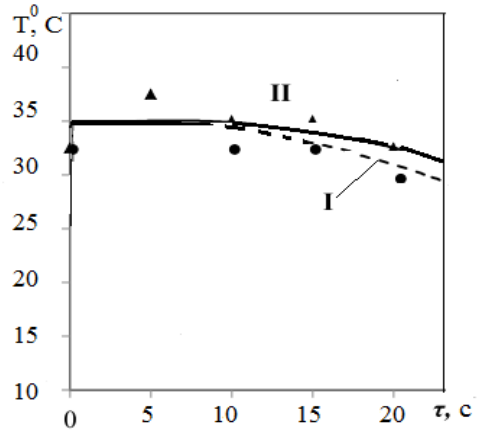


**Figure 9** Change in ambient temperature: 1 – experimental data; 2 – settlement.

during contact and formation of the environment, it is established that their data change by insignificant value. Therefore, the data of temperature formations do not affect the change in the viscosity of the medium but allow to more active influence the biochemical processes under the action of the working bodies of the machine.

***Heat and mass transfer during the interaction of flour with liquid components.***

The heat of reaction from the surface of the flour is distributed by thermal conductivity and convection into the environment. The task of determining the surface temperature of flour, temperature distribution in the medium and flour, determining the heat fluxes to the flour and the liquid allows to establish the process in the developed machine.

The hydrodynamics of the process of interaction of components suggests that there is an analogy between these two processes. Thermal phenomena during mass transfer during mixing in the liquid-solid system are determined by the laws of thermal processes. The processes of flour dissolution occurring in the diffusion region are determined mainly by the laws of molecular and convective diffusion. At the same time, each interaction is accompanied by a certain thermal effect, and its values are facilitated by positive values. In some cases, as noted in **(Zanoni, Pierucci and Peri, 1994)** for minor thermal effects, heat release can be neglected and the temperature can be estimated by the average value.

In the first approximation, we can assume that the mass transfer coefficient β changes its value depending on the temperature T according to the linear law **(Ved and Ponomarenko, 2014; Mallyk and Gumnitsky, 1986)**:

$$\beta = \beta_0 (1 + \sigma T_п)$$

Where:

$\beta_0$ – ismass transfer factor for $T_п$; $\sigma$– is coefficient of proportionality.

The amount of heat Q emitted per unit area per unit time is determined from the dependence **(Ved and Ponomarenko, 2014; Orishkevich et al., 2017)**

$$Q= \beta_0 (1 + \sigma T_п) C_R Q_R , \quad (5)$$

Where:

$C_R$ – is concentration; $Q_R$ – is thermal effect of interaction.

The scheme of temperature distribution in flour and in a liquid medium is shown in Figure 10. Given the surface interaction, the highest temperature will be observed on thesurface of the spherical flour. This significantly changes all the physicochemical characteristics used in the calculation of heat transfer and mass transfer coefficients,as well as diffusion coefficients.

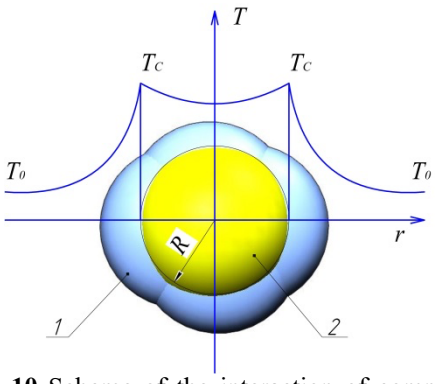


**Figure 10** Scheme of the interaction of components in the formation of a mixture: 1 – liquid components, 2 – particle flour.

The heat distribution in a spherical solid is a determined differential equation of thermal conductivity **(Baum and Bekmuradov, 1976)**:

$$\frac{\partial T}{\partial \tau} = a\left(\frac{\partial^2 y}{\partial r^2} + \frac{2}{r}\frac{\partial T}{\partial r}\right)$$

r>0; 0 < r < R (6)

Where:

T – is temperature; r – is current radius; R – is the radius of flour; a is coefficient of thermal conductivity.

The boundary condition is determined by the distribution of heat released on the surface and which is distributed between the solution and the solid particle is written in the form of an equation **(Baum and Bekmuradov, 1976)**:

$$\beta_0 (1 + \sigma T_п) C_R Q_R = \boldsymbol{\lambda}\left(\frac{\partial T}{\partial r}\right) + \alpha(T_b - T_r)$$

Where:

**λ**– is a coefficient of thermal conductivity of a solid body; a is heat transfer coefficient; $T_b$,$T_r$ – is the surface temperature of the flour and the temperature of the liquid medium.

The initial conditions for this case of interaction will be as follows:

$$T(r,\tau=0) = T_0;\ T_1(\tau) = T_0$$

We enter dimensionless parameters:

$$\varphi = \frac{r}{R}$$

dimensionless radius;

$$F_0 = \frac{a\,\tau}{R^2}$$

Fourier number;

$$B_i = \frac{aR}{\lambda}$$

Bio number;

$$T^* = \frac{\beta_0 C_R Q_R R}{\lambda}$$

parameter having a dimension of temperature.

Hence, we obtain a differential equation with boundary and initial conditions, written in the dimensionless form **(Baum and Bekmuradov, 1976)**:

$$\begin{cases} \dfrac{\frac{\partial T}{\partial Fo} = \frac{\partial^2}{\partial \varphi^2} + \frac{2}{\varphi}\frac{\partial T}{\partial \varphi}}{Bi(T_n - T_0) + \left(\frac{\partial T}{\partial \varphi}\right)_{\varphi-1} = T^1(1+\sigma T_n)} \\ T(\varphi, o) = T_0; T_1(Fo) = 0. \\ \left(\frac{\partial T}{\partial \varphi}\right)_{\varphi-1} = 0. \end{cases} \quad (7)$$

When modeling the formed shape of the drop, the diameter of which is much smaller (d = 0.015 – 0.03 m), it can be considered as an unlimited cylinder. It has its characteristics, which are due to its shape, size and thermophysical properties. In this case, the value of the Fourier test **(Mallik and Gumnitsky, 1986; Orishkevich**

**et al., 2017)** for the minimum value of the diameter (d = 0.015 m.):

$$F_0 = \frac{a\tau}{R^2} = \frac{22\,10^{-8}60}{0.0075^2} = 0.2347$$

Where:
A – is thermal conductivity, $m^2.sec^{-1}$; R – is determining size, m; τ is time, sec.

The Fourier criteria in this context reflect dimensionless time.

At the beginning of the process of interaction of components with the dimensions characteristic of mixing components in a suspended state, they can not be considered as a semi-limited body and neglect the increase in temperature of their center. In the non-stationary process of heat transfer in an unrestricted cylinder of radius R, heat is transferred to the surface of the formed droplet. The condition of uniform initial temperature distribution is accepted.

The system of equations (7) was solved by a method based on Laplace transforms. The solution consists of the zero root and other roots of the characteristic equation, which for this case when the volume of the liquid phase was assumed to be large, has the form:

$$tg\mu = -\frac{\mu}{Bi - T^1\sigma - 1}$$

The heating of a cylindrical droplet is described by the differential equation of thermal conductivity in cylindrical coordinates. The temperature distribution in the droplet-based on the solution of the system of equations (7) has the form:

$$\frac{T-T_0}{(1+\sigma T_0)T^1} = \frac{1}{Bi-T^1\sigma} - \sum_{H-1}^{H} \frac{2(1-Bi+T^1\sigma)}{[\mu_н^2+(1-Bi+T^1\sigma)(T^1\sigma-Bi)cos\mu_n]cos\mu_n} \frac{\sin\mu_n\varphi}{\mu_n\varphi} e^{-\mu_n^2 Fo} \quad (8)$$

In the case when the mass transfer coefficient β changes slightly, the solution (8) is simplified and can be written as:

$$\frac{T-T_0}{T^1} = \frac{1}{Bi} - \sum_{n-1}^{\infty} \frac{2(Bi-1)}{[\mu_n^2+Bi(Bi-1)]cos\mu_n} \frac{sin\mu_n\varphi}{\mu_n\varphi} e^{-\mu_n^2 Fo} \quad (9)$$

Solution (9) makes it possible to theoretically determine the temperature in the center of the droplet particle, which allows us to experimentally test the theoretical solution and assume a slight change in the mass transfer coefficient in the liquid phase. In the center of the value of φ = 0, and:

$$lim \lim_{\varphi\to 0} \frac{sin\mu_n\varphi}{\mu_n\varphi} = 1$$

therefore, the solution for T = Tn will look like this:

$$\frac{T_n - T_0}{T^1} = \frac{1}{Bi} - \sum_{n-1}^{\infty} \frac{2(Bi-1)}{[\mu_n^2 + Bi(Bi-1)]cos\mu_n} e^{\mu_n^2 Fo}$$

Under the conditions of modeling the thermophysical characteristics received by an experimental way take part. They depend on temperature and take into account mass transfer processes when mixing. We determined the nature of changes in the thermophysical characteristics of the vapor. The equations describing the properties of the sponge dough for the temperature range (293 – 305 K) are obtained:

$$C=2866+1.571\ t; \quad (10)$$

$$\lambda=0.219+0.00025t; \quad (11)$$

$$\alpha=6.345+0.003t; \quad (12)$$

We see that the dependence of the coefficients of thermal conductivity λ, thermal conductivity a, and heat capacity c on temperature is linear. Humidity has a greater effect on the experimental coefficients than temperature. The analysis of dependences (10 – 12) shows that their values increase with increasing temperature. The effective density of the formed drop of the medium was determined at t = 25 - 35 °C:

$$\rho(t) = -6.875t + 952.5$$

The relationships between the coefficients of mass transfer and heat transfer are determined by the same hydrodynamic situation that arises due to the interaction of phases formed at the interface of the phases. The only difference is that the mass transfer takes place by transporting the reagent to the surface of the solid phase of the flour, and heat transfer from the surface of the solid phase to the liquid reagent.

The results of the computational experiment of the formed droplet of components with a diameter of 0.002m at a temperature of 29 °C are presented in Figure11.

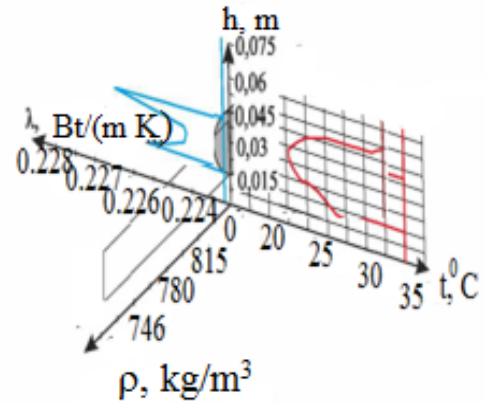


**Figure 11**. The results of a computational experiment of the interaction of components at times τ, p.

For the initial moment of time (τ = 0s) there is a uniform distribution of temperature, density, and thermal conductivity in the cross-section of the droplet. Under the action of the heat flow of the liquid phase on the surface of the flour is heating it with a simultaneous change in thermophysical characteristics, which depend on the temperature of the heated layers. Visualization of the process of droplet formation after 3s mixing is shown in Figure 11.

The obtained experimental values of mass transfer and heat transfer coefficients are of the same type, which allows confirming the analogy between the mass transfer and heat transfer processes for mass transfer processes, which are accompanied by the formation of phase interactions on the surface.

Analysis of the process of preparation of the paste, performed in a suspended state, showed that due to energy dissipation there is no increase in the temperature of the paste during mixing from the initial 32 °C to 32.8 °C, which is acceptable. Temperature regimes occur almost unchanged.

## CONCLUSION

Based on the results of the process of mixing the components, the functions performed by the mixer, first of all, are the even distribution of prescription components (flour, yeast, water) and the creation of favorable conditions for the formation of the structure of the paste. To achieve the desired result - a homogeneous paste - in the considered design of the mixer, the feed rate of the flour should be 2 $m.s^{-1}$, the feed rate of the liquid - 50 $m.s^{-1}$.

From the point of view of kinematics, it is possible to conclude that in the chamber of the car there is a formation of homogeneous liquid mix in the presence of four ways:

- direct flow method, where the speed of liquid components exceeds the speed of flour or their fixed particles;
- gravitational method, where the falling particles of flour are in contact with moving liquid components;
- inertial, where the change in the direction of movement of liquid components and flour occur during their interaction;
- convective method, where the movement of liquid components by the difference in specific gravity, ie, natural convection.

The proposed mathematical model allows the calculation of the duration of the interaction of components depending on the temperature of the medium, thermophysical parameters, and the size of the formed droplet, which helps to determine the rational parameters of the environment of the working chamber in a suspended state. This allows for different shapes and sizes of droplets without conducting a physical experiment to establish the process of mass heat transfer when mixing components.

The results of experimental research and the proposed mathematical model, which takes into account the effective thermophysical characteristics, confirm the feasibility of using a mathematical model to calculate the mixing process.

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# THE EFFECT OF HEAT-MOISTURE TREATMENT CONDITIONS ON THE STRUCTURE PROPERTIES AND FUNCTIONALITIES OF POTATO STARCH

*Chunli Deng, Oksana Melnyk, Yanghe Luo*

## ABSTRACT

Potato starch was modified via heat-moisture treatment (HMT) under various reaction conditions. The effect of HMT on structural, physicochemical, and in vitro digestibility were investigated. HMT led to the rupture, adhesion and partial gelatinization, and agglomeration of the granules which surface became rougher, thereby increasing the particle size and resulting in the hollow structure located at the hilum of potato starch granules. XRD results showed an increased relative crystallinity and transformed crystalline structure from B-type to C-type with the extension of heat moisture treatment. FTIR spectroscopy results indicated that HMT might cause additional interactions between amylose-amylose, and/or amylose-amylopectin and/or amylopectin-amylopectin chains, which eventually leads to the increase of the mass of the carbonyl group and a hydroxyl group. HMT significantly decreased the peak viscosity, hold viscosity, and breakdown viscosity of starch, while the gelatinization temperature increased. The tHMT1, THMT100, and CHMT15 had the lowest content of RDS and there was no significant difference between the three samples, that is these three samples had the highest total content of SDS and RS.

**Keywords:** potato starch; heat moisture treatment; structures properties; physicochemical properties; in vitro digestibility

## INTRODUCTION

Starch is an important plant raw material for food, medicine, and other industrial application due to its advantages of being renewable, nontoxic, biodegradable, and relatively low cost **(Thakur et al., 2019)**. Meanwhile, starch-based derivatives are nowadays used for many applications in food processing to achieve particular technological properties **(Lu et al., 2014)**. Consequently, to meet the requirements of food and other industrial production, various approaches have been applied to enhanced starch properties, including physical, chemical, and enzymatic modifications. Compared with chemical modification, physical modification is a more attractive method due to the absence of extra chemicals and derivative products in the modified starch, **(Xie et al., 2018)**.

Different starch modification techniques regarding properties and applications have been comprehensively discussed in earlier publications **(Li, 2018)**. Heat moisture treatment (HMT) is a physical treatment that heats the starch granules at the low moisture content (<35%) and a high temperature (80 – 140 °C) being above the glass transition temperature, but below the gelatinization temperature **(Sui et al., 2015)**. HMT promotes changes in the structure of starch chains, resulting in granular swelling, thermal stability, and paste property changes **(Colussi et al., 2017)**.

### Scientific hypothesis

Due to the diversity of reaction conditions, including the botanical source, moisture content, temperature, treatment time, heat source, and cooling progress, it is difficult to define the properties of HMT starch. Therefore, this project was carried out to evaluate the effects of moisture levels, heating time, and temperature on morphological, pasting behavior, and structural characteristics as well as vitro digestibility properties of potato starch. We are expecting to provide new knowledge on HMT affecting potato starch, we are also expecting to broaden HMT starch application in the food field.

## MATERIAL AND METHODOLOGY

### Samples

Native potato starch (NS) was obtained from an unknown cultivar of potatoes purchased from a local market with the technology of wet milling extraction in Hezhou, China.

### Chemicals

Porcine pancreatic α-amylase (EC 3.2.1.1, Shanghai Yuanye Bio-Technology Co., Ltd, China).

Amyloglucosidase from Aspergillusniger (EC 3.2.1.3, Shanghai Yuanye Bio-Technology Co., Ltd, China).

### Instruments

Electric thermostatic drying oven (DH411C, Yamto, Japan).

Laser diffraction particle size analyzer (BT-2001, Baxter Instruments Co. LTD, China).

Scanning electron microscopy (JSM-7610F, JEOL, Japan).

X-ray diffractometer (Rigaku Ultima IV, Ultima IVTM, Japan).

FT-IR spectrophotometer (Spectrum 400, Perkin Elmer, USA).

Rapid viscosity analyzer (RVA-Starch Master2, Perten Instruments, Sweden).

**Laboratory Methods**

***Particle size determination***

The particle size parameters of starch samples were detected by a laser diffraction particle size analyzer (BT-2001, Baxter Instruments Co. LTD, China) with a dry method. Air was used as a medium and the optical mode was Mie. A starch sample (3 g) was added to the storage hopper with a shading ratio ranging from 5% – 12%.

***Methods of scanning electron microscopy***

The morphology of starch granule was observed by scanning electron microscopy (JSM-7610F, JEOL, Japan) as **Li (2018)** method with slight modification. The starch samples were sprinkled on a double-sided adhesive tape mounted on the sample stage made of aluminum and coated with gold with for 30 s by using an EDT-2000 ion sputter (2×10 – 4 MPa, 25 mA). Subsequently, all the coated samples were examined at an acceleration voltage of 2.0 kV and the images were captured at ×1500 magnification.

***Methods of X-ray diffraction (XRD)***

An X-ray diffractometer (Rigaku Ultima IV, Ultima IVTM, Japan) with Cu-Kα radiation was used to evaluate the crystalline patterns of starch samples. The measurement was operated at 40 kV and 40 mA with the scanning region of the diffraction ranged between 4° and 40° with a step length of 0.02° and the scan speed was 4°/min **(Zhao et al., 2019)**. The relative crystallinity was calculated by the ratio of the relative area of crystalline peaks to the total area using MDI Jade 6 software as follows:

$$Relative\ crystallinity(\%) = \frac{Area\ of\ crystalline\ peaks}{Total\ area\ of\ crystalline\ and\ amorphous\ peaks} \times 100 \quad (1)$$

***Methods of Fourier transform infrared spectroscopy (FT-IR)***

The Fourier transform infrared spectra of starch samples were recorded on an FT-IR spectrophotometer (Spectrum, Perkin Elmer, USA) with a scanning spectral range from 4000 $cm^{-1}$ to 400 $cm^{-1}$ at 25 °C temperature. The starch powder was placed on to sampling unit and then to be measured directly **(NidhiDangi, Baljeet and Ritika, 2019)**.

***Analysis of the starch viscosity properties***

The starch viscosity properties of starch samples were evaluated using an RVA Starch Master 2 (Perten Instruments, Sweden) with standard RVATM potato starch methodology. A 2.5 g starch sample (corrected to 14% moisture content) was dispersed in 25 mL distilled water in the aluminum RVA canister. The starch suspensions were heated to 50 ℃ in 60 s, heating from 50 ℃ to 95 ℃ in 222 s and maintained at 95 ℃ for 150 s, and then were cooled to 50 ℃ in 228 s, and maintained at 50 ℃ for 108 s. A whole RVA test needed 780 s. The rotational speed was 960 rpm for the first 10 s and followed rotation speed was 160 rpm. The parameters, including gelatinization temperature, peak viscosity, breakdown, setback, and final viscosity were determined.

***Analysis of in vitro digestibility***

The rate of in vitro digestion of native starch (NS) and HMT starch was determined using the Englyst method following the method of **Xie et al. (2018)**. Each Starch sample (200 mg dry basis) was suspended in a 100 mL centrifuge tube containing 10 mL of sodium acetate buffer solution (0.1 M, pH 5.2) by vortexing for 1 min. And then the centrifuge tube was cooked in boiling water for 30 min, then was removed and cooled to 37 ℃. Another 10 mL of sodium acetate buffer solution (0.1 M, pH 5.2) was used as a blank control. Then five glass balls were added to the sample centrifuge tube, and all the experimental samples and blank control samples were incubated at 37 ℃ for 10 min with shaking (160 rpm). Then 5 mL α-glucoamylase enzyme solution (300 $U.mL^{-1}$) and 2 mL amyloglucosidase enzyme solution (225 $U.mL^{-1}$) were added to the sample centrifuge tube, followed by incubation in a water bath at 37 °C with shaking (190 rpm). Aliquots (1 mL) of the hydrolyzed solution were taken at 20 and 120 min and mixed with 20 mL of ethyl alcohol to deactivate the enzymes. The mixed solution was centrifuged at 4000 rpm for 10 min, and the glucose content in the supernatant was investigated using the GOPOD assay kit (Megazyme, Co., Wicklow, Ireland). Based on the hydrolysis rate, starch was defined following digestion speed as follows: rapidly digested starch (RDS, digested within 20 min), slowly digested starch (SDS, digested between 20 and 120 min), and resistant starch (RS, residual starch after 120 min), respectively. The RDS, SDS, and RS contents were calculated following the following respective formulas:

$$RDS(\%) = (G20 - FG) \times 0.9 / TS \quad (2)$$

$$SDS\ (\%) = (G120 - G20) \times 0.9/TS \quad (3)$$

$$RS(\%) = 100 - RDS - SDS \quad (4)$$

Where:

G20 is the glucose content released at 20 min; G120 is the glucose content released at 120 min; FG is the glucose content in the sample before enzymatic treatment (in terms of 0) and TS is the mass of the total starch.

**Description of the Experiment**

**Sample preparation:** The moisture content of native starch powder (NS) was determined before heat- moisture treatment, and then heat-moisture treatment modified potato starch samples were prepared as follow: (1) Different heating time groups (tHMT): 70 g native starch powder was weighted in 500 mL Duran laboratory bottle, and ultrapure water was added to adjust moisture content to 25% and equilibrated for 24 hours at 25 ℃, samples were then heated at 110 ℃ for 1 h, 2 h, 3 h, 4 h,5 h and 6 h ( tHMT1, tHMT2, tHMT3, tHMT4, tHMT5, tHMT6, respectively ) in a hot-air oven. (2) Different heating temperature groups (THMT): Starches were weighted in different spiral blue-moth bottles, and ultrapure water was added to adjust moisture content to 25% and equilibrated for 24 hours at 25 ℃, samples were then heated at 90 ℃, 100 ℃, 110 ℃, 120 ℃, 130 ℃ (THMT90, THMT100, THMT110, THMT120, THMT130, respectively) for 2 h in a hot- air oven. (3)

Different moisture content of starch system groups (CHMT): Starches were weighted in different spiral blue-moth bottle, and different volumes of ultrapure water were added to adjust moisture content to 15%, 20%, 25%, 30%, 35%（CHMT15, CHMT20, CHMT25, CHMT30, CHMT35, respectively）and equilibrated for 24 h at 25 ℃, samples were then heated in an electric thermostatic drying oven for 2 h. All these three groups of treated samples were dried in a drying oven at 45 °C for 24 h to make sure the moisture content of the treated samples was less than 12%. The dried potato starch was pulverized for 45 s using a universal pulverizer, passed through an 80-mesh sieve, vacuum-packed in polyethylene bags, and stored in an airtight container for conducting further studies.

### Statistical Analysis

Results are presented as mean± standard deviation (SDM). Statistical analysis was performed using the analysis of variance tool in the DPS (7.05) environment. The obtained data from the particle size determination, the analysis of the starch viscosity properties and in vitro digestibility were subjected to analysis of variance (ANOVA) and the Duncan Multiple Range Test (DMRT), and the values were considered significantly different when $p \leq 0.05$.

## RESULTS AND DISCUSSION

### Particle size analysis

The particle size distributions of heat moisture treatment starch and native potato starch granules were shown in Table 1 to Table 3. As can be seen from Table 1 to Table 3. The D10, D50, D(4,3), and D (3,2) of HTM starch were significantly higher than that of NS except for the 50 value and D(4,3) of tHMT6, whereas the S.S.A. value of HMT was significantly lower than NS. The temperature and moisture content had a great influence on the D90 value of HMT starch. The large particle size of HMT starch indicated that heat moisture treatment could result in the slight swell of potato starch, which might further lead to the rupture, adhesion and partial gelatinization, and agglomeration of the granules. This result was in agreement with a previous study about lily starch **(Li et al., 2020)**.

### Morphological properties

The microscopic images of the NS and HTM starch samples obtained under SEM were shown in Figure 1. Almost all the NS starch showed elliptical-shaped granules and some round-shaped granules with a smooth surface.

After heat moisture treatment, potato starch granules were damaged and showed a negligible change in terms of morphology at low moisture levels (≤25%), which was in agreement with the results of HMT modified highland barley starch granules at low moisture levels **(Liu et al., 2018)**. Relatively high temperatures (e.g., 120 ℃ and 130 ℃) and high moisture content (e.g., 30% and 35%) led to a coarser and rougher granule surface than that of NS. Similar results were obtained for HMT modified rice starch that the granule surface became slightly rougher than that of NS with the increasing agglomeration degree of the starch granules **(Wang et al., 2019)**.

**Table 1** Effect of heat-moisture treatment on the particle size distribution of potato starch under a different holding time.

| Samples | D10 (μm) | D50 (μm) | D90 (μm) | D(4,3) (μm) | D(3,2) (μm) | S.S.A($m^2 \cdot kg^{-1}$) |
|---|---|---|---|---|---|---|
| NS | 17.30 ±0.49$^{d}$ | 34.12 ±0.40$^{c}$ | 60.24 ±0.47$^{ab}$ | 36.60 ±0.38$^{c}$ | 22.24 ±0.56$^{d}$ | 99.93 ±2.54$^{a}$ |
| tHMT1 | 18.37 ±0.22$^{c}$ | 35.33 ±0.19$^{b}$ | 61.61 ±0.30$^{ab}$ | 37.83 ±0.18$^{ab}$ | 24.02 ±0.14$^{c}$ | 92.50 ±0.52$^{b}$ |
| tHMT2 | 18.67 ±0.03$^{c}$ | 35.30 ±0.14$^{b}$ | 61.51 ±0.59$^{ab}$ | 37.84 ±0.21$^{ab}$ | 24.27 ±0.06$^{c}$ | 91.52 ±0.20$^{b}$ |
| tHMT3 | 19.43 ±0.13$^{ab}$ | 35.23 ±0.94$^{b}$ | 58.49 ±4.10$^{bc}$ | 37.18 ±1.35$^{bc}$ | 25.90 ±1.25$^{ab}$ | 85.99 ±4.17$^{cd}$ |
| tHMT4 | 18.95 ±0.13$^{bc}$ | 36.16 ±0.122$^{a}$ | 63.28 ±0.44$^{a}$ | 38.75 ±0.12$^{a}$ | 25.00 ±0.21$^{bc}$ | 88.87 ±0.74$^{bc}$ |
| tHMT5 | 18.59 ±0.14$^{c}$ | 35.37 ±0.28$^{b}$ | 61.45 ±0.31$^{ab}$ | 37.85 ±0.27$^{ab}$ | 24.48 ±0.19$^{c}$ | 91.63 ±0.48$^{b}$ |
| tHMT6 | 19.80 ±0.41$^{a}$ | 34.73 ±0.46$^{bc}$ | 56.39 ±2.33$^{c}$ | 36.50 ±0.76$^{c}$ | 26.51 ±0.39$^{a}$ | 83.83 ±1.23$^{d}$ |

**Table 2** Effect of heat-moisture treatment on particle size distribution potato starch under different holding temperatures.

| Samples | D10(μm) | D50(μm) | D90(μm) | D(4,3) (μm) | D(3,2) (μm) | S.S.A($m^2 \cdot kg^{-1}$) |
|---|---|---|---|---|---|---|
| NS | 17.30 ±0.49$^{c}$ | 34.12 ±0.40$^{c}$ | 60.24 ±0.47$^{c}$ | 36.60 ±0.38$^{c}$ | 22.24 ±0.56$^{c}$ | 99.93 ±2.54$^{a}$ |
| THMT90 | 18.68 ±0.29$^{b}$ | 35.59 ±0.30$^{ab}$ | 61.78 ±0.33$^{b}$ | 38.06 ±0.27$^{ab}$ | 24.02 ±0.67$^{b}$ | 92.54 ±2.66$^{b}$ |
| THMT100 | 18.62 ±0.21$^{b}$ | 35.60 ±0.39$^{ab}$ | 61.85 ±0.55$^{b}$ | 38.08 ±0.36$^{ab}$ | 24.30 ±0.26$^{b}$ | 91.43 ±0.97$^{b}$ |
| THMT110 | 18.67 ±0.03$^{b}$ | 35.30 ±0.14$^{b}$ | 61.51 ±0.59$^{b}$ | 37.84 ±0.21$^{b}$ | 24.27 ±0.06$^{b}$ | 91.52 ±0.20$^{b}$ |
| THMT120 | 19.36 ±0.42$^{a}$ | 35.49 ±0.35$^{ab}$ | 59.77 ±0.09$^{c}$ | 37.86 ±0.42$^{b}$ | 26.09 ±1.13$^{a}$ | 85.27 ±3.79$^{c}$ |
| THMT130 | 18.84 ±0.17$^{b}$ | 35.87 ±0.16$^{a}$ | 62.69 ±0.47$^{a}$ | 38.46 ±0.18$^{a}$ | 24.92 ±0.19$^{b}$ | 89.15 ±0.68$^{b}$ |

**Table 3** Effect of heat-moisture treatment on the particle size distribution of potato starch under different moisture content.

| Samples | D10(μm) | D50(μm) | D90(μm) | D(4,3) (μm) | D(3,2) (μm) | S.S.A($m^2$/kg) |
|---|---|---|---|---|---|---|
| NS | 17.30 ±0.49$^{c}$ | 34.12 ±0.40$^{d}$ | 60.24 ±0.47$^{e}$ | 36.60 ±0.38$^{d}$ | 22.24 ±0.56$^{c}$ | 99.93 ±2.54$^{a}$ |
| CHMT15 | 18.53 ±0.08$^{b}$ | 35.25 ±0.11$^{c}$ | 61.25 ±0.25$^{de}$ | 37.71 ±0.14$^{c}$ | 24.17 ±0.11$^{b}$ | 91.95 ±0.40$^{b}$ |
| CHMT20 | 18.93 ±0.16$^{b}$ | 35.72 ±0.03$^{c}$ | 62.38 ±0.29$^{bc}$ | 38.33 ±0.02$^{bc}$ | 24.67 ±0.29$^{b}$ | 90.04 ±1.06$^{b}$ |
| CHMT25 | 18.67 ±0.03$^{b}$ | 35.30 ±0.14$^{c}$ | 61.51 ±0.59$^{cd}$ | 37.84 ±0.21$^{c}$ | 24.27 ±0.06$^{b}$ | 91.52 ±0.20$^{b}$ |
| CHMT30 | 18.81 ±0.29$^{b}$ | 36.38 ±0.21$^{b}$ | 63.37 ±0.90$^{b}$ | 38.68 ±0.46$^{b}$ | 24.92 ±0.46$^{b}$ | 89.17 ±1.67$^{b}$ |
| CHMT35 | 20.05 ±0.47$^{a}$ | 41.12 ±0.42$^{a}$ | 78.39 ±0.62$^{a}$ | 45.60 ±0.50$^{a}$ | 27.00 ±0.81$^{a}$ | 82.33 ±2.45$^{c}$ |

Note: all values are the means of triplicate determinations ±*SD*. The means within the same column with different letters are significantly different ($p < 0.05$); D(4,3), particle diameter of volume; D(3,2), particle diameter of surface, S.S.A., specific surface area; D10, D50, and D90 represent the corresponding particle size which is smaller than 10%, 50%, and 90% of the sample particles, respectively.

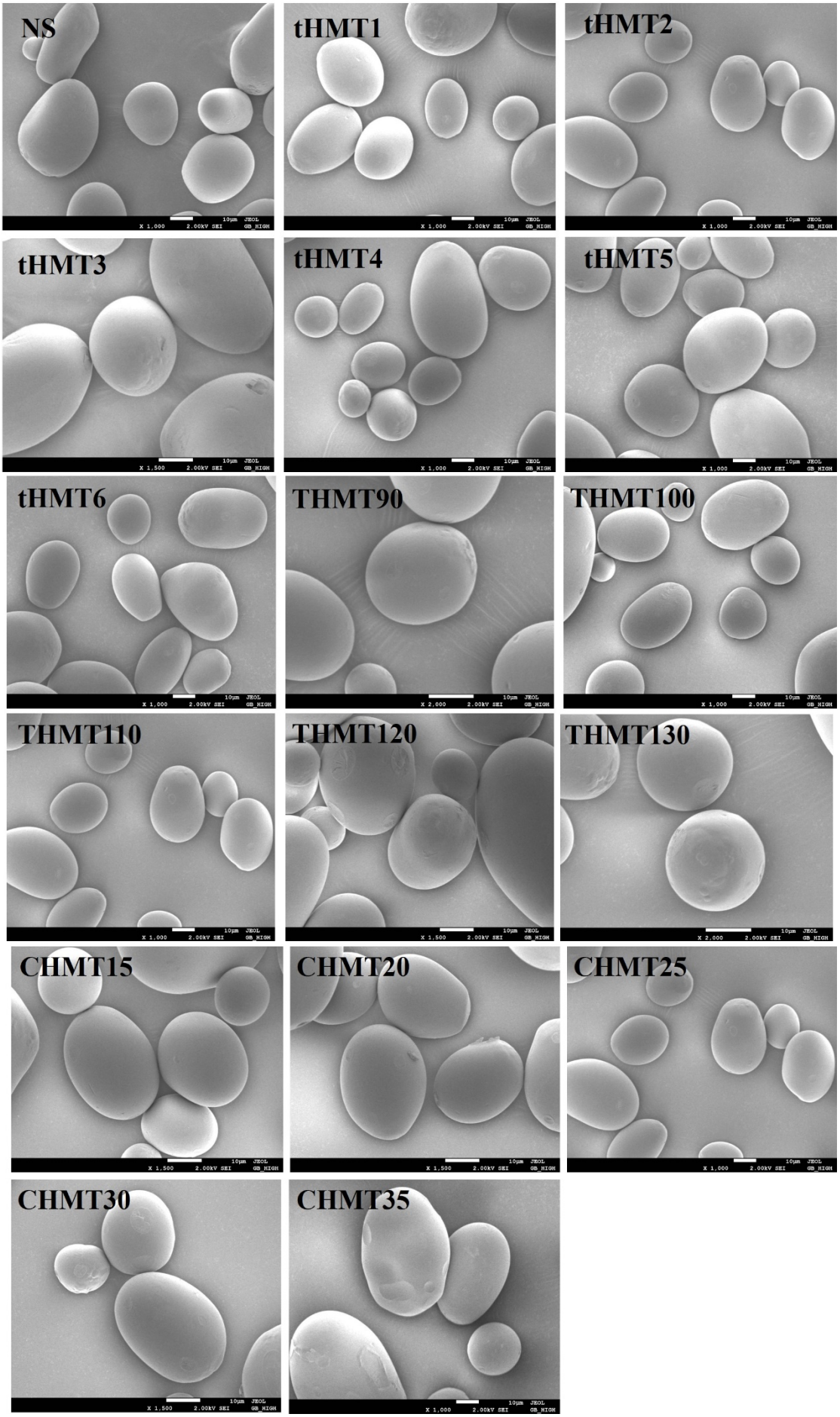


**Figure 1** Morphology of the native potato starch and HMT starch.

## Light microscopy

Normal light microscopy (NLM) and polarized light microscopy (PLM) was used to obtain the microscopic images of NS and HMT modified starch (Figure 2 and Figure 3). Smooth surface and hilum structure were obtained from NS granules under normal light. After HMT modification, the hilum structure of HMT potato starch became hollow. The diameter and depth of the hollow region increased with increasing the length of heating, moisture content, and heating temperature. Similar results were obtained by **Li et al. (2020)**. The location of the hilum structure which was in the amorphous zone led to relative fragility. Furthermore, high temperature and certain moisture conditions during HMT caused partial swelling and disruption of starch granules, which was the reason that the hollow central region became large.

Visible black polarization cross-like "X" and "十" of NS starch granules were observed under polarized light. Maltese cross with the helium was biased toward the small end of the starch granules, and the deviation of large granular starch was higher. However, for HMT starch, the contour of the Maltese cross became gradually unclear and showed a large black area in the middle of the cross. The Maltese cross contour became unclear by short-time HMT modification and became obvious when the heating length was over 5 h. the average orientation of helical structures caused birefringence patterns. The results were consistent with the results of previous research of K. Liu et al **(Liu et al., 2018)**. The decrease in birefringence intensity on HMT indicated a change in the radial orientation of double helices due to increased amylopectin chain flexibility.

## X-ray diffraction (XRD)

The diffraction patterns of NS and HMT starch samples were illustrated in Figure 4. Four main peaks at 5.6° 2θ, 17° 2θ, 22° 2θ and 24° 2θ can be seen from the NS starch pattern, indicating that the crystal form of potato starch was B-type. Some significant changes in the crystalline patterns of HMT starch were observed. With the extension of heat moisture treatment, all HMT starch diffraction peaks gradually disappeared at 5.6° 2θ and 19.5° 2θ, the diffraction peak at 15° 2θ became smooth, and the two independent diffraction peaks at 22° 2θ and 24° 2θ tended to merge into a single peak near 23° 2θ, which was a characteristic of A-type. When treatment time reached 5 hours, the diffraction peak at 23° 2θ divided into two diffraction peaks at 22° 2θ and 24° 2θ again. These results indicated the crystal structure of potato starch gradually changed from B-type to C-type with the extension heat moisture treatment. When treatment time was too long (more than 5 hours), its crystal structure changed to B-type again.

Relative crystallinity for NS was 19.37% and the crystallinity of tHMT starch initially increased and then decreased with the extension of the treatment time, the tHMT3 sample had the highest crystallinity of 24.43% of tHMT group samples. As can be seen from Table 4 – Table 6, all HMT starch had higher crystallinity than that of NS. With the increase of treatment temperature from 90 ℃ to 130 ℃, the crystallinity increased from 21.30% to 25.74%. With the increase of moisture content from 15% to 35%, the crystallinity increased from 21.46% to 25.09%. The results indicated that heat moisture treatment destroyed and rearranged the double helix structure of starch molecules, which strengthened the connection between starch chains, produced a new and more stable crystalline structure, and ultimately led to an increasing crystallinity of starch. The increased X-ray intensity was agreed with previous studies about HMT mung bean starch **(Li, Ward and Gao, 2011)**.

## FTIR spectroscopy

The FTIR spectra of NS and HMT starches were showed in Figure 5. All samples showed the same characteristic peak at 3290, 2920, 1636, and 1344 $cm^{-1}$ and no new absorption peaks were generated, which indicated that the primary structure of starch could not be changed by heat-moisture treatment and proved that heat-moisture treatment was a physical modification method. However, it can be seen from the spectrograms that within the spectrum range, the intensity of the absorption peaks was different with different modification conditions, although the position of absorption peaks did not change. The absorption peaks at 1600 $cm^{-1}$ – 1750 $cm^{-1}$ correspond to the carbonyl (>C=O) stretching vibration area **(Dankar et al., 2018)**. The absorption peaks at 2850$cm^{-1}$ – 3100$cm^{-1}$ correspond to the CH stretching vibration area and the absorption peaks at 3100$cm^{-1}$ – 3700$cm^{-1}$ corresponds to the hydroxyl (OH) stretching vibration area **(Kizil, Irudayaraj and Seetharaman, 2002)**. The absorption peak strength of potato starch increased or decreased, indicating that the internal arrangement and the secondary structure of starch molecular conformation changed in the process of heat-moisture treatment, moreover, HMT might cause additional interactions between amylose-amylose, and/or amylose-amylopectin and/or amylopectin-amylopectin chains, which eventually leads to the increase of the amounts of the carbonyl group and hydroxyl group.

## The starch viscosity properties

The starch viscosity properties are correlated to the gel texture properties, the stability of starch paste, and retrogradation tendency. The RVA profiles of NS and HMT starch were shown in Figure 6 and the gelatinization parameter values were shown in Table 5, Table 6, and Table 7. As shown in Fig.6, the RVA profiles of all HMT samples had a great difference with NS starch, the RVA gelatinization properties of HMT starches were significantly different from each other.

Compared with native potato starch, the peak viscosity, hold viscosity, and breakdown viscosity of HMT starch decreased with the increasing of moisture content, treatment temperature, and treat time, while the gelatinization temperature increased (Table 5, Table 6, and Table 7). It also can be seen from Table 5, Table 6, and Table 7, the final viscosity of HMT starch was higher than peak viscosity, which indicated that potato starch modified by heat-moisture treatment was more prone to retrogradation. The decrease of peak viscosity is supposedly attributed to the strengthening of starch interior interactions by heat moisture treatment enhancing granule integrity, preventing the penetration of water into the starch granules.

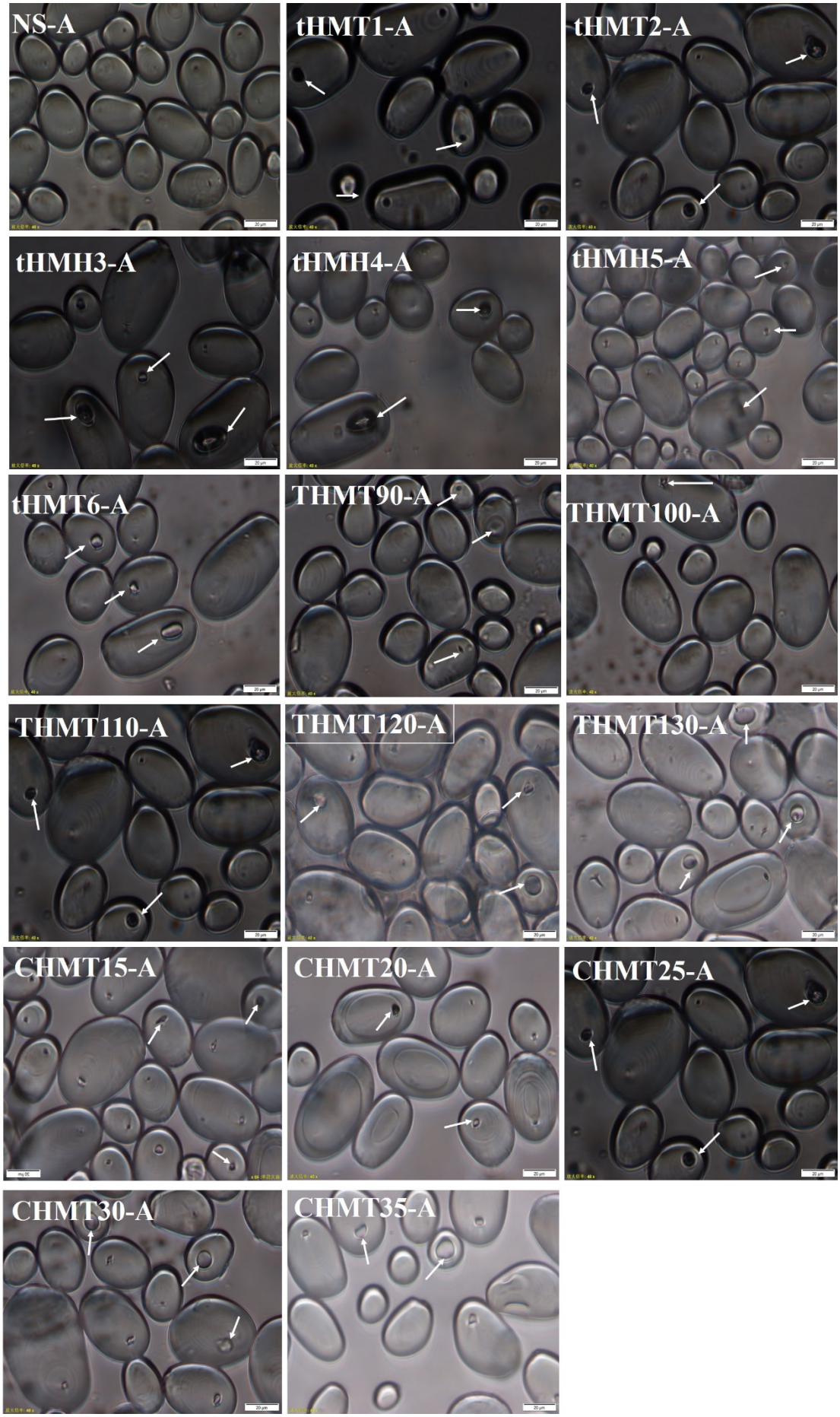


**Figure 2** Morphological characteristics of the heat-moisture treated potato starch granules under normal light × 40 (A).

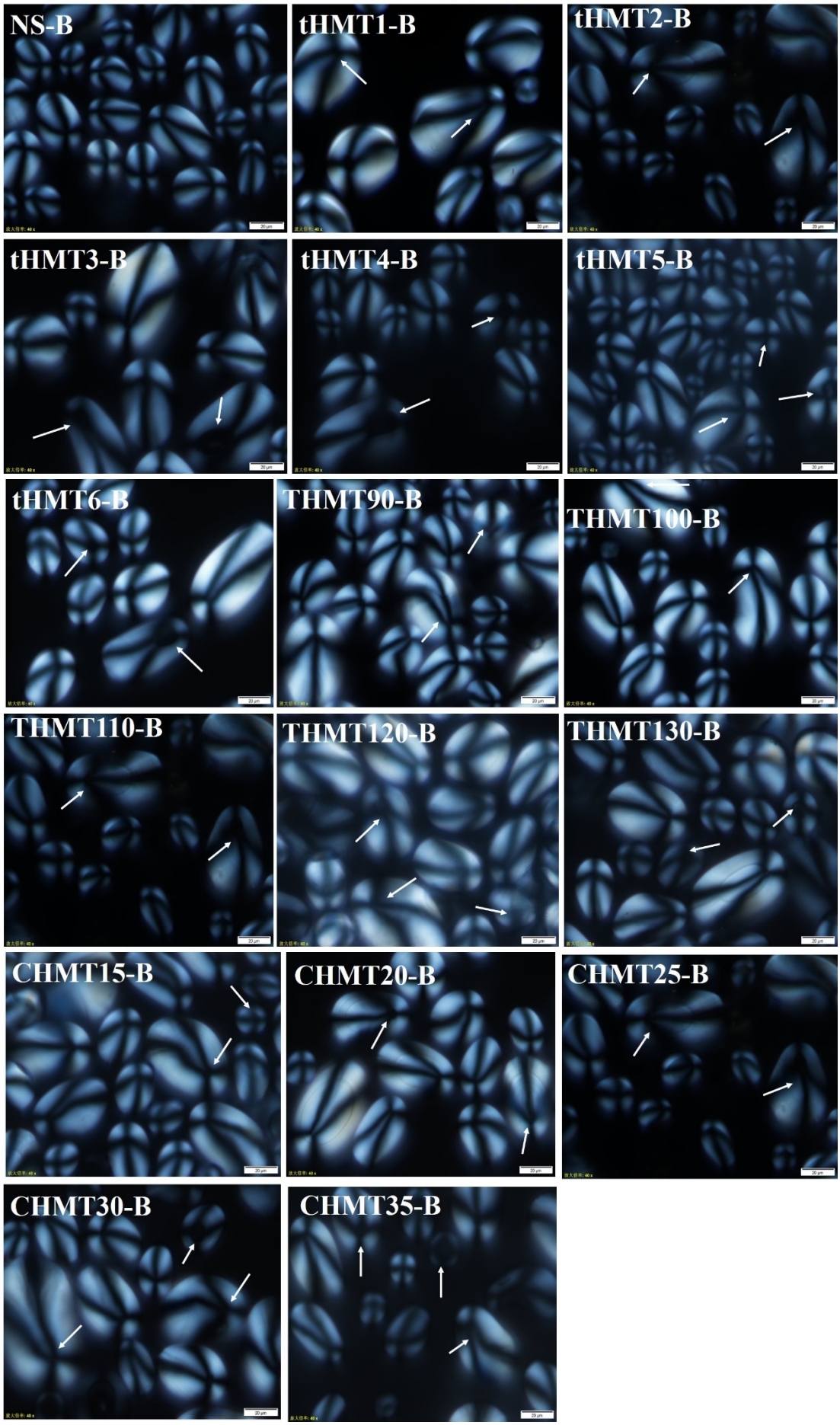


**Figure 3** Morphological characteristics of the heat-moisture treated potato starch granules polarized light × 40 (B).

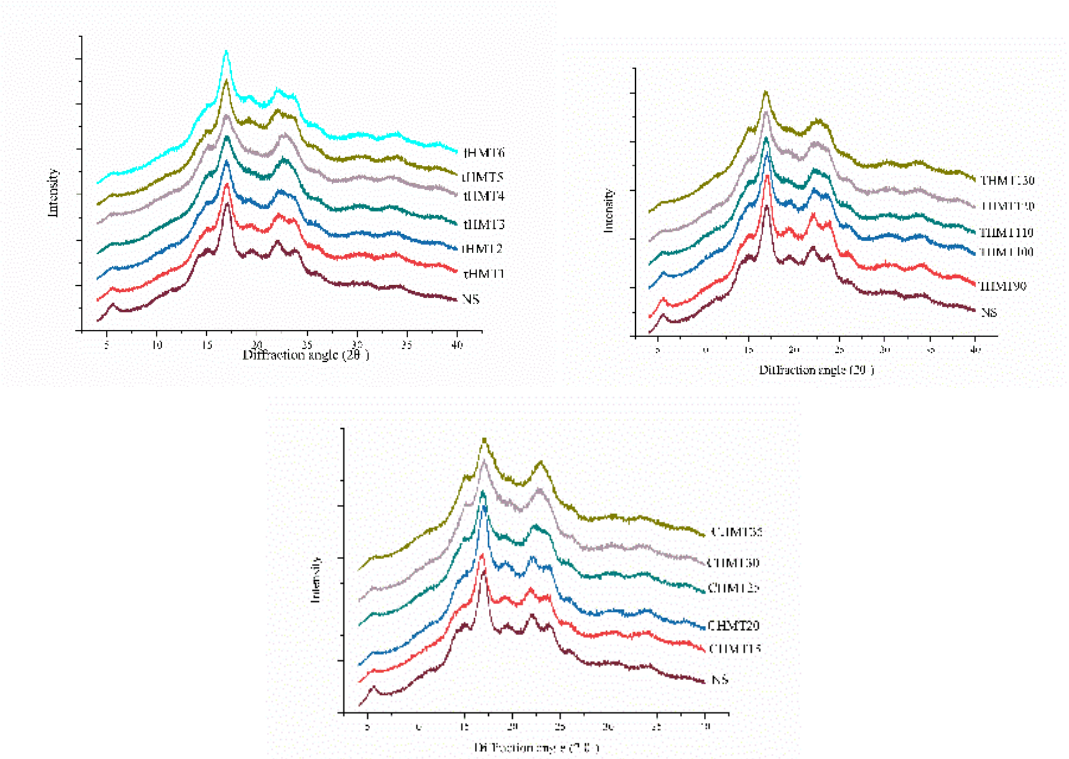


**Figure 4** X-ray diffractograms of native and HMT potato starch.

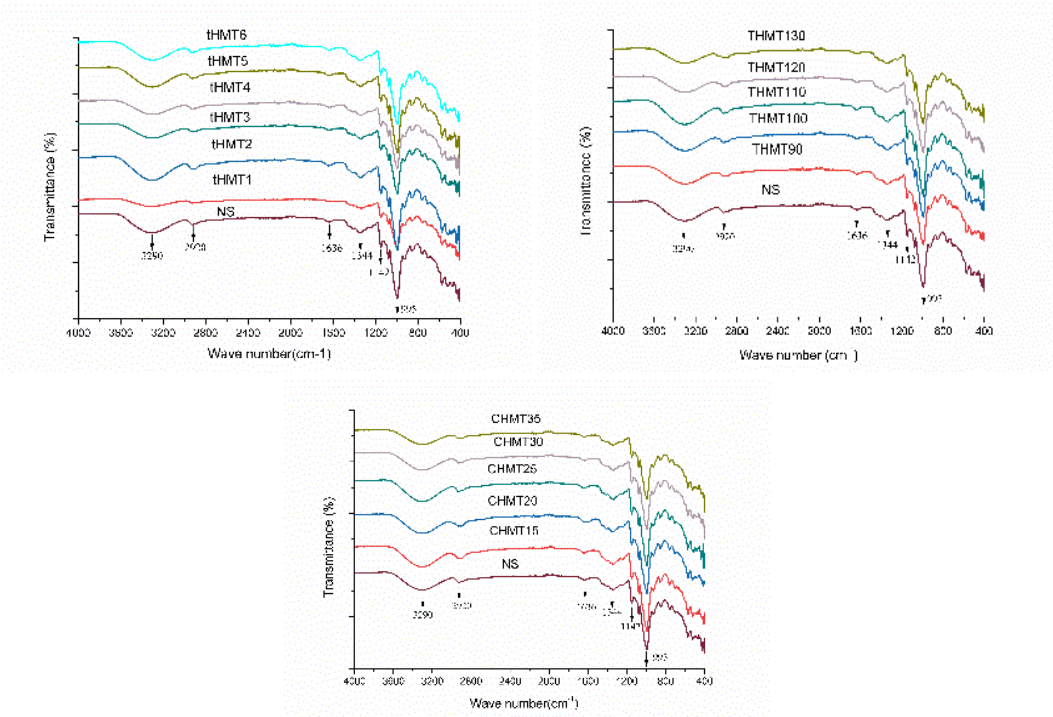


**Figure 5** FTIR spectra of native and HMT potato starch.

The breakdown viscosity is related to the thermal stability of the swollen starch granules in the starch paste during heating and shearing, i.e. lower breakdown indicates more resistance to shear force **(Zhu, Mojel and Li, 2018; Karim et al.,2008)**.

The lower viscosity and higher gelatinization temperature indicated that the structure of starch particles was more stable and the internal molecule force was stronger after heat moisture treatment. Therefore, the starch modified by heat moisture treatment requires more energy to decompose its structure and form paste, thus improving the gelatinization of the sample. The setback viscosity decreased with the extension of treatment time, temperature, and moisture content of the HMT process. The setback viscosity has great relativity to the retrogradation properties of starch and has a great influence on the tensile strength of vermicelli food **(Liao and Wu, 2016)**.

**Table 4** Effect of heat-moisture treatment on rheological properties and relative crystallinity of potato starch under a different holding time.

| Samples | Pasting temperature /℃ | Peak viscosity /cP | Hold viscosity /cP | Final viscosity /cP | Breakdown /cP | Setback /cP | Relative crystallinity (%) |
|---|---|---|---|---|---|---|---|
| NS | 68.53±0.23$^{d}$ | 6598.00±72.75$^{a}$ | 4071.67±25.40$^{a}$ | 4567.33±36.95$^{a}$ | 2526.33±47.34$^{a}$ | 495.67±11.55$^{c}$ | 19.37 |
| tHMT1 | 76.10±1.31$^{c}$ | 2189.00±63.24$^{b}$ | 2145.00±60.90$^{b}$ | 4234.00±71.14$^{b}$ | 44.00±2.65$^{b}$ | 2089.00±25.06$^{a}$ | 20.08 |
| tHMT2 | 76.73±1.62$^{c}$ | 1888.00±14.00$^{c}$ | 1850.00±10.15$^{c}$ | 3736.33±8.62$^{c}$ | 38.00±4.36$^{bc}$ | 1886.33±3.51$^{b}$ | 23.32 |
| tHMT3 | 95.03±0.06$^{a}$ | 173.67±4.51$^{d}$ | 148.67±4.04$^{e}$ | 573.00±9.54$^{d}$ | 25.00±1.00$^{bc}$ | 424.33±5.69$^{d}$ | 24.43 |
| tHMT4 | 97.00±0.00$^{a}$ | 62.67±4.93$^{e}$ | 232.33±22.12$^{d}$ | 232.33±22.12$^{e}$ | 6.67±0.58$^{c}$ | 176.33±17.50$^{e}$ | 22.83 |
| tHMT5 | 95.05±0.07$^{a}$ | 47.33±5.51$^{e}$ | 42.67±3.21$^{f}$ | 145.67±34.43$^{f}$ | 4.67±2.89$^{c}$ | 103.00±31.23$^{f}$ | 20.98 |
| tHMT6 | 90.93±3.97$^{b}$ | 42.33±1.53$^{e}$ | 38.67±0.58$^{f}$ | 109.33±2.08$^{f}$ | 3.67±1.15$^{c}$ | 70.67±2.31$^{g}$ | 20.75 |

**Table 5** Effect of heat-moisture treatment on rheological properties and relative crystallinity of potato starch under different holding temperatures.

| Samples | Pasting temperature /℃ | Peak viscosity /cP | Hold viscosity /cP | Final viscosity /cP | Breakdown /cP | Setback /cP | Relative crystallinity (%) |
|---|---|---|---|---|---|---|---|
| NS | 68.53±0.23$^{d}$ | 6598.00±72.75$^{a}$ | 4071.67±25.40$^{a}$ | 4567.33±36.95$^{b}$ | 2526.33±47.34$^{a}$ | 495.67±11.55$^{f}$ | 19.37 |
| THMT90 | 72.73±1.99$^{c}$ | 3366.67±56.31$^{b}$ | 3299.33±53.29$^{b}$ | 6996.67±80.70$^{a}$ | 67.33±3.05$^{b}$ | 3697.33±71.14$^{a}$ | 21.30 |
| THMT100 | 67.73±3.00$^{d}$ | 1662.00±17.09$^{d}$ | 1617.33±15.04$^{d}$ | 3023.67±13.32$^{d}$ | 44.67±2.08b$^{c}$ | 1406±12.50$^{c}$ | 21.43 |
| THMT110 | 76.73±1.62b | 1888.00±14.00$^{c}$ | 1850.00±10.15$^{c}$ | 3736.33±8.62$^{c}$ | 38.00±4.36$^{bc}$ | 1886.33±3.51$^{b}$ | 23.32 |
| THMT120 | 74.97±1.03b$^{c}$ | 1281.67±35.25$^{e}$ | 1258.00±37.59$^{e}$ | 2364.67±30.43$^{e}$ | 23.67±5.51$^{c}$ | 1106.67±12.10$^{d}$ | 25.77 |
| THMT130 | 81.2±1.61$^{a}$ | 1165.00±25.06$^{f}$ | 1147.00±23.64$^{f}$ | 2001.67±59.21$^{f}$ | 18.00±1.73$^{c}$ | 854.67±35.91$^{e}$ | 25.74 |

**Table 6** Effect of heat-moisture treatment on rheological properties and relative crystallinity of potato starch under different moisture content.

| Samples | Pasting temperature /℃ | Peak viscosity /cP | Hold viscosity /cP | Final viscosity /cP | Breakdown /cP | Setback /cP | Relative crystallinity (%) |
|---|---|---|---|---|---|---|---|
| NS | 68.53±0.23c | 6598.00±72.75$^{a}$ | 4071.67±25.40$^{b}$ | 4567.33±36.95$^{b}$ | 2526.33±47.34$^{a}$ | 495.67±11.55$^{e}$ | 19.37 |
| CHMT15 | 67.80±0.26$^{c}$ | 5594.33±31.26$^{b}$ | 4919.67±48.75$^{a}$ | 5929.67±73.21$^{a}$ | 674.67±41.88$^{b}$ | 1010.00±29.72$^{d}$ | 21.46 |
| CHMT20 | 66.33±3.89$^{c}$ | 1720.33±12.70$^{d}$ | 1685.33±14.47$^{d}$ | 3101.67±34.02$^{d}$ | 35.00±2.00$^{c}$ | 1416.33±26.10$^{b}$ | 21.71 |
| CHMT25 | 76.73±1.62$^{b}$ | 1888.00±14.00$^{c}$ | 1850.00±10.15$^{c}$ | 3736.33±8.62$^{c}$ | 38.00±4.36$^{c}$ | 1886.33±3.51$^{a}$ | 23.32 |
| CHMT30 | 83.80±0.17$^{a}$ | 1377.67±4.73$^{e}$ | 1345.67±7.57$^{e}$ | 2627.33±51.93$^{e}$ | 34.00±2.64$^{c}$ | 1275.00±36.35$^{c}$ | 24.19 |
| CHMT35 | 85.30±1.68$^{a}$ | 1217±9.54$^{f}$ | 1180.00±13.75$^{f}$ | 2190.33±26.58$^{f}$ | 37.00±5.29$^{c}$ | 1010.33±13.80$^{d}$ | 25.09 |

Note: all values are the means of triplicate determinations ±*SD*. The means within the same column with different letters are significantly different ($p$ <0.05).

**Table 7** RDS, SDS and RS contents of native and HMT starch.

| Samples | RDS(%) | SDS(%) | RS(%) |
|---|---|---|---|
| NS | 31.14±0.10$^{a}$ | 55.17±0.17$^{a}$ | 13.69±0.10$^{h}$ |
| tHMT1 | 28.28±0.30$^{fg}$ | 51.46±0.21$^{fg}$ | 20.25±0.10$^{b}$ |
| tHMT2 | 29.91±0.27$^{c}$ | 51.16±0.39$^{g}$ | 18.93±0.25$^{de}$ |
| tHMT3 | 30.46±0.71$^{b}$ | 50.28±0.26$^{h}$ | 19.25±0.16$^{d}$ |
| tHMT4 | 28.73±0.00$^{ef}$ | 49.11±0.16$^{i}$ | 22.16±0.16$^{a}$ |
| tHMT5 | 29.32±0.56$^{d}$ | 51.94±0.47$^{f}$ | 18.74±0.09$^{e}$ |
| tHMT6 | 29.93±0.10$^{c}$ | 50.05±0.17$^{h}$ | 20.02±0.09$^{bc}$ |
| THMT90 | 30.34±0.00$^{bc}$ | 52.59±0.48$^{e}$ | 17.07±0.48$^{g}$ |
| THMT100 | 28.38±0.18$^{fg}$ | 54.13±0.25$^{b}$ | 17.48±0.38$^{f}$ |
| THMT110 | 29.91±0.27$^{c}$ | 51.16±0.39$^{g}$ | 18.93±0.25$^{de}$ |
| THMT120 | 29.89±0.36$^{c}$ | 53.35±0.21$^{cd}$ | 16.76±0.16$^{g}$ |
| THMT130 | 30.30±0.17$^{bc}$ | 52.84±0.26$^{de}$ | 16.86±0.19$^{g}$ |
| CHMT15 | 28.20±0.27$^{g}$ | 53.93±0.18$^{b}$ | 17.88±0.09$^{f}$ |
| CHMT20 | 29.10±0.18$^{de}$ | 53.35±0.22$^{cd}$ | 17.54±0.09$^{f}$ |
| CHMT25 | 29.91±0.27$^{c}$ | 51.16±0.39$^{g}$ | 18.93±0.25$^{de}$ |
| CHMT30 | 28.78±0.50$^{ef}$ | 51.44±0.90$^{fg}$ | 19.79±0.41$^{c}$ |
| CHMT35 | 29.16±0.44$^{de}$ | 53.78±0.12$^{bc}$ | 17.06±0.35$^{g}$ |

Note: all values are the means of triplicate determinations ±*SD*. The means within the same column with different letters are significantly different ($p$ <0.05).RDS :rapidly digested starch(digested within 20 min); SDS: slowly digested starch (digested between 20 and 120 min); RS: resistant starch (residual starch after 120 min).

Therefore, it is necessary to select appropriate heat-moisture treatment conditions for the preparation of vermicelli food by using the HMT potato starch to obtain better edible quality.

### In vitro digestion of starch

The results of RDS, SDS, and RS of native starch and HMT starch sample were shown in Table 7. The values of RDS, SDS, and RS fractions in NS were 31.14%, 55.17%, and 13.69%, respectively. Compared to the native starch HMT starches had less RDS and SDS, but high RS. The total content of SDS and RS of HMT starch was significantly higher than that of NS starch ($p$ <0.05), the holding time of heat-moisture treatment had the greatest effect on the content of RS, indicating that heat moisture treatment rearranged the molecular chain and formed ordered structures (crystallites and helices) and increased the resistance of starch to enzymatic hydrolysis, eventually reduce the digestion of starch by transforming of RDS into SDS and/or RS **(Li, Ward and Gao, 2011)**. From our results, tHMT1, THMT100, and CHMT15 had the lowest content of RDS and there was no significant difference between the three samples, in other words, these three samples had the highest total content of SDS and RS. Therefore, choosing appropriate treatment time, temperature, and moisture content can obtain modified starch with higher content of SDS and RS.

## CONCLUSION

HMT altered the particle size, morphological, structural, rheological, and digestive properties of potato starch at different levels of moisture content, heating time, and heating temperature. HMT led to large particle size, the rough surface of starch granules, and HMT resulted in the hollow structure located at the hilum of potato starch granules. XRD results showed an increased relative crystallinity in HMT starches and transformed crystalline structure from B-type to C-type. FTIR spectroscopy results indicated that the internal arrangement and the secondary structure of starch molecular conformation changed in the process of heat-moisture treatment, which eventually leads to the increase of the mass of the carbonyl group and hydroxyl group. In vitro digestion results showed that the RDS content of HMT starches significantly decreased, whereas the total content of SDS and RS increased, indicating HMT starches could potentially become sources of industrial-resistant starch and as low-calorie food ingredients.

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# REMNANT PHOTOSYNTHETIC PIGMENTS IN TEA DREGS: IDENTIFICATION, COMPOSITION, AND POTENTIAL USE AS ANTIBACTERIAL PHOTOSENSITIZER

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## ABSTRACT

The production of tea dregs is continually increasing along with the growth of people's interest in ready-to-drink beverages. However, the recent development of research on the use of tea dregs is still very limited. The present study was aimed to identify the remnant photosynthetic pigments in tea dregs, determine their composition, and evaluate their potential use as natural antibacterial agents based on light-induced reaction (photosensitization). The tea dregs from six commercial teas, consisting of green and black teas, were analyzed using high-performance liquid chromatography (HPLC) with a photodiode array detector, and the spectroscopic data were analyzed from 350 to 700 nm. Pigment identification was performed based on spectral characteristics, and pigment composition in the extracts from the dregs was determined by a three-dimensional multi-chromatogram analysis method. The dominant pigment fractions in both tea types were pheophytin *a* and its isomers, as well as pheophytin *b*. Although the dregs of black teas generally contain fewer remnant pigments, they possess residual chlorophyll *b*, which is not found in the dregs of green teas. In thirty-minutes illumination under 50 W red light-emitting diode, the presence of pigments from tea dregs caused up to 0.87 and 0.35 log reduction of *Staphylococcus aureus* and *Escherichia coli*, respectively. The disparity of pigments composition among tea types does not strongly influence their photosensitization activity against both bacteria. Hence, upon further application, the amount of total remnant pigments in the dregs could be taken as substantial consideration instead of tea types.

**Keywords:** antimicrobial photosensitization; chlorophyll; pigments; tea dregs

## INTRODUCTION

Besides drinking water, tea is the most favored beverage consumed by at least 65% of people worldwide **(Diby et al., 2017)**. Tea beverages are prepared through the infusion of dried leaves of *Camellia sinensis* (L.) to extract therapeutic compounds, mainly polyphenols, as well as aromatic compounds **(Rodrigues et al., 2015; Chaturvedula and Prakash, 2011)**. The remaining insoluble material is called tea dregs, which can be utilized as feed, fertilizer, or adsorbent **(Chowdhury et al., 2016; Kabir et al., 2021)**. The global market of ready-to-drink tea beverages has continually increased, with a demand of 18 to 20 billion cups of tea per day, and this inevitably impacts the massive production of dregs and improper disposal **(Dubey et al., 2020; Hussain et al., 2018)**.

The other important metabolites in tea leaves are the group of non-polyphenolic pigments, chlorophylls, and carotenoids, which have an important role in capturing light energy for photosynthesis in tea plants. During tea manufacture, these pigments are disrupted into their derivatives as a result of the processing conditions, such as heat, light, and dehydration, as well as enzymatic reactions **(Wei et al., 2020; Roshanak, Rahimmalek, and Goli, 2016)**. This molecular alteration often determines the color, type, and quality of the tea **(Wei et al., 2021; Chaturvedula and Prakash, 2011)**. Nevertheless, these pigments have poor solubility in water during tea infusion and allegedly remain in the dregs **(Donlao and Ogawa, 2019)**. At the time of writing, most studies in the literature provide only data about the total amount of chlorophylls in tea through spectroscopic estimation. Few reports have specifically characterized pigment derivatives in the processed teas **(Suzuki and Shioi, 2003; Yu et al., 2019)**, but none has detailed complete pigment composition in the dregs.

Chlorophyll derivatives have been targeted as potential natural photosensitizers for food disinfection **(Gerola et al., 2011; Ghate, Zhou, and Yuk, 2019)**. Chlorophyll derivatives have the absorption band at the red region (625 – 700 nm). Upon light absorption, the excited energy can be transferred to $O_2$ molecules through triplet-triplet (T-T) energy transfer, and make oxygens become radical species. Such radicals are cytotoxic and able to induce the rapid

inactivation of pathogens **(Indrawati, Lolita, and Limantara, 2021)**. Most investigations used derivatives that were artificially modified from naturally occurring chlorophylls **(Kustov et al., 2018; Zhang et al., 2019; Oktavia, Mulyani, and Suendo, 2021)**. However, natural sources of chlorophyll derivatives would be favored by consumers in food-related products.

Therefore, the present study aimed to (i) identify the photosynthetic pigments that are present in the dregs of teas, determine the composition of remnant pigments, and (ii) evaluate their potential use for bacterial inactivation through photosensitization. The content of pigments in the dregs was compared among several commercial green and black teas. Pigment separation and identification were performed using high-performance liquid chromatography (HPLC) and a diode array detector. The three-dimensional (3D) multi-chromatogram method was adopted to determine the distribution of pigments in a fast and accurate way **(Indrawati et al., 2012; Brotosudarmo et al., 2018; Indrawati et al. 2019)**. Furthermore, the antimicrobial effect upon light induction was verified against *Staphylococcus aureus* and *Escherichia coli*.

## Scientific hypothesis

The study on pigment identification and composition was carried out as descriptive research, whereas the assay of antimicrobial photosensitization was conducted in an experimental framework. The proposed hypotheses were the following: (i) illumination causes a significant effect on the number of viable bacteria treated with dregs extract, and (ii) dregs extract from green and black teas have a different effect on the percentage of photosensitized inactivation.

## MATERIAL AND METHODOLOGY

### Samples

Six commercial teas were purchased from local supermarkets in Malang, Indonesia. The samples are three green teas (GT) and three black teas (BT), which belong to local tea products in Indonesia. The sample code, brand, and manufacturing factory are listed in Table 1.

**Table 1** The identity and code of the tea samples.

| Code | Brand | Manufacturing Factory |
|---|---|---|
| GT1 | Sariwangi | PT Unilever Indonesia |
| GT2 | Teh Jawa | PT Sari Melati Sejahtera |
| GT3 | Kepala Djenggot | PT Gunung Subur Sejahtera |
| BT1 | Sariwangi | PT Unilever Indonesia |
| BT2 | Teh Jawa | PT Sari Melati Sejahtera |
| BT3 | Sosro | PT Sinar Sosro |

### Chemicals

The chemicals used in pigment extraction and liquid chromatographic analysis were acetone, methanol, acetonitrile, and pyridine (Merck KGaA, Darmstadt, Germany, pro-analysis grade). The pigment standard (pheophytin *a*, chlorophyll *b*) was obtained from NatChrom® (Malang, Indonesia). The experiments with microorganisms used Tween-80 (Sigma- Aldrich, France, ≥58.0% oleic acid) and microbiological media (nutrient broth, Mueller–Hinton broth, trypticase soy agar, buffered peptone water) from Merck KGaA (Darmstadt, Germany).

### Biological Material:

The antibacterial assay used *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 8739, acquired from the Department of Pharmacy, Widya Mandala Catholic University, Surabaya, Indonesia.

### Instruments

The analytical instruments used in this study were a UV-visible spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan), an ultra-fast liquid chromatography instrument (LC-20A with an SPD-20MA diode array detector, Shimadzu, Kyoto, Japan) with a C8 column (Waters, Massachusetts, USA) for the separation of photosynthetic pigments, and an HPLC-UV/Vis detector (Agilent Technologies, Waldbronn, Germany) for the determination of polyphenol residues. The equipment used in the photosensitization experiments included a quantum meter (Apogee Instrument, MQ-200, USA) for measuring light photons, an orbital shaker (MaxQ 2000, Thermo Scientific, Iowa, USA), and a red LED lamp (Yomiko YL-2550, 50 W, peak emission at 640 nm).

### Laboratory Methods

#### *Preparation of tea dregs and pigment extraction*

Each tea sample was subjected to the hot infusion (90 °C) for 5 and 20 min at a tea-to-water ratio of 1:100 (w/v). The dregs were then separated after filtration and dried overnight at room temperature to reduce their water content. Pigment extraction was carried out in acetone at a dregs-to-solvent ratio of 1.5:10 (w/v). The extraction was repeated twice until the pellet turned pale. The acetone was subsequently removed by vacuum evaporation, and the pigments were stored in an inert gas ($N_2$) in amber vials at a low temperature (-20 °C) until the time of analysis. The whole extraction procedure was completed under dim light to avoid any possible degradation of pigments.

#### *HPLC separation*

The pigments were first redissolved in acetone, and then an aliquot (20 μL) was injected into the HPLC instrument. The gradient protocol for the mobile phase, lasting for 50 min, was arranged according to a method described earlier **(Zapata, Rodriguez, and Garrido, 2000)**. The diode array detector enabled the recording of the full spectrum of each separated fraction between 200 and 800 nm, and the recordings were saved for later data processing.

#### *Determination of pigment composition*

Ten dominant fractions with clear spectra were chosen for 3D-multi-chromatogram analysis. The mathematical computation was performed using MATLAB R2015a software (MathWorks, Massachusetts, USA), adopting the trapezoidal rule to calculate the total peak area from every single wavelength with a resolution of 1 nm from 350 to 700 nm **(Indrawati et al., 2012)**. The equation of **Lichtenthaler (1987)** was applied to estimate the total content of pheophytins in plant leaves in which the chlorophylls were completely converted into their derivatives.

#### *Polyphenol residue analysis*

The residues of two major polyphenols, epigallocatechin-3-gallate (EGCG) and epigallocatechin (EGC), were detected and quantified using an in-house validated HPLC method by PT Angler Biochemlab (Surabaya, Indonesia).

**Table 2** Peak identification of remnant photosynthetic pigments extracted from the dregs of green and black teas, separated on a C8 HPLC column, and their typical spectra (in the eluent) following diode array detection.

| Peak Number | Pigment | Maximum (nm) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | tR (min) | Observed | | | | | Reported | | | | |
| 1 | pheophorbide *a* | 15.8 | 409 | 508 | 538 | 609 | 665 | 409 | - | - | - | 665 |
| 2 | lutein | 28.4 | (424) | - | 447 | | 475 | (422) | - | 446 | - | 474 |
| 3 | chlorophyll *b* | 33.5 | 462 | - | | 598 | 647 | 461 | - | 533 | 599 | 647 |
| 4 | pheophytin *b* sp. | 34.5 | 434 | - | 524 | 598 | 652 | 436 | - | - | - | 654 |
| 5 | pheophytin *b* | 34.9 | 434 | - | 527 | 598 | 652 | 436 | - | - | - | 654 |
| 6 | pheophytin *a* sp. | 36.0 | 409 | 503 | 533 | 609 | 664 | 409 | 505 | 535 | 608 | 665 |
| 7 | pheophytin *a* | 36.6 | 409 | 506 | 536 | 610 | 665 | 408 | 505 | 535 | 608 | 665 |
| 8 | pheophytin *a* ' | 36.8 | 409 | 506 | 538 | 610 | 666 | 408 | 505 | 535 | 609 | 666 |
| 9 | β-carotene | 37.2 | (425) | - | 454 | - | 476 | - | - | 452 | - | 474 |
| 10 | pyropheophytin *a* | 37.8 | 409 | 508 | 538 | - | 665 | 411 | 505 | 534 | 609 | 667 |

Note: Data in parentheses denote the shoulder. * The data were reported by **Suzuki and Shioi (2003)** the same eluent as used in the present study.

**Description of the Experiment**

**Sample preparation:** The concentration of the pigment standard (positive control) was adjusted to 100 $\mu g.mL^{-1}$ according to the Lambert-Beer equation, with specific extinction coefficients 53.37 $L.g^{-1}.cm^{-1}$ (at 667 nm in acetone) and 62.00 $L.g^{-1}.cm^{-1}$ (at 643.3 nm in diethyl ether) for pheophytin *a* and chlorophyll *b*, respectively **(Jeffrey, Mantoura, and Wright, 1997; Küpper, Spiller, and Küpper, 2000)**. The concentration of the pigments in the dregs (four samples) was equalized with the standard at the maximum absorption (667 nm). Moreover, the solvent was thoroughly evaporated and replaced with an aqueous solution (1% w/v Tween-80) with the aid of sonication. The sterile aqueous surfactant solution was employed as a negative control.

Briefly, from an inoculum of bacteria at $10^8$ $CFU.mL^{-1}$ in nutrient broth medium, a volume of 50 µL was pipetted and added into a 950 µL solution of photosensitizers in an Eppendorf. The suspension was divided into two identical volumes and transferred into the wells of two microplates. One microplate was used for illumination treatment, and the second was covered with aluminum foil for dark treatment. A red LED lamp was set 16 cm above the orbital shaker to give 300 ± 15 $\mu mol.m^{-2}.s^{-1}$ photons and the microplates with samples were gently agitated (100 rpm) for 30 min. Thereafter, each sample was supplemented with Mueller–Hinton broth, diluted, and plated in trypticase soy agar for the enumeration of the total number of viable cells. The percentage of dead cells was calculated based on the difference in the total number of survivors between the dark and illumination treatments.

**Number of samples analyzed:** The number of samples analyzed in the experiment were 84 samples. There were seven couple treatments (dark and illuminated) for each of the two indicator bacteria, which are one negative control, two positive controls using purified chlorophyll *b* and pheophytin *a*, and pigments extracts from the dregs of four tea brands.

**Number of repeated analyses:** Individual analysis of all samples were repeated in duplicate.

**Number of experiment replication:** The data for each treatment were taken from three replication.

**Statistical Analysis**

The experimental data were analyzed with one-way nested ANOVA using Minitab software (Minitab LLC, Pennsylvania, USA) version 17.00 for Windows. The null hypothesis is rejected when the *p-value* is less than 0.05. Furthermore, the comparison and grouping information were given based on Fisher's least significant difference (LSD) method at a 95% confidence level.

## RESULTS AND DISCUSSION

### Identification of pigments

The pigments extracted from the dregs of six commercial teas were separated by HPLC with a diode array detector to record the absorption intensity in the ultra-violet to the visible region. The photosynthetic pigments, chlorophylls, and carotenoids are mainly responsible for harvesting light energy across the visible solar spectrum **(Son et al., 2019)**.

Hence, they are characterized by distinctive absorption spectra in the visible region, which can be used as a reference in identification **(Suzuki, Kamimura, and Hooker, 2015)**. Likewise, any molecular conversion or degradation of pigments can cause a spectral shift **(Chen, 2014; Seifert, Pflanz, and Zude, 2014)**.

In this study, 13 signals were detected in the chromatograms belonging to the group of chlorophylls and carotenoids. Of these, 8 were identified as chlorophylls and 2 as carotenoids based on the reference standard, spectroscopic data, and retention times of pigments in tea leaves **(Suzuki and Shioi, 2003; Zapata, Rodriguez, and Garrido, 2000)**. Figure 1 depicts the chromatograms and absorption patterns of the pigment fractions in the dregs of six commercial green and black teas. Along with it, Table 2 gives the list of fractions in comparison with the reference. The observed pigments, in descending order of polarity, were pheophorbide *a*, lutein, chlorophyll *b*, pheophytins *b*, pheophytins *a*, *β*-carotene, and pyropheophytin *a*. No signal was confirmed as belonging to chlorophyll *a* in all the samples. Some polyphenol residues were detected in the early minutes of separation (1.6 and 7.2 min) at 280 nm, but their presence will be discussed in the next section.

In the photosynthetic apparatus of higher plants, the primary light-harvesting pigments are chlorophyll *a* and *b*, while the accessory pigments are carotenoids **(Croft and Chen, 2017)**.

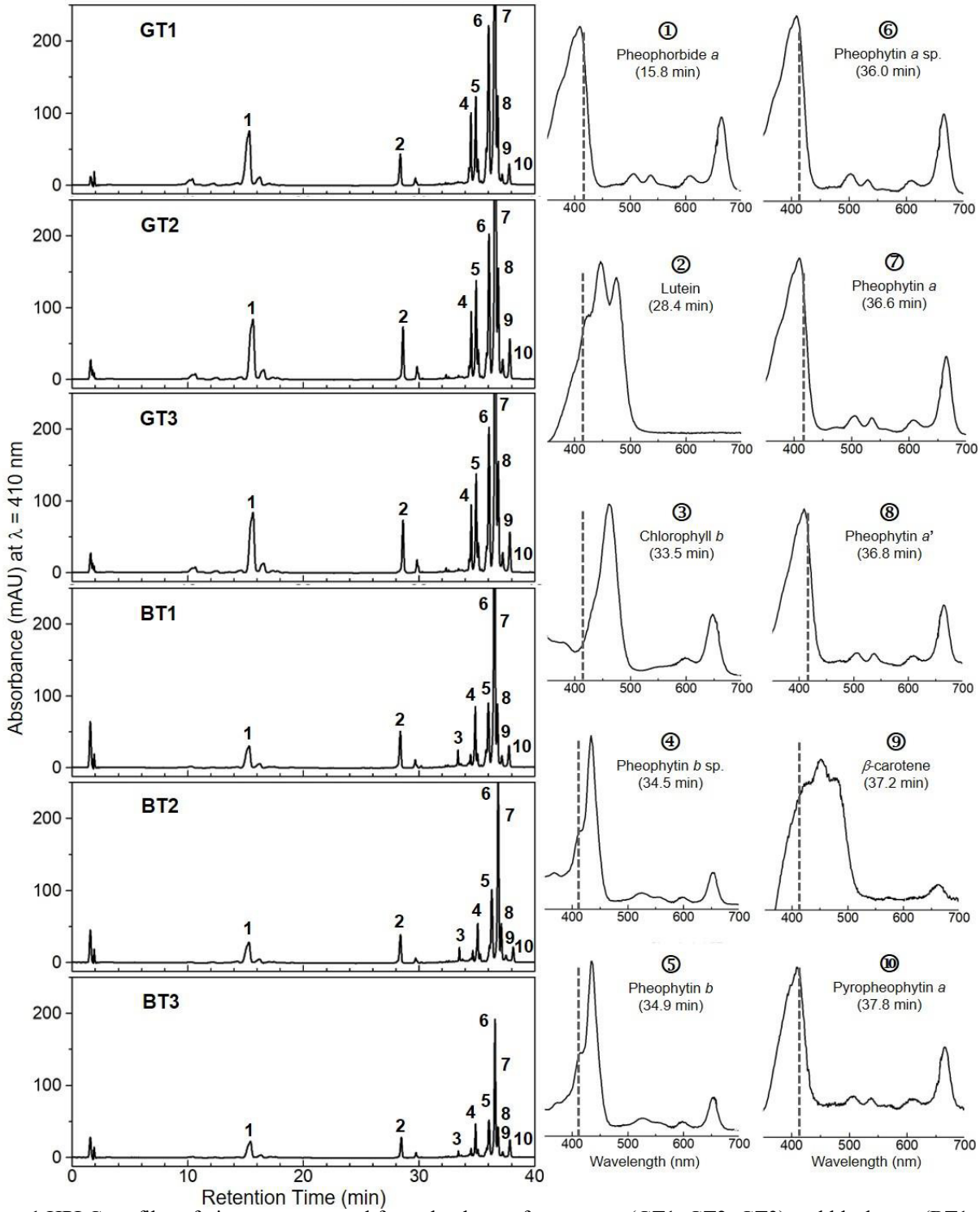


**Figure 1** HPLC profiles of pigments extracted from the dregs of green teas (GT1, GT2, GT3) and black teas (BT1, BT2, BT3), monitored at 410 nm. The visible absorbance spectrum of each pigment in the eluent solution is depicted on the right-hand side, and the identification is listed in Table 1.

Pheophytin is a magnesium-free derivative of chlorophyll, which can be further converted into pheophorbide after detachment of the phytol tail.

These catabolites of chlorophyll are often formed by enzymatic processes during leaf senescence **(Zepka, Jacob-Lopes, and Roca, 2019)**. Besides, these derivatives can be produced by heat and acid treatment during food processing **(Amaya, 2016)**. Strong heat treatment may lead to the decarbomethoxylation of pheophytin, which yields pyropheophytin **(Pareek et al., 2017)**. Heat treatment and the liberation of organic acid from tea leaves are inevitable during the manufacture of green and black teas, so many chlorophyll derivatives can be found in processed tea.

The absence of chlorophyll *a* in all the samples and the intense pheophytins *a* signal confirmed the total derivatization of chlorophyll *a* in both green and black teas. Interestingly, the apparent signal of chlorophyll *b* (peak number 3) was found only in black teas. This difference might be influenced by the different ways of processing tea leaves. The major steps in the manufacturing of green tea

are heating (steaming or roasting), rolling, and drying, whereas those of black tea comprise withering, rolling, oxidation, and drying **(Deb and Pou, 2016)**. The early steaming in green tea processing is presumed to hasten the thermal degradation of both chlorophylls *a* and *b*, whereas the withering in black tea processing leads to a gentler biochemical conversion of chlorophylls. According to the catabolism pathway of chlorophylls, chlorophyll *b* can first be converted into chlorophyll *a* before further derivatization **(Sato et al. 2018)**. Meanwhile, the degradation rate of chlorophyll *a* is 2.5 times higher than that of chlorophyll *b* **(Koca, Karadeniz, and Burdurlu, 2006)**. The presence of chlorophyll *b* residue is more likely than the presence of chlorophyll *a* residue. This finding is in line with a study by **Wijaya et al. (2010)**, who compared the presence of chlorophyll *b* in fresh tea leaves, green teas, and black teas.

From the collation of the elution data, pheophytin *b* existed in two species whose absorption spectra and peak intensities were almost alike, while pheophytin *a* noticeably predominated over its another species and its epimer. **Fu et al. (2012)** and **Hong et al. (2020)** have reported the mass spectra of pheophytin species and epimer, in which the more polar species could be differentiated from pheophytin by the loss of two hydrogen molecules (divinyl pheophytin) or the addition of an oxygen molecule (hydroxy pheophytin). On the other hand, the epimerization of pheophytin *a* into pheophytin *a*' as well as its conversion into a pyro-form derivative (removal of the $-CO_2CH_3$ moiety) is known to reduce its polarity **(Lefebvre et al., 2020)**.

## Pigment composition

As shown in Figure 1, each pigment exhibits a particular absorption pattern, so the selection of the monitoring channel greatly influences the peak intensity that appears in the chromatogram. The 430 nm channel is conventionally used in single detection for both chlorophylls and carotenoids because it gives a peak-rich chromatogram **(Suzuki and Shioi, 2003)**. Some studies applied different channels to optimize the detection: 410 nm for pheophytin *a* and certain carotenes, 430 nm for chlorophyll *a* and pheophytin *b*, and 450 nm for chlorophyll *b* and most carotenoids **(Stinco et al., 2019; Wojdyło et al., 2021)**. Alternatively, the 3D-multi-chromatogram approach, which adds up every single peak intensity at a 1-nm resolution over a specified range of wavelengths, has recently been applied to overcome the need for detection at multiple channels. This method offers higher accuracy and a practical assessment of pigment abundance in extract mixtures without the quantification of individual fractions **(Indrawati et al., 2012; Brotosudarmo et al., 2018; Indrawati et al. 2019)**.

Here, the composition of pigments in the dregs of tea samples was elucidated to determine the differences among tea types and to evaluate their antibacterial activity through light induction. Figure 2 shows the composition of the remnant pigments extracted from the dregs of green and black teas after hot water infusion at 90 °C for 5 and 20 min. The percentage of abundance was calculated relative to the total pigment fractions found in the dregs.

The dominant fraction in all the samples was pheophytin *a*, ranging from 34% to 41%, as the main derivative from chlorophyll *a*. In the group of chlorophylls, the order of abundance of the remnant pigments recovered from the dregs of green teas was as follows: pheophytin *a* > pheophytin *a* sp. > pheophytin *b* > pheophytin *b* sp. > pheophytin *a*' > pheophorbide *a* > pyropheophytin *a* > chlorophyll *b*. This order is slightly different for the remnant pigments recovered from the dregs of black teas, in which either chlorophyll *b* or pheophytin *b* was always found as the second most abundant pigment. This is in line with our previous statement that the different processing of green and black teas affects mainly the remaining chlorophyll *b*. Generally, since the chlorophyll *a*/*b* ratios in vegetation are usually around 2.5 – 4.0 **(Croft and Chen, 2017)**, the total abundance of the derivatives from chlorophyll *a* should normally be higher than that of chlorophyll *b*.

In the group of carotenoids, the percentage of lutein found in the dregs varied among the tea samples, while that of β-carotene was comparable. The presence of lutein and β-carotene has been known to be important in tea grading as the precursors of aroma **(Zhou, Li, and He, 2017)**. These two major carotenoids are usually found at a ratio of 5:3 of lutein to β-carotene in fresh tea leaves **(Zhang et al., 2020)**, but according to the findings in the present study, this ratio might be higher after tea processing, particularly in black tea samples.

Table 3 provides the quantification of the total amount of pheophytins recovered from the dregs of green and black teas. The order of abundance of the pigments was as follows: GT1 > GT2 > GT3 > BT1 > BT2 > BT3. The pigments extracted from the dregs of green teas were nearly twice as abundant as those of black teas. The longer processing steps in the black tea manufacture presumably cause more degradation of leaf pigments. The fermentation and drying procedures are indeed aimed at reducing the chlorophyll content, which can cause black teas to have a grassy aroma and inferior quality **(Pou, Paul, and Malakar, 2019)**.

When the infusion time was increased from 5 to 20 min, the total amount of remnant pheophytins was reduced by about 10%. This reduction could be influenced by the further extraction of tea saponins, which are weak surfactants; hence it can facilitate the dissolution of some photosynthetic pigments **(Suzuki and Shioi, 2003; Safdar et al., 2016)**.

**Table 3** Total amount of pheophytins found in the dregs of green and black teas after hot water infusion at 90 °C for 5 and 20 min.

| Infusion Time | Total Amount of Pheophytins in the Dregs (mg $g^{-1}$ of Dry Basis) | | | | | |
|---|---|---|---|---|---|---|
| | GT1 | GT2 | GT3 | BT1 | BT2 | BT3 |
| 5 min | 3.23 ±0.12 | 3.08 ±0.07 | 2.84 ±0.06 | 1.50 ±0.04 | 1.15 ±0.01 | 0.76 ±0.00 |
| 20 min | 2.90 ±0.04 | 2.76 ±0.14 | 2.14 ±0.02 | 1.24 ±0.25 | 1.07 ±0.02 | 0.69 ±0.00 |

Note: Values are expressed as means ±*SD*.

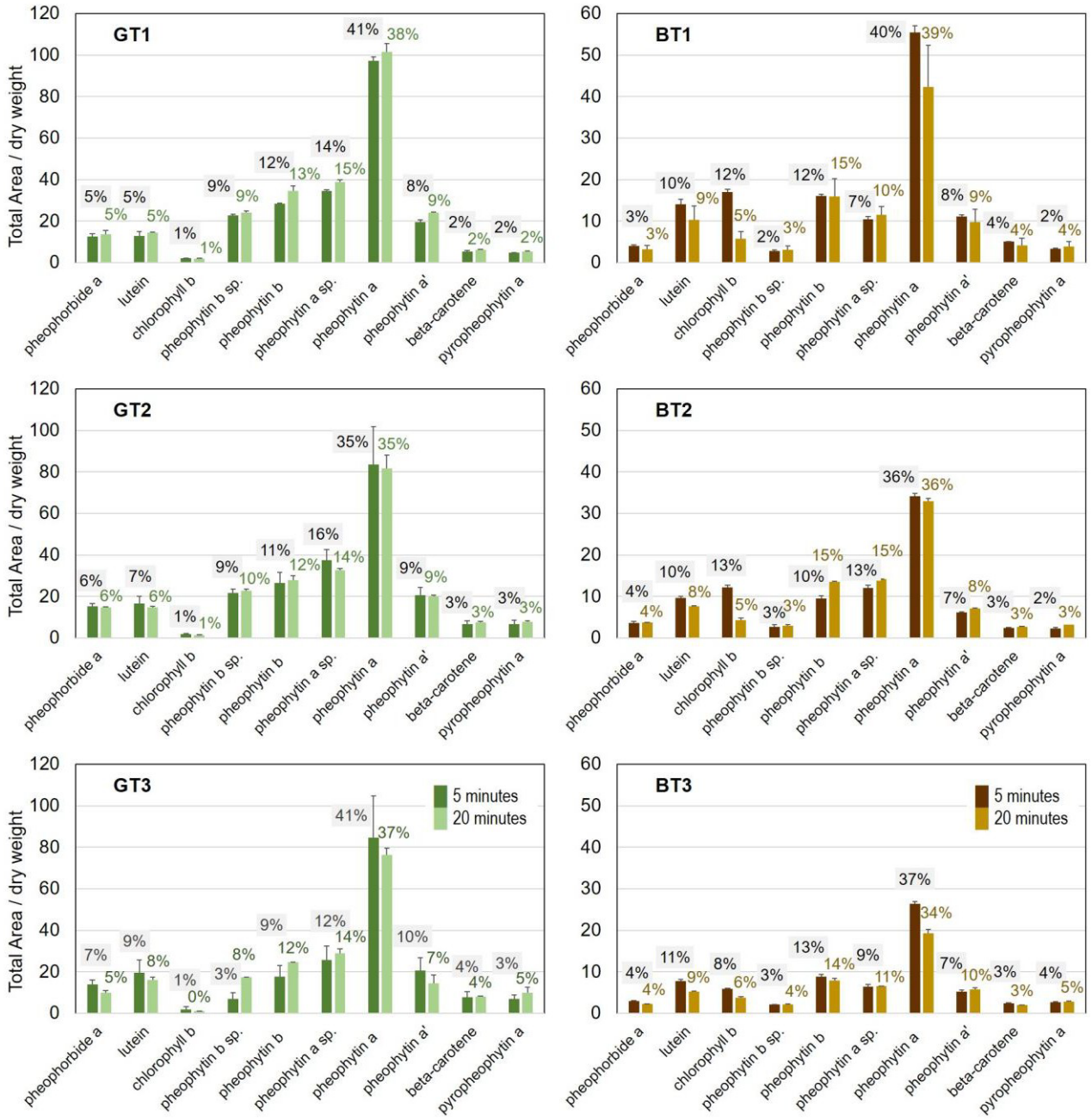


**Figure 2** Composition of the remnant pigments extracted from the dregs of green teas (GT1, GT2, GT3) and black teas (BT1, BT2, BT3) after hot water infusion at 90 °C for 5 and 20 min. For each fraction, the total area was calculated based on a multi-chromatogram approach, summing up consecutive peak areas from 350 to 700 nm. Values are expressed as means ±*SD* of duplicate analyses. The data label denotes the abundance of each fraction in the extract.

**Table 4** Residues of polyphenols jointly extracted with the remnant pigments from the dregs of green and black teas, after hot water infusion at 90 °C for 5 min.

| Fraction | Polyphenol Residue in the Dregs (mg.g$^{-1}$ of Wet Basis) | | | |
|---|---|---|---|---|
| | GT1 | GT2 | BT1 | BT2 |
| EGCG | 4.25 ±0.66 | 3.26 ±0.03 | 0.45 ±0.02 | 0.126 ±0.01 |
| EGC | 0.55 ±0.03 | 0.76 ±0.08 | not detected | not detected |

Note: Values are expressed as means ±*SD*. EGCG – epigallocatechin-3-gallate; EGC – epigallocatechin.

In addition, advanced degradation of chlorophylls into small colorless compounds possibly takes place during prolonged thermal treatment **(Samide and Tutunaru, 2017)**.

Despite such a reduction in the total amount of pheophytins (Table 3), no extreme change in the distribution of pigment fractions was observed, as can be seen in Figure 2. Some alterations in the percentage of

pigment fractions from 5 to 20 min tea brewing were probably the result of intraspecies conversion or epimerization in the group of pheophytins *a* and *b*.
Likewise, further derivatization of chlorophyll *b* in samples of black teas compensated the increase in the percentage of pheophytin *b*.

From these results, two samples with the highest content of pheophytins were chosen among each group of green and black teas (i.e., GT1, GT2, BT1, and BT2) for the antibacterial assay. The dregs extract was used as a mixture of pigments with a determined composition. As mentioned before, some residual polyphenols were jointly extracted with the pigments and detected in the UV region (280 nm) of the chromatogram. Table 4 provides the quantification of the polyphenol residues in the dregs extracts.

## Antibacterial photosensitization

The principle of antibacterial photosensitization also referred to as photodynamic inactivation, is based on interactions among light, a photosensitizer, and oxygen, which produce radical oxygen species that are highly cytotoxic **(Cieplik et al., 2018)**. This method has been intensively explored not only in medicine but also in the fields of fisheries, pest control, and food disinfection **(Alves et al., 2015)**. The light source can be an incandescent lamp, a laser, or a LED that emits light in the same wavelength range as the absorption spectrum of the photosensitizer. Moreover, the photosensitizer itself must be non-toxic, and a naturally occurring compound is preferred when it is used in food products **(Ghate, Zhou, and Yuk, 2019)**.

In the present study, the dregs extracts were applied to bacterial cultures and parallelly incubated under dark and illumination conditions. Purified pheophytin *a* and chlorophyll *b* were used as positive controls, representing the most dominant fractions in the remnant pigments from the dregs as well as in the residual pigments from tea leaves. The dispersing medium, an aqueous solution of 1% (w/v) Tween-80, was used as a negative control to verify the light effect against bacteria in the absence of a photosensitizer. The numbers of living cells after 30 min treatments are provided in Figure 3.

After incubation under dark conditions, there was no reduction in the total number of viable cells of *S. aureus* and *E. coli* in the negative and positive controls and the treatments using dregs extracts. This result revealed that in the dark, the pigments at a concentration of 100 μg $mL^{-1}$ had no toxic effect against the pathogen.

The red LED, emitting at 640 ±60 nm, was applied in the illumination treatment to stimulate the photosensitization of pheophytins and chlorophyll *b*, which also have an absorption spectrum in the red region. The exposure to red light was expected to have a low antibacterial effect. The longer wavelength of visible light has lower energy and hence little impact on bacterial growth **(Ghate et al., 2013; Prasad et al., 2020)**.

Figure 3 shows the apparent reduction in the number of living cells when the photosensitizer was applied to the bacterial suspension, both with the positive controls and with the dregs extracts. The occurrence of antibacterial photosensitization was confirmed by nested ANOVA analysis, which gave a *p-value* <0.05 for the illumination treatment. In other words, the first hypothesis was accepted, stating that illumination causes a significant effect on the number of viable bacteria treated with dregs extract. The differences in the number of survivors between dark and illumination treatments were 0.16 – 0.87 log and 0.22 – 0.35 log for *S. aureus* and *E. coli*, respectively. The greatest effect was found in the dregs extract of GT2, which caused a reduction of nearly one log cycle in the *S. aureus* culture. Furthermore, the magnitude of photodynamic inactivation was calculated as the percentage of dead cells in illumination treatments, in which the dark treatment was assumed to have zero death (Table 5). The percentage of dead cells in the negative control groups was below 5%, whereas that in the positive controls and the treatments with dregs extract was higher. The small photo-killing effect observed in the negative control group was probably due to antibacterial photosensitization by an endogenous photosensitizer in the bacterial cells.
Several metabolites inside bacterial cells, such as protoporphyrin IX, coproporphyrin III, and uroporphyrin, have been recognized as visible-light sensitive compounds **(Hoenes et al., 2020; Fyrestam et al., 2015)**.

The presence of an exogenous photosensitizer caused significantly greater inactivation of bacterial cultures, as confirmed by Fisher's LSD test against the negative control group. Nevertheless, the magnitude of photosensitization among the four tea types was comparable in both bacteria, with the same alphabetical notation and acceptance of the null hypothesis. Despite the antimicrobial photosensitization of GT2 being somewhat higher than that of the other samples, the small difference in pigment composition between the dregs extracts of green and black teas might not greatly affect their inactivation capacity. In this experiment, the concentration of the dregs extracts has been calibrated so that they have a light absorption intensity equal to that of pheophytin *a* at 100 μg $mL^{-1}$. However, the percentage of dead cells in the treatments of *S. aureus* with dregs extract was markedly higher than that in the positive controls. A similar difference was found in the experiments with *E. coli*, although it was not statistically significant. This finding was presumably caused by the synergistic effect of several chlorophyll derivatives in the dregs extract. **Acedo et al. (2014)** reported the beneficial effect of using a combination of photosensitizers to increase the efficacy of photosensitization. In addition, another possible ancillary effect came from the presence of residual EGCG, which enhanced the cytotoxic effect of photosensitization **(Raish et al., 2010; Senapathy, George, and Abrahamse, 2020)**. This ancillary effect could be investigated in further studies.

In general, the attractiveness of antimicrobial photosensitization in modern research arises from its potency in rapid non-thermal disinfection using non-UV light **(Purushothaman and Mol, 2021)**. Investigations into novel photosensitizers are ongoing to increase the efficacy of photosensitization, along with the determination of the optimum photosensitizer concentration, irradiation dose, and incubation time **(Amaral, Azevedo, and Perussi, 2018)**. Although photodynamic treatment is capable of killing a broad spectrum of pathogenic bacteria, the sensitivity varies depending on the membrane characteristics of the bacteria. The Gram-positive bacteria are often more susceptible to photosensitization because of their single-cell wall with high porosity.

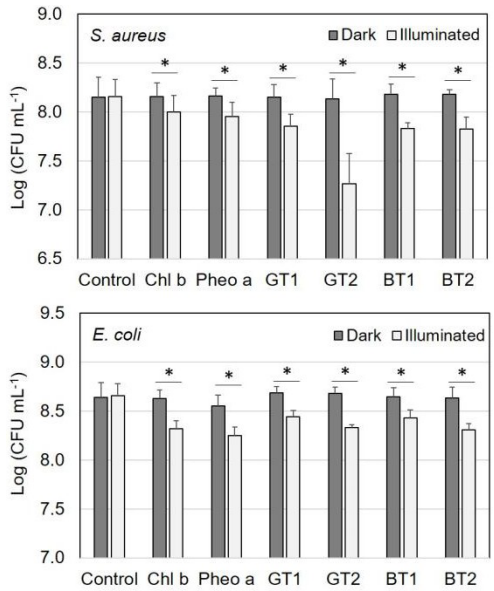


**Figure 3** Total number of viable cells after 30-min incubation with 1% w/v Tween-80 (Control), chlorophyll *b* (Chl *b*), and pheophytin *a* (Pheo *a*), as well as the pigments extracted from the dregs of green teas (GT1, GT2) and black teas (BT1, BT2), in the dark and illuminated by a red, LED light. The error bar shows the standard error of the mean ($n = 3$). The star indicates a significant difference ($p < 0.05$) between treatments. based on nested ANOVA analysis.

**Table 5** Percentage of dead cells after 30-min incubation with pigments extracted from dregs of green and black teas under red LED illumination.

| Treatment | Dead Cells (%) | |
|---|---|---|
| | *S. aureus* | *E. coli* |
| Control | 4.9 ±2.6$^{d}$ | 3.9 ±1.3$^{b}$ |
| Chlorophyll *b* | 31.0 ±9.8$^{c}$ | 27.6 ±9.7$^{a}$ |
| Pheophytin *a* | 38.9 ±7.2$^{bc}$ | 29.3 ±7.2$^{a}$ |
| GT1 | 49.5 ±6.1$^{abc}$ | 42.8 ±2.0$^{a}$ |
| GT2 | 67.4 ±4.5$^{a}$ | 40.5 ±3.4$^{a}$ |
| BT1 | 51.2 ±1.6$^{abc}$ | 39.2 ±2.3$^{a}$ |
| BT2 | 55.2 ±9.2$^{ab}$ | 34.3 ±6.6$^{a}$ |

Note: Values are expressed as means ±SEM of three repetitions. Different letters indicate statistically significant differences between groups of treatments with the same targeted bacteria ($p < 0.05$).

The complexity of the cell wall in Gram-negative bacteria might hamper the attachment and absorption of photosensitizers **(Mesquita et al., 2018)**. Our findings are in line with this, as *S. aureus* (Gram-positive) exhibited a higher susceptibility than *E. coli* (Gram-negative) to antimicrobial photosensitization.

## CONCLUSION

In conclusion, the presence of eight chlorophylls and two carotenoids has been identified in the dregs extracts from six commercial green and black teas. The total amount of remnant pigments in the dregs of green teas was generally higher than that of black teas. The pigment distribution in both tea types was comparable, except for the presence of residual chlorophyll *b* in black teas. The most dominant pigment in all the samples was pheophytin *a*, followed by other species of pheophytins *a* and *b*, the epimer of pheophytin *a*, and pyropheophytin. The dregs extract had a significant effect on the antimicrobial photosensitization against *S. aureus* and *E. coli*. The magnitude of the killing effect was comparable among the tea types and could even be higher than that of a single purified pigment. In the utilization of pigments from dregs, the suggested selection ought to be pointed to the amount of total remnant pigments rather than tea types.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE NUTRITIONAL VALUE, BACTERIAL COUNT AND SENSORY ATTRIBUTES OF LITTLE TUNA (*EUTHYNNUS AFFINIS*) FLOSS INCORPORATED WITH THE BANANA BLOSSOM

*Hartati Kartikaningsih, Yahya, Yuniar Tri Hartita, Abdul Aziz Jaziri, Wahidu Zzaman, Rovina Kobun, Nurul Huda*

## ABSTRACT

The study aimed to evaluate the addition of banana blossom (12.5, 25, 37.5, and 50% w/w) on nutritional quality, histamine content, bacterial count, and sensory characteristic in the fish floss prepared from little tuna (*Euthynnus affinis*). The crude protein content, essential amino acids, lipid, and polyunsaturated fatty acids (PUFA) steadily decreased ($p$ <0.05), while the crude fibre, carbohydrate, and ash components of the tuna floss, increased significantly ($p$ <0.05) with increasing levels of banana blossom. The contents of protein, fat, ash, fibre, carbohydrate, and moisture ranged 28.13 – 30.27%, 14.79 – 18.02%, 4.45 – 5.68%, 2.6 – 3.5%, 27.81 – 31.01, and 16.45 – 17.39%, respectively, and most of them met the Indonesian National Standard. For essential and non-essential amino acids, the level varied about 102.82 mg.g$^{-1}$ to 206.76 mg.g$^{-1}$ and 79.71 mg.g$^{-1}$ to 138.76 mg.g$^{-1}$, respectively in the treated tuna flosses. Moreover, ranging 13.72 – 16.29% of PUFA was found in all treated flosses. The most significant effect was found in the histamine levels of the tuna flosses, especially in the 50% added floss sample. Moreover, bacterial counts and heavy metals content were lower than the maximum limits regulated by the Indonesian National Standard. For sensory evaluation, the banana blossom-added samples significantly increased ($p$ >0.05) the acceptability score for all attributes assessed. Taken together, the tuna floss added with 37.5% of banana blossom may be potentially developed as a low-histamine tuna-based product with high ffibre andEPA+DHA, as well as highly acceptable for consumers.

**Keywords:** floss; little tuna; banana blossom; incorporation; characterizations

## INTRODUCTION

Banana blossom (*Musa acuminata*), also commonly called banana flower, is considered as a leftover product after cultivation, which is widely consumed as a vegetable in the Southeast Asian countries, including Indonesia, Malaysia, Thailand, and the Philippines **(Liyanage et al.,2016; Wahab, Ismail and Abidin, 2020)**. Banana blossom contains high nutritional quality, especially dietary fibre. According to **Sharma, Shukla and Golani (2019)**, the banana blossom (100g) contained dietary fibre (5.74g), protein (1.6g), fat (0.6g), carbohydrate (5.7g), calcium (73.3mg), phosphorous (56.4mg) and vitamin E (1.07mg). Dietary fibre plays an essential role in lowering serum cholesterol levels, preventing obesity, normalizing blood glucose and insulin levels **(Bhaskar et al., 2012; Elaveniya and Jayamuthunagai, 2014)**. In addition, banana blossom possesses some bioactive components, such as vitamin C, saponin, tannin, flavonoid, alpha-tocopherol, and myoinositol phosphates, which could promote health benefits **(Somsub et al., 2008; Sheng et al., 2010)**. Thus, due to being rich in a range of nutrients and biologically active components, the banana blossom has gained considerable attraction as an alternative source of functional food ingredients.

*Abon*, also known as shredded fish or fish floss, is one of the traditional dried meat products popular among Indonesia and the Asian community. It is recognized by different local names such as *serunding* in Malaysia, *mahu* in the Philippines, *moo yong* in Thailand, *thitheotieu* in Vietnam, and rousing in China **(Hang, 2015)**. Popular raw materials for producing floss are beef and chicken; however, some fish species are also suitable materials for shredded meat processing. The processing of floss product generally begins with steaming of washed meat until it is tender. The steamed meat is then shredded finely and mixed with some formulated spices, followed by adding coconut milk. After that, the mixture is fried and stirred continuously under heat until the mixture is dry. Finally, excessive oil in the dried meat product is then separated and removed automatically **(Huda et al., 2012)**. Shredded

meat is consumed as part of a daily dish or served as filler of *lemper* (traditional food made of glutinous rice and wrapped in a banana leaf) **(Suryani, Abdurrachim and Alindah, 2016)**. As a dried meat product, it contains a range of nutrients, including protein/amino acids, fat/fatty acids, and ash/mineral. Protein and fat found in meat floss products are generally high at around 23.99 – 32.93% and 18.31 – 32.30%, respectively **(Huda et al., 2012; Wazir et al., 2019; Fahmi and Purnamayati, 2020)**. A high level of fat is mainly caused by cooking oil absorbed during frying. As reported by **Wazir et al. (2019)**, the meat floss contained approximately 87.73 – 91.65% of saturated fatty acids. For ash value, it contains around 3.17 – 6.67%. Among raw materials, a meat floss made of fish is much preferred by many people following the study from **Huda et al. (2012)**, which revealed that fish floss has the highest score in overall sensory parameters compared to other meat floss samples.

Little tuna (*Euthynnusaffinis*) belongs to the Scombridae family that is categorized as a medium-sized tuna **(Ahmed et al., 2015)**. It is one of the most commercially important marine fish species in Indonesia. According to the **Ministry of Marine and Fisheries Affairs (2021)**, the total production of little tuna significantly increased around 61.27%, from 366,900 tonnes in 2010 to 592,056 tonnes in 2019. In terms of nutritional value, little tuna is rich in protein and contains the amount of polyunsaturated fatty acids (PUFA). **Kannaiyan et al. (2019)** reported the protein content found in the white and dark muscle of little tuna was around 23.15% and 23.12%, respectively, while PUFA was around 51.86% and 55.87% in the white and dark muscle, respectively. Due to nutrients rich, little tuna is extensively employed as an essential raw material for sashimi and canned products in the seafood processing industry. Also, other diversifications from little tuna have been developed, including shredded meat, nugget, dumplings, fish ball, and fish cakes **(Suprayitno, Adi and Sulistiyati, 2016; Hizbullah et al., 2020)**. Fish floss derived from little tuna or other fish species mainly uses muscle part with adding some condiments. As a result, it has high protein content, but low dietary fibre. Moreover, histamine content would be high in the end-product because of the raw materials prepared particularly from the dark muscle of tuna and it could give rise to food-borne poisoning **(Lee et al., 2016; Colombo et al., 2018)**. Therefore, there has been increasing interest in formulating food products with functional ingredients to deal with the unbalanced nutrition and undesirable component. Previous studies reported different quality aspects of banana blossom added nugget, noodle, biscuit, and meat floss **(Wahab, Ismail and Abidin, 2020; Elaveniya and Jayamuthunagai, 2014; Komal and Kaur, 2019; Novidiyanto et al., 2020; Puspita, Kartikaningsih and Dayuti, 2019)**. Among them, the added banana blossom could elevate the amount of dietary fibre, antioxidant, and other functional properties.

### Scientific hypothesis

There is a lack of information regarding the use of banana blossom incorporated with tuna floss in increasing fibre content and decreasing histamine levels. Therefore, this study hypothesized that incorporating little tuna floss with banana blossom may give favorable nutritional features, particularly enhancing fibre content and lowering histamine levels. Banana blossom was added up to 500 $g.kg^{-1}$ in the formulation of little tuna floss. Nutritional aspects such as chemical composition, amino acids, fatty acids, and dietary fibre contents of the formulated little tuna floss were analyzed. Heavy metals content as safely required in the food developments was evaluated. The histamine content and pathogenic bacteria test were also determined. In addition, the sensory properties of the little tuna floss were studied to provide a basis for consumer acceptance and commercial applications. This study may stimulate further study in developing healthy fish floss products for nutritional and functional applications.

## MATERIAL AND METHODOLOGY

### Samples

Little tuna (*E. affinis*) used in this study was obtained from Sedang Biru fish market (Malang, East Java, Indonesia). The size of little tuna samples was approximately 202.8 ±3.3 g and 27.6 ±1.9 cm for weight and length, respectively. Banana blossom (*M. acuminata*) was purchased from a local market (Malang, East Java, Indonesia), and its weight was around 2.2 ±0.2 kg.

### Chemicals

All chemicals and reagents used were of analytical grade. $H_2SO_4$, petroleum ether and nitric acid (Merck, Germany) were supplied from a local supplier. Kjeldahl catalyst selenium tablet (Fisher Chemical, USA).

### Bacteria and biological material

Bacteria used, including *Escherichia coli*, *Salmonella* sp. and *Staphylococcus aureus were* obtained from the Indonesian Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Sciences (LIPI). Microbial media used in a recent study were Oxoid (Basingstoke, UK)-based Nutrient agar (NA), MacConkey agar (MCA), Rappaport-Vassiliadis broth (RV), Xylose Lysine Deoxycholate (XLD) agar and Baird Parker agar (BPA).

### Instruments

The instrument used consists of the oven (M720, Germany), Soxtec 2050 (FOSS Analytical, Denmark), ultra-pressure liquid chromatography (UPLC) (Waters, US), gas chromatography (GC) (Agilent Technologies, California, US), High-Performance Liquid Chromatography (HPLC) (Agilent Technologies, California, US) and Atomic Absorption Spectrophotometer (AAS) (GBC Scientific Equipment, USA).

### Description of the experiment

#### *Sample preparation*

Around 15 kg of little tuna samples were purchased from Sendang Biru fish market (Malang, East Java, Indonesia). Fish samples were kept under cold conditions in an insulated box with ice during transportation (around 2-3 h). Upon arrival, the little tuna samples were washed in running water and were then filleted manually using a sharp knife. The filleted samples, including white and dark muscles, were stored in a freezer until use. For banana blossom, about 5 pieces of samples used were was obtained from a local market (Malang, East Java, Indonesia). Due to fresh banana blossom samples used, the

detailed preparation was described in the following procedure for addition into little tuna floss.

***Preparation of little tuna floss incorporated with banana blossom***

Table 1 presents the distinct formulations for all little tuna floss samples. The banana blossoms added with banana blossom in preparing little tuna floss products were 12.5% to 50% based on the weight of the whole little tuna sample (Figure 1). The little tuna meat used consists of white and dark muscles with distinct proportions *i.e.,* 80% and 20%, respectively. These treatments were chosen in compliance with the formulated proportion for fish floss recommended by the previous study from **Puspita, Kartikaningsih and Dayuti (2019)**. The control little tuna floss (0%) was used as a control and compared to the treated little tuna floss samples (12.5 – 50%) to evaluate the distinct characteristics of the formulation process and its corresponding attributes on the treated little tuna floss produced. During preparation, the thawed little tuna muscles were steamed at 100°C for 15 min (Halco, Indonesia). Separately, the banana flowers were cut into small sizes and then steamed at the same condition of fish treatment. After the steaming process, the steamed tuna was shredded using a fork and all ingredients used were thoroughly mixed into the pan. Afterward, the mixture samples were fried and stirred continuously under heat until the mixtures were dried. The excessive oil in the dried fish floss products was then separated and removed automatically using a spinner machine (Spiner Abon, Indonesia). All experimentations were carried out in triplicate.

## Nutritional attribute

***Proximate analysis***

The proximate analysis of little tuna floss samples was determined according to the method of the Association of Official Analytical Chemists **(AOAC, 2000)**. The Kjeldahl method was used to obtain the crude protein from all dried fish floss samples, while fat content was analyzed using the Soxhlet extractor method. The ash and moisture contents of the samples were examined by the gravimetric method. Carbohydrate content was measured by subtracting total crude protein, fat, ash, and moisture contents from 100%. For energy value (kcal.100g$^{-1}$), the calculation used the following formula provided by **AACC (2000)**:

Energy value = (4 × carbohydrate content) + (4 × protein content) + (9 × lipid content) (1)

***Dietary fibre analysis***

The dietary fibre analysis used in this study referred to the method of AOAC 985.29 **(AOAC, 2000)**. The little tuna floss samples were dried at 100 °C to constant weight using air-oven (M720, Binder, Germany). Around 0.5 g of all samples were added α-amylase enzyme and kept in accordance with its optimum condition (*viz*. pH 6; at 100 °C, for 30 min). Afterward, the treated samples were added protease with incubating at 60 °C for 30 min at optimum pH (7.5). The protease-treated samples were then added amyl-glucosidase and incubated at pH 6 for 30 min at optimum temperature. Total dietary fibre of all treated samples was measured by precipitating using ethanol, followed by washing and drying. The obtained residues were then weighed manually.

***Amino acids analysis***

The amino acids composition was analyzed using ultra-pressure liquid chromatography (UPLC) (Waters, US) according to the method of **Nollet and Fidel (2015)**. About 5 mL of hydrochloric acid 6 N was mixed to 0.1 g of samples. The prepared samples were then hydrolyzed at 110 °C for 24 h (M720, Binder, Germany). The hydrolysed samples were transferred to prepared distilled water. After that, the mixtures were filtered with a 0.45 µm filter paper. The 500 µL filtrates were mixed with 40 µL of 2-Amino-4-boronobutanoic acid (ABBA) and 460 µLof double distilled water. Then, 10 µL of the solution was mixed with 70 µL of AccQ Fluorine Borate and 20 µL of fluorine. The homogenized solutions were incubated at 55 °C for 10 min. Finally, the solutions were injected into the UPLC system to calculate the amino acid composition.

***Fatty acids analysis***

The fatty acid composition of little tuna floss was analysed by PT. Saraswanti Indo Genetech, Bogor Indonesia. Fatty acid methyl esters (FAMEs) from extracted fat of tuna floss samples were prepared by basic transesterification following the official method **(AOAC, 2000)**, using hexane and hydroxide potassium 2N.

**Table 1** Formulation for preparation of little tuna floss products.

| Ingredients | Control little tuna floss | Banana blossom addition | | | |
|---|---|---|---|---|---|
| | | 12.5% | 25% | 37.5% | 50% |
| Little tuna meat (g) | 1000 | 870.5 | 750 | 620.5 | 500 |
| Banana blossom (g) | 0 | 120.5 | 250 | 370.5 | 500 |
| Coconut milk (L) | 1 | 1 | 1 | 1 | 1 |
| Onion (g) | 40 | 40 | 40 | 40 | 40 |
| Garlic (g) | 10 | 10 | 10 | 10 | 10 |
| Ginger (g) | 10 | 10 | 10 | 10 | 10 |
| Sugar (g) | 10 | 10 | 10 | 10 | 10 |
| NaCl (g) | 80 | 80 | 80 | 80 | 80 |
| Candlenut (g) | 5 | 5 | 5 | 5 | 5 |
| Brown sugar (g) | 100 | 100 | 100 | 100 | 100 |
| Lemongrass leaves (10 g) | 20 | 20 | 20 | 20 | 20 |
| Lime leaves (8 pieces) | 8 | 8 | 8 | 8 | 8 |

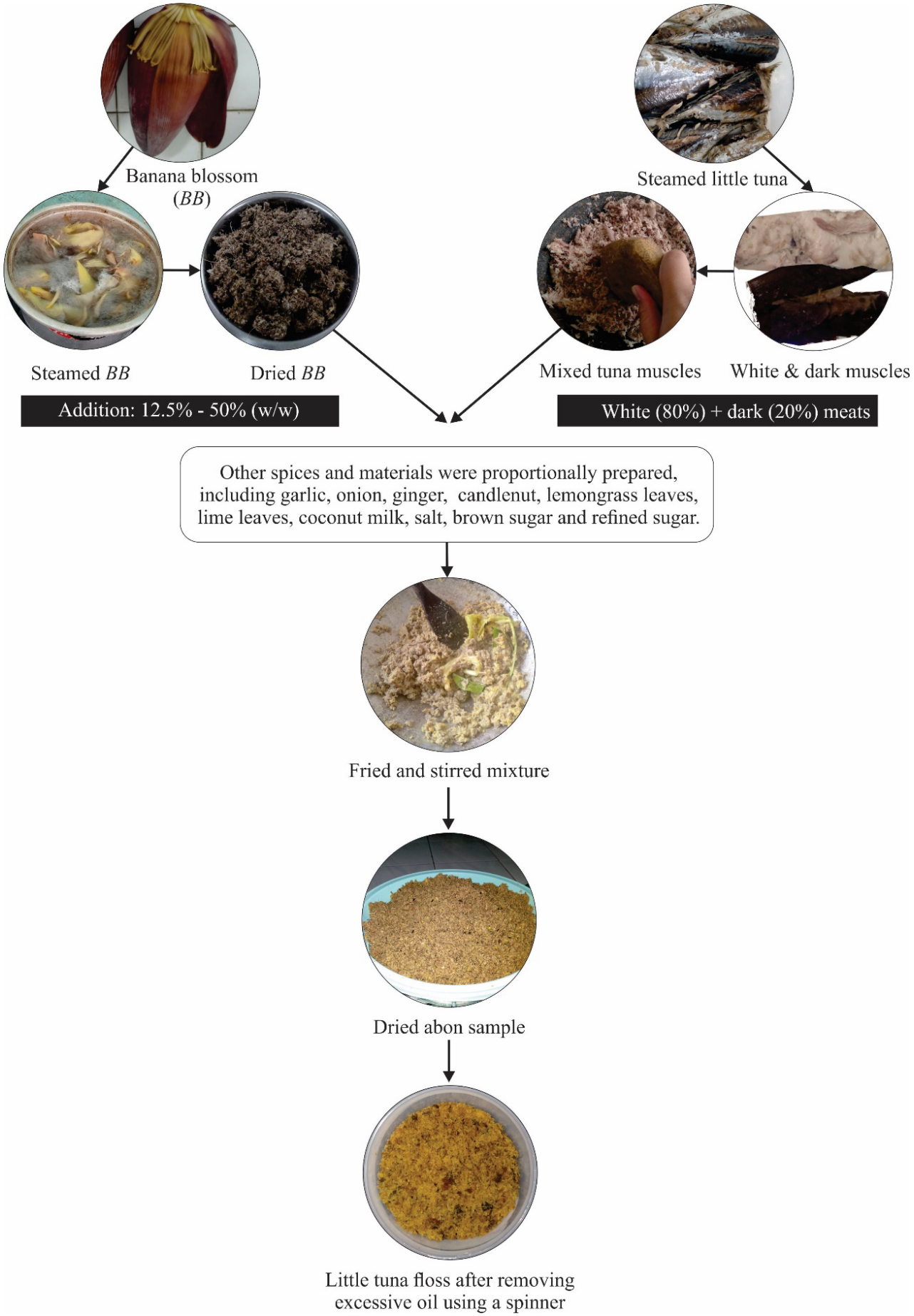


**Figure 1** Preparation of tuna floss incorporated with banana floss.

FAMEs were analyzed by gas-chromatography (GC) (Agilent Technologies, California, US) described by **Aquilani et al., (2018)**. The GC with a flame ionization detector (FID) was used to analyze the amount of fatty acids in the little tuna floss samples. The GC analysis was conducted using capillary column polyethylene glycol, equivalent variants CP-Wax 52 CB (30m × 0.25mm × 0.25µm film thickness). The carrier gas was nitrogen at a flow rate of 1 mL.min$^{-1}$. The initial oven temperature was set at 120 °C with an increase of 5 °C per minute until reached 240 °C. The injector and detector temperatures were 260 °C. Individual peaks were measured by comparison of their retention times (RT) with those of standards. Peak areas were calculated, and FAMEs were expressed as the area percentage of total area FAMEs (%).

***Heavy metal analysis***

Heavy metals analyzed in this study were composed of cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg), and tin (Sn). These microelements were conducted by the method of **AOAC (2000)** using the Atomic Absorption Spectrophotometer (AAS) (GBC Scientific Equipment, USA).

***Histamine determination***

The histamine content was carried out by High-Performance Liquid Chromatography (HPLC) (Agilent Technologies, California, US) according to the method from **Muscarella et al., (2005)**. Around 10 g of homogenized little tuna floss was extracted by 20 mL of 5% trichloroacetic acid (TCA). After filtration, 1 mL of the extracted sample was purified with 2 mL of chloroform solution. The mixture was then centrifuged and 20 µL of the supernatant was analyzed by HPLC. The HPLC column used was a Supelcosil LC-ABZ 4.6 × 150 mm, 5 µm film thickness (Supelco, Bellefonte, PA, USA). The decansulphonate phosphate buffer pH 6.9/acetonitrile mixture (85/15) was used as a mobile phase. Isocratic elution was used; the flow rate was 1.2 mL.min$^{-1}$. The UVDAD was regulated at the absorption wavelength of 210 nm; the injection loop was 20 µL.

## Bacterial count

Bacterial evaluations in the tuna floss consist of total bacteria count, total coliform, *Escherichia coli*, *Salmonella* sp., and *Staphylococcus aureus* with the methods developed by **Anang et al. (2018)** with some modifications. For total bacteria count, serial dilutions of 10 – 1 to 10 – 5 were prepared by diluting around 1 g of the little tuna floss samples into 9 mL of distilled water. Approximately 0.1 mL of aliquots from 10 – 3 to 10 – 5 dilutions were inoculated into Petri dishes containing the Nutrient Agar media. The plates were then incubated at 37 °C for 24 h. After incubation, bacteria colonies formed were counted using the colony counter and recorded as total viable counts. Total coliform was analyzed by dropping 0.1 mL of diluted samples into MacConkey broth with Durham tubes and incubated at 37 °C for 24 h. After incubation, the inoculated tubes were identified as positive total coliform by changing color from purple to yellow, and gas was collected in the Durham tubes. The positive tubes were then transferred into a 5 mL test tube of tryptone solution and incubated at 44 °C for 24 h to test *E. coli*. Afterward, a drop of Kovacs' reagent was added into the treated tubes. The suspected *E. coli* in each tube was shown by a red ring color development, indicating the presence of indole. *Salmonella* sp., the count was evaluated using pre-enrichment of bovine peptone water, followed by enrichment of Rappaport-Vassiliadis (RV) broth. After enrichment, around 0.1 mL of aliquots were inoculated into Xylose Lysine Deoxycholate (XLD) agar. The suspected *Salmonella* colonies appear colorless with a black center because of $H_2S$ production. For S. aureus count, about 1 g of fish floss samples were added into test tubes containing 9 mL of distilled water and the mixtures were serially diluted from 10 – 1 to 10 – 5. Around 0.1 mL of aliquots (10 – 3 to 10 – 5) were inoculated into plates prepared with Baird Parker Agar media. Afterward, the inoculated samples were incubated at 36 °C for 48 h. After the incubation period, colonies formed were identified with the suspected colonies were round, convex with a diameter of 2 – 3 mm, greyish black color with a clear circle (halo).

## Sensory evaluation

Sensory parameters, including appearance, aroma, color, taste, and texture were evaluated using 30 untrained sensory panelists randomly selected among students and this method was adopted from **Sęczyk, Świeca and Gawlik-Dziki (2016)**. The panelists were initially presented with the little tuna floss samples in identical but labeled containers with a three-digit code for their evaluation. Before the sensory session, the tuna floss samples were prepared in triplicate in a randomized permutation. This study used a hedonic test with a 9-point scale to obtain the acceptability score of the little tuna floss products. The likeness scale was arranged in accordance with the above sensory parameters was as follows: 1 = dislike extremely; 2 = dislike very much; 3 = dislike moderately; 4 = dislike slightly; 5 = neither like nor dislike; 6 = like slightly; 7 = like moderately; 8 = like very much; 9 = like extremely.

## Statistical analysis

The experimental design applied in this study was a completely randomized design (CRD). All measurements were performed in triplicate. Data were expressed as the mean values ± standard deviation (SD). The differences were determined using a one-way analysis of variance (ANOVA), followed by Duncan's test. The significant difference was established at $p < 0.05$ using SPSS, Version 27, statistical software program (SPSS Inc., Chicago, Ill., USA).

# RESULTS AND DISCUSSION

## Nutritional attributes

***Proximate composition***

The proximate composition of little tuna floss (as a control) and floss samples incorporated with banana blossom with different levels (12.5 – 50%) is presented in Table 2. Results showed a significant ($p < 0.05$) decreased in protein and fat contents of treated tuna floss samples with the increase in the percentage of banana blossom.

**Table 2** Proximate composition (g.100g$^{-1}$), energy value (Kcal) and histamine content (mg.kg$^{-1}$) of the control sample and added little tuna floss with banana blossom.

| Parameter | Control little tuna floss | Banana blossom addition | | | | SNI* |
|---|---|---|---|---|---|---|
| | | 12.5% | 25% | 37.5% | 50% | |
| Moisture | 16.03±0.01$^{a}$ | 16.45±0.38$^{a}$ | 16.74±0.27$^{a}$ | 17.23±0.16$^{b}$ | 17.39±0.06$^{b}$ | <7 |
| Crude protein | 43.08±1.14$^{d}$ | 30.27±1.41$^{c}$ | 29.51±1.24$^{b}$ | 28.68±0.30$^{a}$ | 28.13±0.16$^{a}$ | >15 |
| Crude fat | 18.70±0.31$^{c}$ | 18.02±0.07$^{c}$ | 16.62±0.06$^{b}$ | 15.20±0.18$^{a}$ | 14.79±0.08$^{a}$ | <30 |
| Ash | 4.12±0.03$^{a}$ | 4.45±0.11$^{a}$ | 4.52±0.06$^{a}$ | 4.78±0.22$^{a}$ | 5.68±0.13$^{b}$ | >7 |
| Crude fibre | 1.8±0.42$^{a}$ | 2.6±0.21$^{a}$ | 2.8±0.50$^{b}$ | 3.1±0.43$^{b}$ | 3.5±0.59$^{c}$ | <1 |
| Carbohydrate | 15.07±0.58$^{a}$ | 27.81±1.04$^{b}$ | 29.61±0.43$^{c}$ | 31.11±0.27$^{d}$ | 31.01±0.20$^{d}$ | - |
| Energy | 271.66±1.01$^{a}$ | 303.69±1.84$^{d}$ | 297.53±0.52$^{c}$ | 289.92±0.25$^{b}$ | 285.28±0.14$^{b}$ | - |
| Histamine | 319.48±121$^{e}$ | 144.16±1.18$^{d}$ | 110.56±1.68$^{c}$ | 85.09±1.68$^{b}$ | 52.25±1.35$^{a}$ | <200** |

Note: Values are given as mean ± standard deviation from triplicate determinations ($n$ = 3). Different superscript letters in the same row indicate significant differences ($p$ <0.05). * SNI 01-3707-1995 is used for fish floss specification. ** Histamine limit referred to **FAO/WHO (2012)**.

However, the protein and fat contents of all little tuna floss samples agreed with the Indonesian National Standard (SNI) values and were even much better than a limit recommended by the standard **(SNI 7988, 2009)** and for protein content, all treated samples were in accordance with other fish flosses **(Huda et al., 2012; Wijaya et al., 2016; Romadhon, Amalia and Anggo, 2019; Fahmi and Purnamayati, 2020)**.

The higher protein indicates the developed tuna floss samples in this study are potentially used as a filler for low-protein food products, such as glutinous rice rolls, bread, and pastel products. The most significant effect of the formulation is the improvement in crude fibre content of the control little tuna flosses, which increased by 44.44 – 94.44% with increasing the percentage of banana blossom from 12.5 – 50%. The increase in crude fibre content of the control tuna floss with the increase in the percentage of banana blossom was similar to the results obtained for floss incorporated with other fibre sources **(Bujang et al., 2016; Candra and Tunoq, 2018; Poonsri et al., 2019)** and its fibre content was higher than the values determined by SNI and other fish floss products. The higher fibre content is an indication of the better quality of the products with the potential health benefits as above mentioned. The contents of ash, carbohydrate, and moisture increased significantly ($p$ <0.05) with incorporating banana blossom in the little tuna flosses. In terms of ash content, the formulated floss samples agreed with the SNI requirements (>7) for the standardized fish floss product. Moreover, the energy values of treated samples (285.28 – 303.69 kcal) were higher than the control (271.66 kcal); but these values were slightly lower than the dietary requirements recommended by the **FAO/WHO/UNU (2007)** for children and young adult foods. The high energy level and protein content in the tuna floss added with banana blossom indicates its suitability as a supporting diet for children and young adults.

The effect of the addition of banana blossom into a tuna floss formula on histamine content is also depicted in Table 2. Interestingly, the histamine contents of treated tuna floss samples decreased significantly ($p$ <0.05) with increasing the level of banana blossom. Compared to the control sample (319.48 mg.kg$^{-1}$), around 2-fold (144.16 mg.kg$^{-1}$) to 6-fold (52.25 mg.kg$^{-1}$) of histamine content decreased with the increment of at around 12.5% to 50% into tuna floss samples, respectively. The reduction of histamine in the treated tuna floss might be due to the ratio of banana blossom and tuna as main materials in the development of little tuna floss products, in which a higher banana blossom was added, a lower content of histamine found in the tuna floss samples. These results were lower than found in the tuna dumpling (1608 mg.kg$^{-1}$) studied by **Chen et al. (2008)**, while the level of histamine in the present study showed a higher compared to the study from **Peivasteh-Roudsari et al. (2020)** in the canned tuna (34.46 mg.kg$^{-1}$). Nonetheless, the histamine concentration in all treated flosses was below the 200 mg.kg$^{-1}$ allowable limitation suggested by the **FAO/WHO (2012)**, which would not cause an adverse effect. This histamine limit is also under the European Union Regulation (EC) No 2073/2005 **(EC, 2005)** for fishery products. It can be inferred that all treated tuna floss samples are safe as a food product.

***Amino acids composition***

Table 3. presents the profile of amino acids found in the control sample and the tuna floss incorporated with different concentrations of banana blossom. Results exhibited that glutamic acid (Glu) was the most dominant amino acid in all little tuna flosses. However, 15-amino acid analyzed in the control sample was a higher amount of total amino acids both essential and non-essential amino acids than found in the treated tuna floss products. This is due to the lower amino acids content observed in banana blossom incorporated with tuna floss samples (Table 3). In terms of essential amino acids, the control and treated samples showed higher values compared to the non-essential amino acids. Furthermore, the content of essential amino acids, including arginine, histidine, isoleucine. leucine, lysine, phenylalanine, threonine, and valine, found in the treated tuna floss samples did not meet the standard recommended by Food and Agriculture Organisation/World Health Organisation/United Nations

University (FAO/WHO/UNU) for children and adult humans **(FAO/WHO/UNU, 2007)**.

Nevertheless, fish floss is a non-staple food product like bread, noodle, and rice, which is routinely consumed in large quantities to provide adequate energy, but it is generally combined with other staple foods to enhance nutritional values.

***Fatty acids composition***

The effect of the formulation of tuna floss with banana blossom on the fatty acid composition is presented in Table 4. The content of fatty acids in the tuna floss samples, both control and treated samples with different levels of banana blossom, varied considerably ($p$ <0.05). The most abundant fatty acids in all treated floss samples were oleic acid (C18:1) (34.03 – 40.63%), followed by palmitic acid (C16:0) (27.19 – 31.84%) and linoleic acid (C18:2) (11.20 – 13.30%), but these fatty acids were slightly lower compared to the control sample's fatty acids. It might be due to a low-fatty acids content observed in the banana blossom (0.39 – 1.28%). In addition, all tuna floss incorporated with banana blossom had lower content of saturated (33.84 – 41.44%), monounsaturated (34.37 – 41.00%), and polyunsaturated (13.72 – 16.29%) fatty acids than those contained in the control. However, among the identified fatty acids, omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids seem to be the most important, due to their multiple biological roles, such as reducing oxidative stress, influencing the inflammatory cascade, presenting neuroprotection, and cardiovascular protection. The total *n*-6 and *n*-3 in the treated samples ranged at around 11.28 – 13.40% and 2.44 – 3.23% respectively, with an n-6/n-3 ratio of about 3.89 – 4.62%. These values within the range (1 – 5) of omega-6 and omega-3 ratio per day recommended by some food experts that should be consumed to prevent undesirable diseases related to the lack of essential fatty acids intake **(EFSA, 2010)**. Moreover, amongst *n*-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are required essentially by the human body and approximately 0.2-2.0 g/day recommended by most health organizations **(Desai et al., 2018)**, and all formulated tuna floss samples met the requirements.

***Heavy metal content***

Heavy metals analyzed in this study are cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg), and tin (Sn). These are classified as toxic metals and endanger human health if the total content of the metals exceeds the recommended exposure limits **(Sajib et al., 2014; Lukáčová et al., 2014; Timorácká, Vollmannová and Ismael, 2017)**. As presented in Table 5, the formulation of tuna floss with different levels (12.5 – 50%) of banana blossom had a wide range of metal element compositions such as Cd (<16×10 – 4 mg.kg$^{-1}$), Pb (<20×10 – 4 mg.kg$^{-1}$), As (8×10 – 4 mg.kg$^{-1}$), Hg (1×10 – 4 mg.kg$^{-1}$) and Sn (36.15 – 37.67 mg.kg$^{-1}$).

**Table 3** Amino acids composition (mg.g$^{-1}$ protein) of control sample and tuna floss incorporated with banana blossom.

| Amino acids | Banana blossom | Control tuna floss | Banana blossom addition | | | | RDA* |
|---|---|---|---|---|---|---|---|
| | | | 12.5% | 25% | 37.5% | 50% | |
| Ala | 0.88±0.00 | 12.60±0.04$^{e}$ | 10.43±0.03$^{d}$ | 6.92±0.03$^{c}$ | 6.43±0.01$^{b}$ | 6.27±0.02$^{a}$ | - |
| Arg | 1.36±0.01 | 32.81±0.05$^{e}$ | 26.73±0.07$^{d}$ | 16.05±0.04$^{c}$ | 14.68±0.29$^{b}$ | 11.13±0.03$^{a}$ | 20 |
| Asp | 1.58±0.00 | 34.90±0.13$^{e}$ | 28.94±0.07$^{d}$ | 20.54±0.10$^{c}$ | 19.65±0.16$^{b}$ | 17.71±0.01$^{a}$ | - |
| Glu | 2.08±0.02 | 55.88±0.17$^{e}$ | 46.82±0.12$^{d}$ | 32.25±0.08$^{c}$ | 30.86±0.05$^{b}$ | 28.80±0.06$^{a}$ | - |
| Gly | 1.05±0.01 | 24.43±0.05$^{e}$ | 20.34±0.03$^{d}$ | 12.76±0.01$^{c}$ | 11.82±0.23$^{b}$ | 10.17±0.02$^{a}$ | - |
| His | 0.62±0.01 | 25.31±0.10$^{e}$ | 19.71±0.09$^{d}$ | 13.22±0.06$^{c}$ | 10.43±0.14$^{b}$ | 8.55±0.02$^{a}$ | 15-16 |
| Ile | 0.94±0.01 | 24.29±0.08$^{e}$ | 19.81±0.05$^{d}$ | 11.93±0.04$^{c}$ | 11.55±0.11$^{b}$ | 10.46±0.02$^{a}$ | 30-31 |
| Leu | 1.11±0.01 | 40.84±0.15$^{e}$ | 33.57±0.08$^{d}$ | 20.25±0.08$^{c}$ | 19.23±0.03$^{b}$ | 18.08±0.02$^{a}$ | 59-61 |
| Lys | 0.78±0.01 | 32.12±0.10$^{e}$ | 26.41±0.08$^{d}$ | 18.57±0.06$^{c}$ | 17.96±0.01$^{b}$ | 17.26±0.03$^{a}$ | 45-48 |
| Phe | 0.76±0.01 | 26.69±0.15$^{e}$ | 21.02±0.03$^{d}$ | 13.63±0.00$^{c}$ | 11.09±0.57$^{b}$ | 8.63±0.03$^{a}$ | 38 |
| Pro | 0.90±0.01 | 15.14±0.05$^{d}$ | 12.76±0.04$^{c}$ | 8.11±0.03$^{b}$ | 7.40±0.02$^{a}$ | 7.38±0.02$^{a}$ | - |
| Ser | 1.40±0.01 | 24.42±0.08$^{e}$ | 19.47±0.06$^{d}$ | 11.53±0.03$^{c}$ | 10.62±0.08$^{b}$ | 9.37±0.02$^{a}$ | - |
| Thr | 0.86±0.01 | 28.99±0.09$^{e}$ | 23.60±0.09$^{d}$ | 14.09±0.06$^{c}$ | 13.63±0.06$^{b}$ | 10.80±0.03$^{a}$ | 23-25 |
| Tyr | 0.67±0.00 | 16.27±0.07$^{e}$ | 13.67±0.03$^{d}$ | 8.25±0.04$^{c}$ | 6.46±0.03$^{b}$ | 5.24±0.03$^{a}$ | - |
| Val | 0.94±0.01 | 27.32±0.09$^{d}$ | 22.24±0.09$^{c}$ | 13.98±0.02$^{b}$ | 12.84±0.06$^{a}$ | 12.67±0.01$^{a}$ | 39-40 |
| TEAA** | 8.05 | 254.56$^{e}$ | 206.76$^{d}$ | 129.97$^{c}$ | 118.35$^{b}$ | 102.82$^{a}$ | - |
| TNEAA*** | 7.89 | 167.37$^{e}$ | 138.76$^{d}$ | 92.12$^{c}$ | 86.27$^{b}$ | 79.71$^{a}$ | - |

Note: Values are given as mean ± standard deviation from triplicate determinations ($n$ = 3). Different letters in the same row indicate significant differences ($p$ <0.05). * RDA: Recommended dietary allowance for children and adult humans by **FAO/WHO/UNU (2007)**. ** TEAA: total essential amino acids (arginine, histidine, isoleucine. leucine, lysine, phenylalanine, threonine and valine). *** TNEAA: total non-essential amino acids (aspartic acid, glutamic acid, serine, tyrosine, glycine and alanine).

**Table 4** Fatty acids profile (%) of control tuna floss and treated tuna floss with addition of banana blossom.

| Fatty acids | Banana blossom | Control tuna floss | Banana blossom addition | | | |
|---|---|---|---|---|---|---|
| | | | 12.5% | 25% | 37.5% | 50% |
| C6:0 | n.d. | 0.04 ±0.000$^{b}$ | 0.03 ±0.001$^{a}$ | 0.04 ±0.000$^{b}$ | n.d. | n.d. |
| C8:0 | n.d. | 0.38 ±0.007$^{c}$ | 0.37 ±0.006$^{c}$ | 0.44 ±0.009$^{d}$ | 0.23 ±0.001$^{b}$ | 0.17 ±0.000$^{a}$ |
| C10:0 | n.d. | 0.25 ±0.002$^{c}$ | 0.26 ±0.003$^{c}$ | 0.33 ±0.006$^{d}$ | 0.16 ±0.002$^{b}$ | 0.13 ±0.001$^{a}$ |
| C12:0 | n.d. | 2.15 ±0.051$^{c}$ | n.d. | 2.68 ±0.063$^{d}$ | 1.35 ±0.005$^{b}$ | 1.14 ±0.000$^{a}$ |
| C14:0 | 0.02 ±0.000 | 1.71 ±0.014$^{a}$ | 1.76 ±0.041$^{a}$ | 1.85 ±0.012$^{b}$ | 1.20 ±0.012$^{b}$ | 1.25 ±0.001$^{c}$ |
| C15:0 | n.d. | 0.05 ±0.000$^{b}$ | 0.04 ±0.000$^{a}$ | 0.04 ±0.000$^{a}$ | 0.04 ±0.001$^{a}$ | 0.03 ±0.000$^{a}$ |
| C16:0 | 0.92 ±0.010 | 32.52 ±0.056$^{c}$ | 31.84 ±0.433$^{c}$ | 28.83 ±0.115$^{b}$ | 27.47 ±0.224$^{a}$ | 27.19 ±0.016$^{a}$ |
| C16:1 | 0.02 ±0.000 | 0.19 ±0.000 | 0.17 ±0.004 | 0.16 ±0.001 | 0.16 ±0.001 | 0.15 ±0.001 |
| C17:0 | n.d. | 0.12 ±0.000 | 0.10 ±0.001 | 0.10 ±0.000 | 0.10 ±0.001 | 0.09 ±0.000 |
| C17:1 | n.d. | 0.04 ±0.001 | 0.03 ±0.000 | 0.03 ±0.001 | 0.02 ±0.000 | 0.03 ±0.000 |
| C18:0 | 0.12 ±0.000 | 4.53 ±0.004$^{e}$ | 4.31 ±0.061$^{d}$ | 3.93 ±0.004$^{c}$ | 3.68 ±0.024$^{b}$ | 3.47 ±0.002$^{a}$ |
| C18:1n9 | 0.39 ±0.001 | 41.99 ±0.020$^{e}$ | 40.63 ±0.323$^{d}$ | 35.86 ±0.001$^{c}$ | 34.97 ±0.139$^{b}$ | 34.03 ±0.012$^{a}$ |
| C18:2n6 | 1.28 ±0.000 | 14.02 ±0.008$^{d}$ | 13.30 ±0.106$^{c}$ | 12.42 ±0.001$^{b}$ | 11.25 ±0.042$^{a}$ | 11.20 ±0.000$^{a}$ |
| C18:3n3 | 0.36 ±0.000 | 2.45 ±0.000$^{e}$ | 2.17 ±0.023$^{c}$ | 2.28 ±0.002$^{d}$ | 1.68 ±0.008$^{a}$ | 1.79 ±0.000$^{b}$ |
| C20:0 | 0.24 ±0.003 | 0.38 ±0.006$^{d}$ | 0.35 ±0.007$^{c}$ | 0.30 ±0.002$^{b}$ | 0.29 ±0.007$^{b}$ | 0.27 ±0.001$^{a}$ |
| C20:1 | 0.14 ±0.000 | n.d. | 0.17 ±0.004 | 0.16 ±0.000 | 0.16 ±0.002 | n.d. |
| C20:4n6 | n.d. | 0.11 ±0.001$^{c}$ | 0.10 ±0.002$^{b}$ | 0.12 ±0.002$^{d}$ | 0.12 ±0.003$^{d}$ | 0.08 ±0.001$^{a}$ |
| C20:5n3 | n.d. | 0.20 ±0.003$^{c}$ | 0.18 ±0.004$^{a}$ | 0.19 ±0.002$^{b}$ | 0.21 ±0.003$^{c}$ | 0.17 ±0.000$^{a}$ |
| C21:1 | n.d. | 0.18 ±0.001 | n.d. | n.d. | n.d. | n.d. |
| C22:6n3 | n.d. | 0.64 ±0.012$^{c}$ | 0.55 ±0.008$^{b}$ | 0.76 ±0.006$^{d}$ | 0.67 ±0.016$^{c}$ | 0.48 ±0.000$^{a}$ |
| C24:0 | 0.02 ±0.000 | 0.13 ±0.002 | 0.10 ±0.001 | 0.10 ±0.002 | 0.12 ±0.000 | 0.10 ±0.001 |
| ∑ SFA | 1.38 ±0.005 | 42.25 ±0.031$^{e}$ | 41.44 ±0.443$^{d}$ | 38.63 ±0.030$^{c}$ | 34.65 ±0.197$^{b}$ | 33.84 ±0.027$^{a}$ |
| ∑ MUFA | 0.62 ±0.001 | 42.40 ±0.019$^{e}$ | 41.00 ±0.323$^{d}$ | 36.21 ±0.001$^{c}$ | 35.31 ±0.139$^{b}$ | 34.37 ±0.012$^{a}$ |
| ∑ PUFA | 1.64 ±0.000 | 17.42 ±0.009$^{d}$ | 16.29 ±0.143$^{c}$ | 15.77 ±0.008$^{b}$ | 13.93 ±0.073$^{a}$ | 13.72 ±0.001$^{a}$ |
| ∑n6 | 1.28 ±0.000 | 14.13 ±0.004$^{d}$ | 13.40 ±0.004$^{c}$ | 12.54 ±0.001$^{b}$ | 11.37 ±0.004$^{a}$ | 11.28 ±0.001$^{a}$ |
| ∑n3 | 0.36 ±0.000 | 3.29 ±0.007$^{e}$ | 2.90 ±0.011$^{c}$ | 3.23 ±0.005$^{d}$ | 2.56 ±0.009$^{b}$ | 2.44 ±0.000$^{a}$ |
| PUFA/SFA | 6.54 ±0.025 | 2.27 ±0.001$^{b}$ | 2.16 ±0.042$^{a}$ | 2.25 ±0.003$^{b}$ | 2.21 ±0.024$^{ab}$ | 2.23 ±0.002$^{ab}$ |
| EPA+DHA | n.d. | 0.84 ±0.015$^{c}$ | 0.73 ±0.012$^{b}$ | 0.95 ±0.009$^{d}$ | 0.88 ±0.019$^{c}$ | 0.65 ±0.000$^{a}$ |
| n6/n3 | 3.56 | 4.29 | 4.62 | 3.89 | 4.44 | 4.62 |

Note: Values are given as mean ± standard deviation from triplicate determinations ($n$ = 3). Different superscript letters in the same row indicate significant differences ($p$ <0.05).

**Table 5** Heavy metals content (mg.kg$^{-1}$) of control and added tuna floss samples with banana blossom.

| Elements | Control little tuna floss | Banana blossom addition | | | | SNI* |
|---|---|---|---|---|---|---|
| | | 12.5% | 25% | 37.5% | 50% | |
| Cd | <0.0016 | <0.0016 | <0.0016 | <0.0016 | <0.0016 | <0.50 |
| Pb | <0.0020 | <0.0020 | <0.0020 | <0.0020 | <0.0020 | <2.00 |
| As | <0.0008 | <0.0008 | <0.0008 | <0.0008 | <0.0008 | <1.00 |
| Hg | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.05 |
| Sn | 27.53±0.41$^{a}$ | 36.15±0.120$^{b}$ | 34.21±0.10$^{b}$ | 37.67±0.20$^{b}$ | 37.3±0.20$^{b}$ | <40.00 |

Note: Values are given as mean ± standard deviation from triplicate determinations ($n$ = 3). Different superscript letters in the same row indicate significant differences ($p$ <0.05). *SNI 01- 3707-1995 is used for fish floss specification.

Compared to the control treatment, all identified elements were relatively the same in the contents except amount of Sn, which the content of Sn increased significantly ($p$ <0.05). However, all heavy metals contained in the treated samples agreed with the Indonesian National Standard for fish floss specification **(SNI 01-3707, 1995)**. It indicates that the tuna floss incorporated with a banana blossom is potentially safe for human beings.

## Bacterial test

Table 6. shows the bacterial counts for all tuna floss samples evaluated with different tests, including total count, *Salmonella* sp., *E. coli*, *S. aureus,* and total coliform. For total bacterial count, a non-selective media was used, and the results presented that no significant difference ($p$ >0.05) was exhibited in the total bacterial count and slightly higher compared to scad fish (*Decapterus* sp.) floss **(Kasmiati et al., 2020)**. However, all treated samples did not exceed the maximum permissible limits of microbial contamination in food products regulated by the Indonesian National Standard **(SNI 7988, 2009)**. The identification of *Salmonella* sp., *E. coli*, *S. aureus* in the tuna floss samples using specific media showed no growth (negative) after incubation and these results agreed with the requirement for microbial contamination in the dried fish product **(SNI 7988, 2009)**. Furthermore, all treated samples, both augmented and un-augmented with banana blossom, showed a similar count (<3 MPN.g$^{-1}$) to the total coliform. These results revealed that the total coliform in all tested samples were within the acceptable microbial quality of the Indonesia National Standard. From those microbial evaluations, the tuna floss product used in the present study agrees with the SNI 7988: 2009.

## Sensory attribute

Sensory evaluation is a suitable tool for developing food products with assessing consumer's acceptance **(Anang et al., 2018; Fiorentini, Kinchla and Nolden, 2020; Witczak, Jaworska and Witczak, 2020)**.

**Table 6** Bacterial count of control tuna floss and tuna flosses added with banana blossom.

| Bacterial test | Control tuna floss | Banana blossom addition | | | | SNI* |
|---|---|---|---|---|---|---|
| | | 12.5% | 25% | 37.5% | 50% | |
| Total count (cfu.g$^{-1}$) | 3.4 × 10$^4$ | 1.6 × 10$^4$ | 3.3 × 10$^4$ | 5.2 × 10$^4$ | 1.1 × 10$^4$ | <1× 10$^5$ |
| *Salmonella* sp. (cfu.g$^{-1}$) | negative | negative | negative | negative | negative | negative |
| *E. coli* (cfu.g$^{-1}$) | negative | negative | negative | negative | negative | < 3 |
| *S. aureus* (cfu.g$^{-1}$) | negative | negative | negative | negative | negative | <1×10$^2$ |
| Total coliform (MPN.g$^{-1}$) | <3 | <3 | <3 | <3 | <3 | <10 |

Note: *SNI 7988: 2009: maximum permissible limits of microbial contamination in food products.

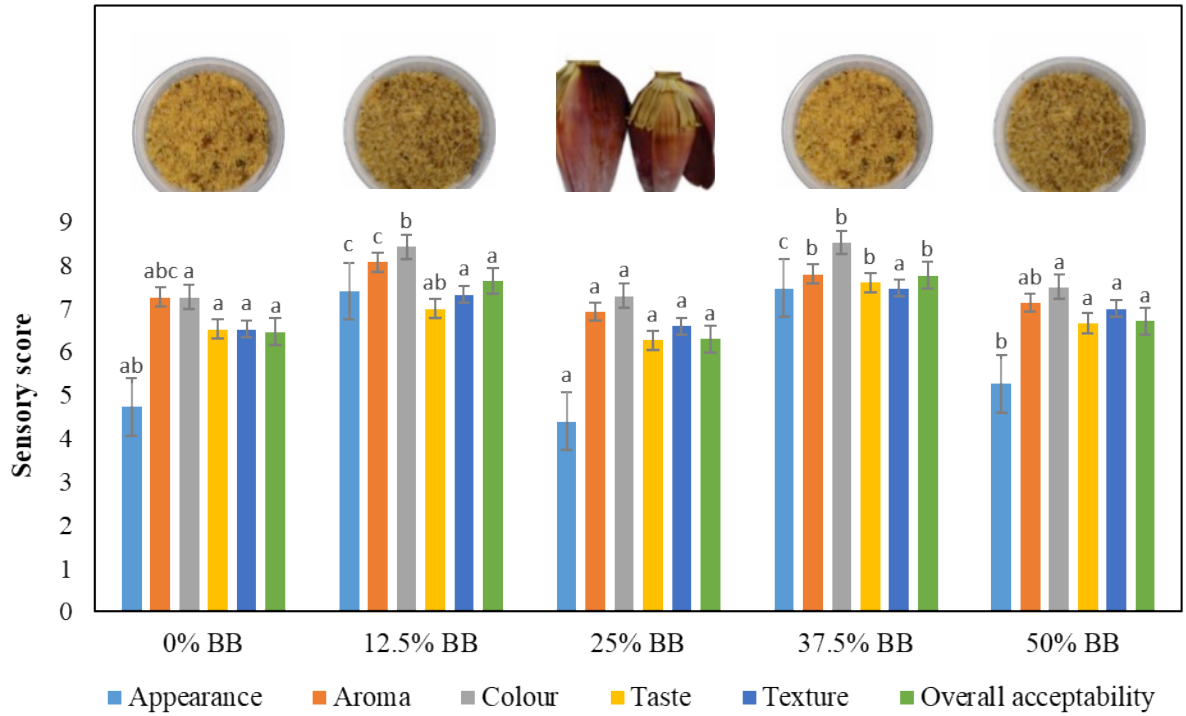


**Figure 2** Physical appearance and sensory attributes of control tuna floss and tuna floss incorporated with different levels (12.5 – 50%) of banana blossom. The same letters denote the lack of statistically significant differences between the results at $p$ <0.05 ($n$ = 30).

Assessment of sensory attributes (appearance, aroma, color, taste, texture, and overall quality) of tested tuna floss by the panelist is depicted in Figure 2. Generally, the addition of banana blossom at the level from 12.5% to 50% to tuna flosses had a significant influence ($p$ <0.05) on its sensory characteristics and consequently on consumer acceptance. Amongst these formulations, the 37.5% added floss showed the highest scores in almost assessed attributes compared to other treatments and control samples.

In addition, the mean score of overall acceptability in the tested floss samples was approximately 7.11, which implies that the treatment of tuna flosses is preferred moderately among the consumers. This acceptability value agreed with the requirement recommended by the Indonesian National Standard (5) **(SNI 01-3707, 1995)**, and even much higher than the score accepted by the SNI. Also, these results are in accordance with the studies from **Candra and Tunoq (2018)**, **Puspita, Kartikaningsih and Dayuti (2019)** and **Novidiyanto et al., (2020)** used the addition of banana flowers into snakehead (*Channa striata*), Indian scad (*Decapterusrusselli*) and chicken floss products, respectively.

## CONCLUSION

Taken together, the addition of tuna floss with different levels (12.5% – 50%) of banana blossom reduced histamine level and increased the dietary fibre content in the little tuna floss samples. In addition, microbial and toxic elements were permissible limits regulated by the Indonesian National Standard (SNI) in all the treated tuna flosses. Our results suggested that 37.5% of banana blossom incorporated into the tuna flosses was selected as an appropriate formula due to its attributes, such as being more acceptable for overall sensory evaluation, high contents of EPA+DHA, and dietary fibre, as well as a low histamine content. However, our further research concerns the nutritional value of the selected tuna floss sample, especially on the enrichment of essential amino acids composition through fortification strategy. Also, functional properties like antioxidant and antimicrobial activities will be further studied.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE SYSTEM OF MONITORING OF INHIBITORY DRUG RESIDUES IN RAW COWS' MILK IN SLOVAKIA

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## ABSTRACT

This article provides brief information on the system of monitoring raw cows' milk for the presence of inhibitory veterinary drug residues in the Slovak Republic. We are describing in detail how the monitoring is carried out and what laboratory methods are used for this monitoring. We also deal with the issue of the disposal of contaminated milk. The presence of inhibitory veterinary drugs like antibiotics or residues of these drugs in milk in an amount exceeding maximal residual limits defined in legislation is illegal. Milk supplies containing detectable concentrations are not acceptable. The reputation of milk as a healthy and safe food should be protected. Dairy companies and consequently consumers want to be confident that milk and milk products are free of inhibitory veterinary drugs contamination. Small amounts of certain antimicrobial agents may affect antibiotic resistance in the human population, some percentage of the population is hypersensitive to antibiotics and other drugs. The presence of drug residues in milk affects the technological process of the production of fermented dairy products. Each country should implement a system of monitoring these drugs' residues in food. The system, which is applied in Slovakia, is effective, it can detect and the presence of these substances before milk processing in dairies. The total incidence of these substances in 2020 was 0.025% of all 12,181 samples tested in central testing laboratories. During the last 20 years.

**Keywords:** raw milk; drug residues; cow milk; antibiotics

## INTRODUCTION

The system of monitoring of raw cow's milk for the presence of residues of inhibitory substances in Slovakia is performed in several stages of the food chain. This is a mandatory obligation arising from Commission Regulation (EC) No 1662/2006 **(EC, 2006)**. For this Regulation, food business operators must initiate procedures to ensure that raw milk is not placed on the market if:

a) it contains antibiotic residues in quantities which, in the case of any of the substances listed in Annexes I and III to Regulation (EEC) No. 2377/90 (**EEC, 1990)** exceeds the value permitted under that Regulation, or
b) the combined total antibiotic residue level exceeds any maximum permitted level.

Regulation EEC No. 2377/90 (**EEC, 1990)** was repealed by regulation (EC) No. 470/2009 **(EC, 2009a)**, but the anexes present in repeald regulation remain in force.

If raw milk does not meet these conditions, the food business operator must, following Commission Regulation (EC) No. 1662/2006 **(EC, 2006)** to notify the appropriate authority (Regional Veterinary and Food Administration of the Slovak Republic) and take measures to immediately solve this problem.

In the Slovak Republic, therefore, food business operators ensure compliance with the requirements of this Regulation through a multi-level control system.

The use of antibiotic therapy to treat and prevent udder infections in cows is a key component of mastitis control in Slovak Republic **(Zajác, Golian, Vázciová, 2006**; **Zajác, Golian, Čapla, 2007a**; **Zajác, Golian Vázciová, 2007b**; **Kološta, 2007)**, Czech Republic **(Navrátilová, 2008)** and many other countries **(Hillerton et al., 1999)**.

Factors to be considered to choose the most suitable method of residue detection are the type of antibiotic used, expected time limitations, sensitivity of the test, and costs **(Senyk et al., 1990)**.

The antibiotic residue detection assay systems that are currently available use different methods and test organisms **(Van Eenennaam et al., 1993)**.

Microbiological assays for the detection of antibiotic residues utilize the genus *Bacillus* sp. because of its high sensitivity to the majority of antibiotics. The *Bacillus stearothermophilus var. calidolactis* is routinely used by the dairy industry to screen antibiotic residues. Delvotest SP is a multiple microbial inhibitor test used to detect antimicrobial agents such as beta-lactams and sulpha compounds **(Suhren and Beukers, 1998)**.

Microbiological tests Copan Milk Test and Delvotest SP-NT can be used for screening antimicrobial substances in milk **(Le Breton, Savoy-Perroud and Diserens, 2007)**.

In general monitoring of antimicrobial agent residues, microbial growth inhibition methods, and rapid tests are used. Microbial inhibitor screening methods are easy to perform and enable the detection of a wide spectrum of agents. The microbial inhibitor screening methods do not attain with some antibiotics the sensitivity at the levels specified by the MRLs (maximum residue level). For the determination of these agents, other methods should be used (e.g. immunochemical or receptor). Another disadvantage is the long period needed to perform the test and the occurrence of falsely positive results if the tests are used for the analysis of individual samples containing higher levels of naturally occurring antimicrobial agents. Rapid tests enable to obtain the result of a test in several minutes and they are highly specific. A majority of the rapid assays were aimed above all, at the detection of β-lactam antibiotics. Even in these tests, falsely positive results can occur **(Navrátilová, 2008)**.

The performance criteria described by the European Decision 2002/657/EC have to be used for validation of these tests **(Le Breton, Savoy-Perroud and Diserens, 2007)**.

The quantification of these substances is performed by High-Performance Liquid Chromatography/Tandem Mass Spectrometry **(SVFI, 2021)**.

## Scientific Hypothesis

The number of positive samples decrees during the observed period. On-farm milk testing with rapid veterinary drug residue screening tests by farmers or dairies is an effective tool to reduce the risk of processing milk contaminated with veterinary drugs.

## MATERIAL AND METHODOLOGY

### Samples

Raw cow milk samples were sampled on the farm by trained and certified personnel from dairy factories. These persons were trained by the Veterinary and Food Institute in Slovakia in National Reference Laboratory for Milk and Milk Products. The training course "Sampling of raw milk manually and by autosamplers for milk payment as part of self-inspection" is accredited by the Ministry of Education, Science, Research and Sport of The Slovak Republic **(MESRS, 2020)**.

### Chemicals

Samples were not preserved or preserved by Azidiol 0.02% (PanReac AppliChem).

### Animals and Biological Material:

This research does not involve the use of animals or biological materials.

### Instruments

Laboratories used water-baths 40 °C for sample preparation and incubators 64 °C ±0.5 °C and 65 ±0.5 °C for samples incubation.

### Laboratory Methods

The following laboratory method was used:

- Delvotest® SP-NT (Supplier: Skar s.r.o. Bratislava, Slovakia);
- Eclipse 50 test (Supplier: Merck, Slovakia).
- CMT+ or formally Copan Milk test (Supplier: Climax, s.r.o., Bratislava);
- Twinsenzor (Supplier: Merck Slovakia);

To determine residues of inhibitory substances, the instructions provided by the suppliers and requirements of the ISO/TS 26844 **(ISO, 2006)** were fulfilled.

In the Delvotest® SP-NT ampoules (Figure 1) with a culture medium containing *Bacillus stearothermophilus var. calidolactis* were used. The ampoules were incubated for 3 h at 64 ±0.5 °C and the color change was evaluated. Samples were considered as negative if the agar change the color from violet to yellow **(MP, 2020; MARD, 2006)**.

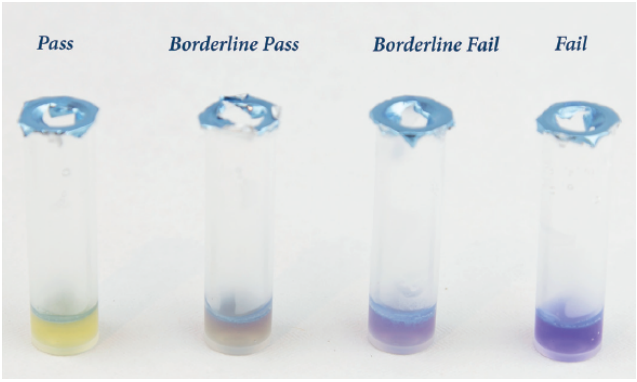


**Figure 1** Reading results in Delvotest® SP-NT.

ECLIPSE 50 combines the principle of agar diffusion tests with a change in the color of the indicator due to the active metabolism of the test microorganism in the absence of an inhibitor. The Eclipse 50 kit has format microplates. The test sample is dispensed into a well-filled agar medium containing *Bacillus stearothermophillus var. calidolactis.* Incubation at 65 °C, at which normal growth of the test strain, causes the color of the pH indicator to change from blue to yellow. If they are under investigation in a sample of the growth inhibitory substance of the test strain, the color of the indicator remains blue-violet **(MARD, 2009a)**.

### Description of the Experiment

**Sample preparation:** samples were heated to 40 ±1 °C in the water bath and gently mixed.

**Number of samples analyzed:** The total number of analyzed raw cows' milk samples was 452,731.

**Number of repeated analyses:** Each sample was analyzed once. If a positive result was found, the analysis was repeated.

Laboratory samples were sampled manually according to the international standard ISO 707 **(ISO, 2008)** or by autosamplers according to the Slovak technical standard STN 57 0005 **(STN, 2000)** with calibrated autosampler.

Samples were stored at 5 °C and sent to one of two Central testing laboratories authorized by the Ministry of Agriculture and Rural Development of the Slovak Republic (MILEX Progres, a.s. Bratislava and EXAMINALA – Dairy Research Institute, Žlina).

Samples were analyzed by one of the above-mentioned laboratory methods. Results of the analysis were evaluated as positive or negative. Overall Results were sent to the Veterinary and Food Institute Bratislava, National reference laboratory for milk and milk products in Nitra, Slovakia to be processed and statistically evaluated.

**Statistical Analysis**

Firstly, the total number of analyzed samples per year was calculated. Secondly, the percentage of positive samples was calculated. These results were plotted on the graph (Figure 2). The reliability equation and the coefficient of $R^2$ were calculated by Microsoft Excell.

We have used ONE-WAY ANOVA to evaluate the effect of the rapid drug residues tests used by farmers. The results were statistically significant at a p-value less than 0.05.

The group of samples from 2001 was considered as a comparison group of samples because rapid tests were not yet widely used in Slovakia in this period.

The results of the group of samples from 2020 were analyzed against this comparison group of samples from 2001.

The statistical evaluation was performed by XLSTAT 2021.2 (Addinsoft).

## RESULTS AND DISCUSSION

The results of the experiment are present in Table 1 and Figure 2. The overall number of analyzed samples per year decrease continuously from 34,795 (2001) to 12,181 (2020). This fact results from a change in legislation due to the accession of the Slovak Republic to the European Union, which took place on 1 May 2004, as well as from the subsequent decline in the number of dairy farms and the number of dairy cows. In 1989, 559 thousand dairy cows were bred in Slovakia and milk production reached more than two billion liters. At present, only less than a quarter of dairy farms are bred in Slovakia, and milk production has halved **(Pravda 2019)**. The number of drug residues positive samples significantly ($p$ <0.05) decrees from 299 (2001) to 3 (2020). This is because, since about 2004, rapid tests to detect the presence of residues of inhibitory substances have become more widely used in Slovakia. Already in 2004, a significant decrease in positive samples to 114 was recorded. This trend continued in the following years and is related to the use of rapid tests. In 2019 and 2020, only 3 positive cases per year were recorded in central testing laboratories, which represents 0.025% of all tested samples. For comparison in 2001, the percentage of positive samples was 0.859%. However, we must note that these statistics are partly distorted because after the accession of the Slovak Republic to the EU the Regulation of the Government of the Slovak Republic No. 312/2003 **(GOV SR, 2003)** has been replaced by European legislation **(EC, 2006)** and the obligation to report positive samples by Regional Veterinary and Food Administrations to the National reference laboratory for milk and milk products has lapsed in Slovakia. These data are available in individual Regional Veterinary and Food Administrations but are not further statistically evaluated. Finally, our statistic contains only data reported by central testing laboratories and does not include positive cases reported directly by dairies to the Regional Veterinary and Food Administrations of the Slovak Republic.

In the following parts of the discussion, we describe in detail the system of monitoring these substances in Slovakia. From a theoretical point of view, this system takes place on several levels. Even based on strict control, we can state that the risk of the presence of residues of inhibitory substances in dairy products in Slovakia is minimal. Dairies only process milk without these substances.

**Milk control system for the presence of inhibitory substances**

***The first level of control***

The first stage of control is carried out directly on the farms using rapid screening tests carried out by the farmer or by the driver of the tanker truck before milk is pumped to the tanker and transported to the dairy. However, not all farmers or tanker truck drivers check freshly milked milk directly on the farm. From the dairy's point of view, this level of control directly at the farmer is interesting because it eliminates the problem of contamination of milk from other farmers in the truck, and also it eliminates the transfer of risk and potential problems with the presence of veterinary drug residues to the next stage of the food chain. Food business operators carry out this control for their own money to eliminate the risk of supplying milk containing antibiotic residues. The purchase of rapid screening tests is provided directly by farmers or by dairies and distributed to farmers or milk tankers drivers.

***The second level of control***

The second stage of control takes place at the entrance to the dairy as a self-verification check. Following EC Regulation No 852/2004 **(EC, 2004a)**, the established HACCP system and monitoring for the presence of inhibitory substances are performed routinely during milk intake. Each dairy has a customized monitoring system. In most cases, the laboratory worker will firstly take samples from individual farms sampled by the truck driver or autosampler. Also, it is necessary to take the "tanker sample" which contains milk from all farmers. Usually, this sample is sampled automatically on farms if the autosampler is used. Milk from all farmers is collected in one big bottle. In the case of manual sampling, laboratory workers need to take the sample from the tanker cistern or each tanker cistern chamber. This can be made following ISO 707 **(ISO, 2008)**. Sometimes this tanker sample is sampled by dripping milk from the pipe valve leading to the receiving tank. Milk is waiting in the truck tanker or the designated intake tanker and can not be processed until is analyzed for drug residues presence. The sample is examined by a rapid 5 – 10 minute test (e.g. BetaStar, BetaStar Combo, ROSA Charm 3 SL3, ROSA Charm SLB, Twinsensor BT, SNAP BT ST test, etc.).

The sensitivity of rapid tests is either at the level of maximum residue limits or even higher **(Zajác et al., 2006, 2007a, 2007b)** and is suitable for milk testing by dairies.

If the residues of inhibitory substances are detected, then all samples from individual farms are tested. This is how the farm that caused the contamination is found. These positive samples are tested by the dairy only with a long 3-hour tests Delvotest® SP-NT **(MARD SR, 2006)**, Eclipse 50 **(MARD SR, 2009a),** or Kalidos TB MP **(MARD SR, 2009b)**. The dairy reports this situation to the appropriate Regional Veterinary and Food Administration, but as this is a self-verification check, these results are not statistically evaluated by this institution.

Some dairies do not perform a long 3-hours test but they send positive samples directly to the accredited laboratory for confirmation, or they automatically consider the milk to be contaminated and therefore intended only for disposal.

**Table 1** Number of samples analyzed in central testing laboratories and number of drug residues positive samples in Slovakia.

| Year | Number of analyzed samples | Number of negative samples | Number of positive samples | Percentahe of possitive samples |
|---|---|---|---|---|
| 2001 | 34 795 | 34 496 | 299 | 0.859 |
| 2002 | 36 050 | 35 869 | 181 | 0.502 |
| 2003 | 33 402 | 33 210 | 192 | 0.575 |
| 2004 | 30 572 | 30 458 | 114 | 0.373 |
| 2005 | 30 324 | 30 250 | 74 | 0.244 |
| 2006 | 27 646 | 27 547 | 99 | 0.358 |
| 2007 | 26 024 | 25 992 | 32 | 0.123 |
| 2008 | 26 297 | 26 275 | 22 | 0.084 |
| 2009 | 23 313 | 23 286 | 27 | 0.116 |
| 2010 | 21 371 | 21 360 | 11 | 0.051 |
| 2011 | 19 475 | 19 458 | 17 | 0.087 |
| 2012 | 18 754 | 18 746 | 8 | 0.043 |
| 2013 | 17 965 | 17 959 | 6 | 0.033 |
| 2014 | 18 918 | 18 913 | 5 | 0.026 |
| 2015 | 17 440 | 17 436 | 4 | 0.023 |
| 2016 | 15 270 | 15 267 | 3 | 0.020 |
| 2017 | 15 321 | 15 317 | 4 | 0.026 |
| 2018 | 14 196 | 14 191 | 5 | 0.035 |
| 2019 | 13 417 | 13 414 | 3 | 0.022 |
| 2020 | 12 181 | 12 178 | 3 | 0.025 |

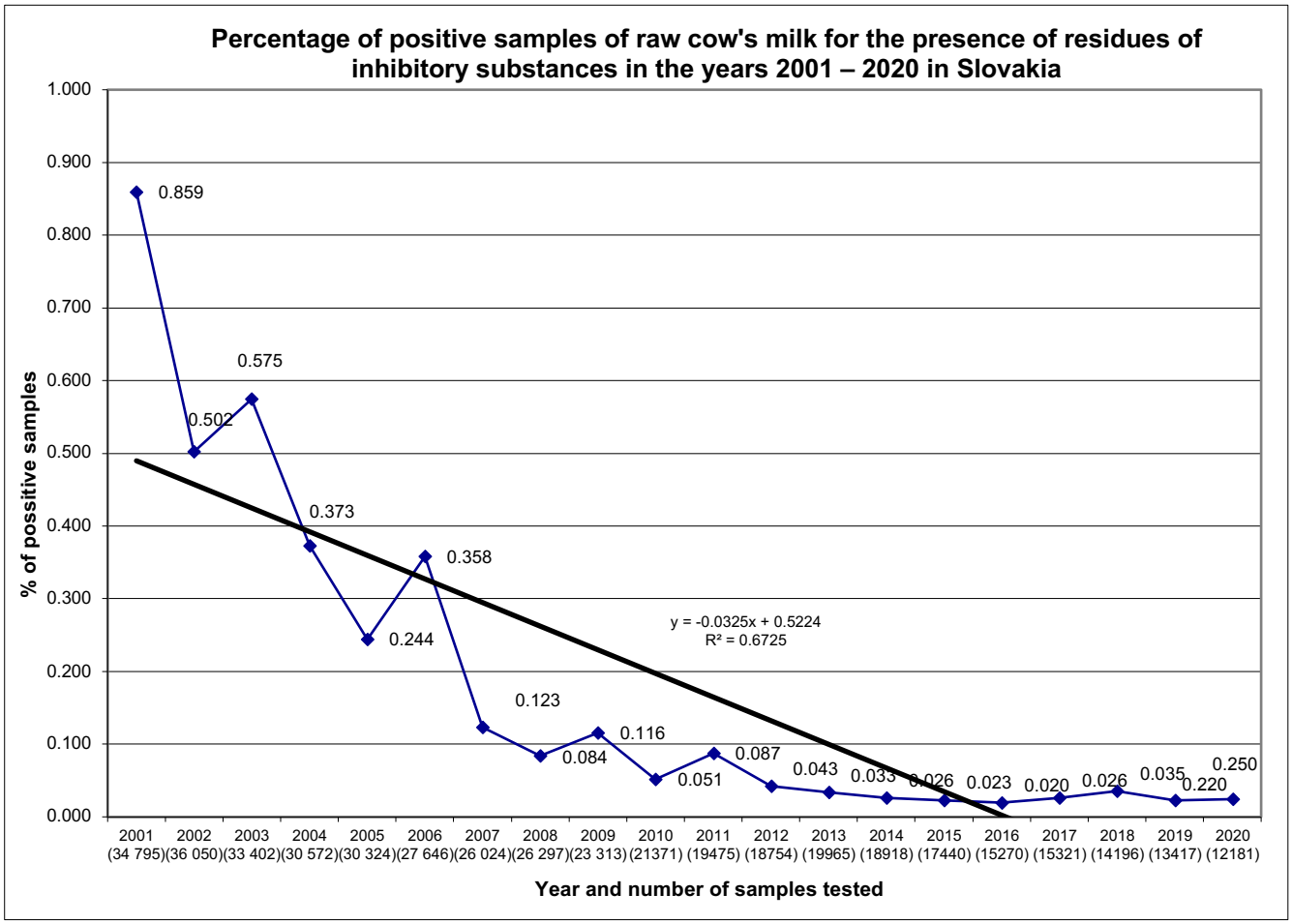


**Figure 1** Percentage of positive samples of raw cow's milk for the presence of residues of inhibitory substances in the years 2001 – 2020 in Slovakia.

The damage must be paid by the farmer who caused such contamination. Usually, the incurred damage is resolved as an insured event.

In Slovakia or European Union, the legislation does not specify which laboratory should test samples **(EC, 2006)**. Even the legislation does not require accreditation of this laboratory! In practice, this situation can be handled directly by the legislation of the country or by the supplier-customer contract. In Slovakia, it is usually tested in one of the two accredited central testing laboratories specifically intended for milk analysis or in the National Reference Laboratory for Milk and Milk Products in Lužianky or by the National Reference Laboratory for Antibacterial Substances and Other Pharmacologically Active Substances Dyes and Some Prohibited Substances in Dolný Kubín **(MARD, 2020)**.

In practice, dairies usually use the services of accredited laboratories for confirmation of positive samples only rarely. They usually immediately consider the sample to be positive. This is how it is solved in most supplier-customer contracts. Confirmation of positive samples can be performed in a contracted accredited central testing laboratory or one of the above-mentioned national reference laboratories. When the accredited laboratory confirms the positivity of the sample, then milk is legally disposed of by the authorized company under a decision of the Regional Veterinary and Food Administration.

Some dairies in Slovakia have recently started sending milk samples for analysis of milk composition, total bacterial count, somatic cells count, and drug residues to laboratories in Hungary, Austria, Germany, or the Czech Republic. The results from these laboratories are then not statistically processed by National Reference Laboratory for Milk and Milk Products. Only the results from central testing laboratories in Slovakia are processed by NRL.

The dairy reports the presence of a positive case of residues of inhibitory substances to the Regional Veterinary and Food Administration, which will carry out an inspection at the farmer who caused the contamination.

After the correction, the farmer will request a Regional Veterinary and Food Administration for sampling the milk, which will then be examined in an accredited testing laboratory. Only after this analysis can the farmer deliver the milk to the dairy again.

***The third level of control***

The third stage of control is performed in central testing laboratories or any other laboratory. Dairies in Slovakia are usually sent milk samples for the inhibitory drug residues substances test to the contracted one central testing laboratory. The frequency of testing is not prescribed in European legislation **(EC, 2006)**. Usually, two samples per month are collected from each farm. This testing is performed as a part of the verification process based on the HACCP plans. Also, the frequency of testing is mentioned in the supplier-purchaser contract. The testing is generally carried out in conjunction with the examination of the freezing point of the milk because it is recommended to analyze not preserved milk samples. On the other hand, for some tests, preserved samples may be used.

Persons taking milk samples for payment purposes, whether drivers or directly the purchaser's staff, must be trained by the Veterinary and Food Institute, National Reference Laboratory for Milk and Milk Products. These persons periodically undergo an accredited educational activity every five years and hold a “milk sampler's certificate”, which entitles them to take milk samples. In principle, such samples are taken in duplicate. One sample remains on the farm and the other is used by a dairy company. The sampling plan created by the dairy for payment must be secret. Farmers must not know the date of this sampling. Sampler notifies the farmer immediately when this sample is collected. All samples are sent to a contracted laboratory (usually a central testing laboratory).

If residues of inhibitory substances are detected in the second or third stage of control, the dairy shall trace the source of the contamination to identify the farm which caused the contamination.

The farm that caused the contamination will then provide financial compensation for milk and costs associated with the legal disposal of the contaminated milk to all farmers affected by this contamination. From a technical point of view, single-chamber or multi-chamber tanks can be used to transport milk. In most cases, the milk is contaminated in the relevant chamber of the entire milk tank into which the milk is pumped is contaminated. In some cases, contamination of several chambers in the milk tank may occur if the capacity of the currently filled chamber is insufficient or by cross-contamination from residues of contaminated milk left in the hoses and distribution system of the milk tank truck. In the case of using a multi-chamber tank, it is, therefore, possible to save part of the milk supply, since the positive milk is usually only found in one of the chambers of the tank.

In the case of confirmation of a "positive" result for the presence of antibiotic residues, the dairy plant shall carry out the following:

- does not accept (reject) the milk and sends it for disposal following the rules laid down in Regulation (EC) No. 1069/2009 **(EC, 2009b)** on animal by-products and derived products not intented for human consumption. The disposal of Category 3 material is carried out as follows:
- applied to land without processing,
- it can also be disposed of by composting or transformation into biogas.
- Such disposal is possible only in that case if the competent authority does not consider it a risk of any disease communicable through that product to humans or animals.

According to the Decree of the Ministry of Industry and Trade of the Slovak Republic No. 148/2012 **(MIT SR, 2012)**, such disposal must be carried out only under the official supervision of a veterinary inspector with his consent. According to EC Regulation no. 142/2011 **(EC, 2011)**, the removal of milk by the wastewater stream is prohibited.

Depending on how the buyer has stated in the supplier-customer contract with the farmer, he can ensure that another confirmatory test is performed, which identifies and quantifies the antibiotic and proves whether or not the maximum residue limit for this particular antibiotic is exceeded. These confirmation tests are usually performed in accredited testing laboratory: Veterinary and Food Institute Dolný Kubín, National Reference Laboratory for Antibacterial Substances and Other Pharmacologically

Active Substances, Dyes and Certain Prohibited Substances.

If the maximum residue limit is exceeded, the milk and milk products are disposed of legally. If the maximum residue limit is not exceeded, milk and products made from it are considered safe.

According to **Kološta (2007)**, the biggest problem is mainly residues of antibiotics and sulfonamides, as these are mostly used in the treatment of dairy cows. The reference method for the detection of antibiotics and sulphonamides in raw milk and heat-treated milk is the CD 91/180 / EEC method on the principle of microbial inhibition. The mentioned method was also taken over into the system of Slovak technical standards STN 57 0531 Determination of antibiotics and sulfonamides in raw and heat-treated milk **(STN, 2001)**.

The journals of the Ministry of Agriculture and Rural Development of the Slovak Republic state which laboratory methods can be used for the official control of the presence of inhibitory substances in milk. These are methods based on the principle of agar diffusion tests with a change in the color of the indicator due to the active metabolism of the test microorganism (*Bacillus stearothermophilus var. Calidolactis*) in the absence of an inhibitor. The determination of residues of inhibitory substances in milk and milk products can therefore be carried out in central testing laboratories by the following methods approved by the Ministry of Agriculture and Rural Development of the Slovak Republic:

- Delvotest® SP-NT **(MARD SR, 2006)**,
- Eclipse 50 **(MARD SR, 2009a)** or
- Kalidos TB MP **(MARD SR, 2009b)**.

Also, there is a

- CMT+ test **(Climax, 2021)**, which has been verified by the National Reference Laboratory for Milk and Milk products, but it is not listed in the list of approved methods of the Ministry of Agriculture and Rural Development of the Slovak Republic.

When testing raw cow's milk for payment, central testing laboratories in the Slovak Republic (MILEX Progres, as Bratislava and EXAMINALA – Dairy Research Institute in Žilina) use a diffusion test to determine residues of inhibitory substances, which lasts 3 hours: Delvotest® SP-NT. This test is used also by the National Reference Laboratory for Milk and Milk Products in Nitra (NRLM) **(Zajác and Čapla, 2010)**.

However, it is not possible to determine the exact amount of antibiotic residues or a specific species using these microbiological or antigen methods.

Veterinary and Food Institute Dolny Kubin, National Reference Laboratory for Antibacterial Agents and Other Pharmacologically Active Substances, Dyes and Certain Prohibited Substances quantify veterinary drug residues by HPLC/MC method **(SVFI, 2021)**.

***The fourth degree of control***

The State Veterinary and Food Administration of the Slovak Republic annually compiles the National Plan for the Control of Residues in Live Animals and Products of Animal Origin following the applicable legislation of the EU **(EC, 2017; EC, 2019)**. The aim of the control through this plan is to obtain an overview of the current state of contamination of individual components of the food chain, to implement effective measures to eliminate the identified deficiencies and prevent their further occurrence. Long-term monitoring of the presence of inhibitory substances in milk leads to the finding that almost 95% of cases of secondary contamination with inhibitory substances are antibiotic contamination, approximately 2.5% of contamination is due to other drugs, especially sulfonamides and residual detergent and disinfectant residues, 1% may cause mycotoxins from feed (nuclear, bulk), 1% may be caused by agrochemical residues or some preservatives and then only about 0.5% remains on natural inhibitors in milk **(Kološta, 2007)**.

Commission Regulation (EC) No. 853/2004 **(EC, 2004b)** contains specific hygiene rules for food of animal origin.

Commission Regulation (EC) No 1662/2006 **(EC, 2006)** defines health requirements for the production of raw milk. Raw milk must come from animals to which no unauthorized substances have been administered and the products (medicines or substances) administered have complied with the withdrawal periods prescribed for those products. This Regulation also defines the requirements for milking, transport, and transport hygiene. According to the Regulation, it is necessary to identify animals that have undergone treatment which could cause the excretion of drug residues in milk and colostrum, and that milk and colostrum obtained from such animals should not be used for human consumption before the expiry of the prescribed withdrawal period. Dips or sprays pods will be used only after authorization or registration. Based on these requirements, primary milk producers must put in place systems to control the treatment of dairy cows.

In the past, the State Veterinary and Food Administration published a guideline for control of raw milk in farms for the production of raw milk **(SVFA, 2009)**, today the control is based on the requirements of the current European legislation **(EC, 2017; EC, 2019)**, Title IV, Article 49 Control of milk and colostrum production holdings and Article 50 Control of milk and colostrum.

The Regional Veterinary and Food Administration checks the food business operator whether, according to EC Regulation no. 1662/2006 **(EC, 2006)** has procedures in place to ensure that raw milk is not placed on the market if it contains antibiotic residues in quantities which, for any of the substances listed in Annexes I and III to Regulation (EEC) No. 2377/90 **(EC, 1990)** exceeds the value permitted under that Regulation or if the combined total antibiotic residue level exceeds any maximum permitted value.

During the official control, the inspector fills in the prescribed official control record, in which he states the number of reports of positive cases of residues of inhibitory substances in raw milk. It also checks whether the supplier and the relevant regional veterinary and food administration of the Slovak Republic have been informed about unsatisfactory results of raw milk. In case of unsatisfactory results of raw milk, the inspector checks the documentation, the date of detection (notification from the Central testing laboratory, the State Veterinary, and Food Institute, the own laboratory, or any laboratory), the date of written notification to the relevant Regional Veterinary and Food Administration and the raw milk supplier.

The food business operator must therefore take measures to remedy this situation and should not place milk

containing residues of inhibitory substances on the market. In the case of residues of inhibitory substances, the legislation does not further specify what measures the food business operator has to take. The legislation does not specify how the control body should proceed, nor does it specify whether samples are to be taken to prove remediation and where (what laboratory) these are to be tested. However, according to the methodological guideline **(SVFA SR, 2009)**, inspectors of Regional Veterinary and Food Administrations are instructed as follows: the producer of raw milk must start procedures to ensure that raw milk is not placed on the market, i.e. unless corrective measures have been taken and it should be proved that raw milk does not exceed the maximum permitted level of the combined total antibiotic residues. Food business operators must not place such milk on the market. In practice, this means that after the situation has been remedied, the farmer must have a sample of milk examined at his own expense in the laboratory (it is not defined what kind of laboratory, but usually it is agreed with the inspector what laboratory should be used for this testing. Almost it is an accredited central testing laboratory or any accredited laboratory of the Veterinary and Food Institute – NRL for milk and milk products or NRL for Antibacterial Substances). If the result of the test shows that the milk does not contain antibiotic residues, it may place it on the market.

Control of antibiotic residues is necessary to protect the reputation of milk as safe food and to prevent exposure of the consumers to drug residues. Governmental authorities which are obliged to organize supervision and control systems by state supervision agencies must provide these requirements **(Popelka, et al., 2003)**.

EU countries must monitor food of animal origin for the presence of residues. The legislation governing the design and implementation of residue monitoring plans is as follows **(EC, 2021)**:

- Directive 96/23/EC - Annexes are in force and establish the sampling frequency and range of substances to be tested for.
- Decision 97/747/EC - Annexes are in force and establish additional sampling frequencies for milk.
- Regulation (EU) 2021/808 - Rules for the validation of analytical methods used in the residue monitoring plan and official sample treatment (Laboratory Analysis).

Specific EU legislation outlines the laboratory analysis and correct interpretation of results:

- Regulation (EU) 2021/808 establishes Criteria and procedures to validate analytical methods, ensuring the quality and comparability of the results of official laboratories. Common criteria for the interpretation of test results. Minimum required performance limits (MRPL) for analytical methods to detect substances without a maximum limit. This is relevant for substances that are not authorized or that are specifically prohibited in the EU. Also sampling procedures and official sample treatment.

Commission Decision 97/747/EC lays down that the annual number of samples taken should be one per 15,000 tonnes of annual milk production, with a minimum of 300 samples **(EFSA, 2019)**.

The overview of the non-compliant results for prohibited substances is present in Table 2. Chloramphenicol is the most dominant prohibited substance detected in milk in European Union.

**Table 2** Prohibited substances detected in milk in EU.

| Year | Drug residue | Number of non-compliant results | Countries reporting non-compliant results | Source |
|---|---|---|---|---|
| 2019 | Chloramphenicol | 3 | Italy, Latvia, Poland | **(EFSA, 2021)** |
| 2018 | Chloramphenicol | 2 | Poland, Spain | **(EFSA, 2020)** |
| 2017 | Chloramphenicol | 1 | Croatia | **(EFSA, 2019)** |
| 2017 | AHD (1-aminohydantoin) | 1 | Croatia | **(EFSA, 2019)** |
| 2017 | AMOZ (5-methylmorpholino-3-amino-2-oxazolidone) | 1 | Croatia | **(EFSA, 2019)** |
| 2017 | AOZ (3-amino-2-oxazolidone) | 1 | Croatia | **(EFSA, 2019)** |
| 2017 | SEM (semicarbazide) | 1 | Croatia | **(EFSA, 2019)** |
| 2016 | Chloramphenicol | 3 | Poland | **(EFSA, 2018)** |
| 2016 | Hydroxymetronidazole (MNZOH) | 1 | Germany | **(EFSA, 2018)** |
| 2015 | Chloramphenicol | 2 | Latvia, Poland | **(EFSA, 2017)** |
| 2014 | Chloramphenicol | 2 | Latvia, Poland | **(EFSA, 2016)** |
| 2013 | Chloramphenicol | 3 | Latvia, Slovenia, Slovakia | **(EFSA, 2015)** |
| 2012 | Chloramphenicol | 1 | Spain | **(EFSA, 2014)** |
| 2011 | Chloramphenicol | 1 | Spain | **(EFSA, 2013)** |
| 2010 | Chloramphenicol | 3 | Czech Republic, Spain | **(EFSA, 2012)** |
| 2009 | Chloramphenicol | 1 | Slovakia | |

Note: the list of the reports is present on the European Commission website **(EC, 2021)**.

The specific species of residues detected are listed in Table 3. There are several specific groups of residues according to the EU legislation: A (1-6), B (1-3). European Union member states report all positive samples from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products including milk **(EFSA, 2021)**.

The Slovak Republic has performed the monitoring and the targeted sampling according to the EU legislation. The number of positive samples was:

- 2013: Chloramphenicol (1 positive sample) **(EFSA, 2015)**,
- 2012: Benzylpenicillin (Penicillin G) (1 positive sample) **(EFSA, 2014)**,
- 2009: Chloramphenicol (1 positive sample) **(EFSA, 2011)**.

Positive samples present in Table 1 are not included in these reports (Table 2), because they were detected in the self-control by dairies and were not sampled as a part of the targeted monitoring performed by the Veterinary and Food Administration of the Slovak Republic.

The monitoring system in Slovakia is functional and will detect possible contamination of milk with residues of veterinary drugs. Dairies in Slovakia process only milk that does not contain residues of veterinary drugs.

**Table 3** Overview of the inhibitory substances detected in milk in EU 2015 – 2019.

| Year | Overview of the substances detected | Source |
|---|---|---|
| 2019 | In group A, there were three non-compliant samples and non-compliant results reported in group A6 (chloramphenicol), by three countries. For antibacterials (B1), seven countries reported a total of 11 non-compliant samples and results. In the group B2, there were 5 non-compliant samples and results for anthelmintics (B2a) and 19 noncompliant samples and results for NSAIDs (B2e), reported by three and eight countries, respectively. In the group B3, there were three non-compliant samples and results for mycotoxins (B3d), relating to Aflatoxin M1, reported by two countries. For organochlorine compounds, including PCBs (B3a) there was one non-compliant sample (2 non-compliant results) reported by one country. More information on the specific substances identified and the number of non-compliant results reported by each country is given in Appendix A. Detected substances: Chloramphenicol, Aminosidin (Paromycin, Paromomycin), Amoxycillin, Ampicillin, Benzylpenicillin (Penicillin G), Dihydrostreptomycin, Doxycycline, Rifaximin, Closantel, Ivermectin, Moxidectin, Oxyclozanide, Acetaminophen (Paracetamol), Antipyrin-4-Hydroxy, Diclofen (Diclofenac), Salicylic acid, TEQ Dioxin-like PCBs LB, Aflatoxin M1. | **(EFSA, 2021)** |
| 2018 | In the group A, there were two non-compliant samples (two non-compliant results) for group A6 (chloramphenicol), reported by two Member States. For antibacterials (B1), four Member States reported a total of four non-compliant samples (and results). In the group B2, there were 18 non-compliant samples and results: 6 for anthelmintics (B2a) and 12 for NSAIDs (B2e). In the group B3, there was three non-compliant samples and results for chemical elements (B3c), relating to lead, reported by two Member States. More information on the specific substances identified and the number of non-compliant results reported by each Member State is given in Appendix A. Detected substances: Chloramphenicol, Aminosidin (Paromycin, Paromomycin), Amoxycillin, Cloxacillin, Oxytetracycline, Closantel, Ivermectin, Albendazole, Sulphoxide, Albendazole sulphone, Albendazole 2-amino sulphone, Diclofen (Diclofenac), Salicylic acid, Lead (Pb). | **(EFSA, 2020)** |
| 2017 | In the group A, there were two non-compliant samples (five non-compliant results) in group A6 reported by one Member State (one result each for AHD, AMOZ, AOZ, chloramphenicol and SEM). For antibacterials (B1), seven Member States reported a total of 19 non-compliant samples (20 noncompliant results). In the group B2, there were 54 non-compliant samples and results: 11 for anthelmintics (B2a) and 43 for NSAIDs (B2e). In the group B3, there were 11 non-compliant samples and results for mycotoxins (B3d) (all aflatoxin M1). More information on the specific substances identified and the number of non-compliant results reported by each Member State is given in Appendix A. Detected substances: AHD (1-aminohydantoin), AMOZ (5-methylmorpholino-3-amino-2-oxazolidone), AOZ (3-amino-2-oxazolidone), Chloramphenicol, SEM (semicarbazide), Ampicillin, Benzylpenicillin (Penicillin G), Cefalonium, Ciprofloxacin, Cloxacillin, Doxycycline, Florfenico, Oxytetracycline and its 4-epimer, tetracycline and its 4-epimer, Trimethoprim, Tulathromycin, Clorsulon, Closantel, Ivermectin, Levamisole, Nitroxinil, Ketotriclabendazole, Oxfendazole sulphone, Triclabendazolsulfon, Diclofen (Diclofenac), Salicylic acid, Aflatoxin M1. | **(EFSA, 2019)** |
| 2016 | Detected substances: Chloramphenicol, Hydroxymetronidazol (MNZOH), Amoxycillin, Benzylpenicillin (Penicillin G), Danofloxacin, Gentamicin, Tetracycline, Tilmicosin, Ivermectin, Triclabendazole, Diclofen (Diclofenac), Salicylic acid, 2,4-Dichlorophenoxybutyric acid, Lead, Aflatoxin M1. | **(EFSA, 2018)** |
| 2015 | Detected substances: Chloramphenicol, Amoxycillin, Benzylpenicillin (Penicillin G), Cefalexin (Cefalexin Anhydrate), Cefquinom, Cloxacillin, Enrofloxacin, Kanamycin, Spiramycin, Tetracycline, Albendazol, Closantel, Ivermectin, Diclofen (Diclofenac), Lead Pb, Aflatoxin M1. | **(EFSA, 2017)** |

## CONCLUSION

The system of monitoring of inhibitory drug residues in raw cow's milk applied in Slovakia is effective, it can detect the presence of these substances before milk is processed in dairies. The total incidence of these substances in 2020 was 0.025% of all 12,181 samples tested. A total number of 452,731 samples have been tested over the last twenty years in central testing laboratories. The number of drug residues positive samples significantly ($p$ <0.05) decrees from 299 (2001) to 3 (2020). This is because, since about 2004, rapid tests for the detection of residues of inhibitory drug substances have become more widely used in Slovakia. The system of monitoring effectively reducing the risk resulting from the occurrence of residues of inhibitory substances in raw cow's milk on human health. As it is not possible to test samples from all milk deliveries in central testing laboratories, the responsibility for control and testing remains on the dairies. Every supply of raw cow's milk is tested for the presence of residues of inhibitory substances in each dairy company in Slovakia. The role of the State Veterinary and Food Administration of the Slovak Republic is to monitor and control whether food business operators implement measures to prevent the placing on the market of milk if it contains residues of inhibitory substances. This institution annually compiles the National Plan for the Control of Residues in Live Animals and Products of Animal Origin following the applicable legislation. Only the three positive samples were detected in targeted monitoring during the last ten years.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE ROLE OF GAMBIER FILTRATE AND RED PALM OIL IN THE FORMATION OF CANNA STARCH BASED-FUNCTIONAL EDIBLE FILM

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## ABSTRACT

This study aims to analyze the role of gambier filtrate and red palm oil in the formation of functional edible film and to determine whether the gambier filtrate and red palm oil are synergistic or antagonistic in the formation of functional edible films. The study design used a factorial randomized block design with two treatment factors and each treatment consisted of three levels, namely: gambier filtrate concentration (A): 20, 30, and 40% (v/v) as well as red palm oil concentration (B): 1.2 and 3% (v/v). The observed parameters were thickness, elongation percentage, water vapor transmission rate, antioxidant activity, and antibacterial activity. Gambier filtrate and red palm oil were capable to improve the antioxidant and antibacterial properties of canna starch-based edible film. Gambier filtrate plays a role in increasing the elongation percentage, thickness, and water vapor transmission rate of edible film.

**Keywords:** antibacterial; antioxidant; edible film; gambier; canna starch

## INTRODUCTION

The oxidative reaction and pathogenic microbial contamination are the main factors for the deterioration of food products **(Lee et al., 2004)**. Food packaging is usually used in order to maintain food product quality and to increase the storage life of food products. Most of the food packaging material used currently is plastics packaging material.

In relation to awareness toward environmental deterioration due to plastics packaging waste, then packaging materials had been developed with characteristics of environmentally friendly and safe to be consumed by human-made of biopolymers or edible film **(Zhang et al., 2018)**. The main objectives of edible film application are decreasing weight loss, the danger of physical damage, improvement of appearance, color, flavor, and nutrients of products **(Franssen and Krochta, 2003)**. Edible film function can be improved by incorporating antimicrobial and antioxidant materials to protect the product from deterioration caused by microbial and oxidative deterioration which results in increasing storage life and product safety **(Rojas-Graü et al., 2006; Jung, Chung, and Lee, 2006)**.

Gambier is an extraction product from gambier plant containing catechin compound with a magnitude of 67.55 to 72.02% and gambier extract has other important compounds such as catechin gallate, gallocatechin, gallocatechin gallate, epicatechin, epicatechigallateat, epigallocatechin, epigallocatechin gallate, catechin tannat, and quercetin which have antibacterial and antioxidant characteristics. Catechin compound has weak acid (pKa 1 = 7.72 and pKa 2 = 10.22), it is less soluble in water and very unstable in the open air. It is easily oxidized at pH close to neutral (pH 6.9) and stable at lower pH (pH 2.8 and 4.9). This compound is also easily decomposed by light with a higher reaction rate at low pH (3.45) than at high pH (4.9) **(Pambayun et al., 2007)**.

Red palm oil is palm oil obtained from CPO processing yield without going through blanching process with an objective to maintain its carotenoid content. According to **Van Rooyen et al. (2008)**, red palm oil contains 500 ppm of carotenoid consisting of 37% of α-carotene, 47% of β-carotene, 11.5% of lycopene, and 6.9% of cis- α-carotene. **Bohn (2008)** had described that in addition to having functioned as free radicals catcher, some carotenoids also have vitamin A activity such as α-carotene, β-carotene, γ-carotene and β-cryptoxanthin.

Canna starch is an extraction product from tubers of the canna plant (*Canna edulis* Kerr) as one of the starch sources that have the potential to be developed. Canna starch concentration is 62.92 to 67.32% with amylose content of 21.14 – 24.44% and amylopectin content of 75.56 – 78.86%. Canna starch based-edible film [4% (b/v)] had elongation percentage in the range of 84.4 to 87.78% and water vapor transmission rate in the range of 8.52 to 11.77 $g.m^2.d^{-1}$ **(Santoso et al., 2016)**. **Santoso et al. (2019)** had added that canna starch based-composite edible film incorporated with protein extract of paddy field eel had produced elongation percentage and water vapor transmission rate of 58.84% and 18.85 $g.m^2.d^{-1}$, respectively.

The incorporation of bioactive materials that have antibacterial and antioxidant characteristics on edible film

has been widely developed. **Santoso et al. (2018)** had revealed that corn starch based-edible film incorporated with gambier extract had characteristics of antibacterial (diameter of inhibition with a magnitude of 6.67 to 7.67 mm) and antioxidant (IC50 with a magnitude of 258.14 to 469.32 ppm). This finding showed that antioxidant characteristic was classified as very weak with IC50 value much higher than 50 ppm. Therefore, it is important to add biopolymer materials having antioxidant characteristics such as red palm oil.

The research objectives were as follows to analyze the role of gambier powder extract and red palm oil in formation of functional edible film and to determine whether gambier powder extract and red palm oil have synergistic or antagonistic characteristics in the formation of functional edible film.

## Scientific hypothesis

The addition of gambier filtrate and red palm oil had a significant effect on the functional properties of the edible film with physical and chemical properties according to Japan International Standards **(JIS, 1975)**.

## MATERIAL AND METHODOLOGY

### Sample

The edible film made from biopolymer materials such as canna starch, glycerol, and CMC added with gambier filtrate and red palm oil.

### Chemical

Canna starch from CV Warung Panganku Jakarta, gambier powder extract was obtained from Indralaya traditional market, red palm oil was obtained from PT Hindoli Banyuasin, South Sumatra, bacterial culture of *Staphylococcus aureus* from Agricultural Product Chemical Laboratory, Sriwijaya University, Indonesia, 1,1 diphenyl 2 picrilhydrazil (DPPH), 6) nutrient agar (NA) media, 7) glycerol, and 8) carboxymethyl cellulose (CMC).

### Instruments

Hot plate merk Torrey Pines Scientific, analytical balance (Ohaus Corp. Pine Brook, N.J. USA), vacuum pump (model; DOA-P504-BN), NDH-200 series haze meters, Nipon Denshoku Kogyo Co Ltd, micrometer (Roch) (A281500504, Sisaku SHO Ltd, Japan), testing machine MPY (Type: PA-104-30, Ltd Tokyo, Japan), and water vapor transmission rate tester Bergerlahr.

### Laboratory Methods

Laboratory methods were used according to **Santoso et al., 2018**; **ASTM, 1997**; **AOAC, 2012**, **Laohakunjit and Noomhorm, 2004**; **Maesaroh, Kurnia and Anshori, 2018**; **Trisia, Philyria and Toemon, 2018**.

### Description of the Experiment

#### Sample preparation

Processing of gambier powder filtrate was done according to the modified procedure by **Santoso et al. (2018)**. Dry gambier is pounded until smooth by using a mortar and sieved by using 80 mesh siever. Subsequently, 20 g of fine dry gambier is taken and put into a 250 mL Erlenmeyer flask. The next step is the addition of aqua dest up to the border mark of 100 mL and heated at a temperature of 55 to 60 °C for 10 minutes while stirring by using a magnetic stirrer. Finally, the mixture was centrifuged at speed of 1000 rpm and the filtrate is taken with a magnitude of 1/3 part of the total volume.

Processing of functional edible film was done according to the modified procedure by **Santoso et al. (2018)**. Canna starch with a magnitude of 12 g is put into Beaker glass and added with aqua dest up to border mark 300 mL followed by stirring with a magnetic stirrer. Starch suspension is heated by using a hot plate at a temperature of 65 °C while stirring until perfect gelatinization is produced. The next step is the addition of aqua dest with a magnitude of 2% (v/v) from the total volume. Subsequently, gambier filtrate at concentration according to treatments [20, 30, and 40% (v/v)] and addition of CMC 1% (b/v) are put into Beaker glass gradually while constantly stirring. Red palm oil at concentration according to treatments [1, 2, and 3% (v/v)] is added and stirred until a homogenous suspension is developed. Suspension is degassed by using a vacuum pump for about one hour. Suspension with a magnitude of 40 mL is poured and leveled on a petri dish having 20 cm in diameter followed by drying within an oven at a temperature of 65 °C for 24 hours. The edible film is removed from the petri dish and put into a desiccator for one hour and finally, the edible film is ready to be analyzed.

#### Number of samples analyzed

This research used two treatment factors. Treatment factors were as follows: 1) concentration of gambier filtrate [$A_1$ = 20%, $A_2$ = 30% and $A_3$ = 40% (b/v)] and 2) concentration of red palm oil [$B_1$ = 1%, $B_2$ = 2% and $B_3$ = 3% (v/v)] with the number of samples analyzed as many as 9 samples.

#### Number of repeated analyzed

Three repetitions for each treatment factor were performed. The total amount of analyzed samples was 27.

## Statistical Analysis

This research used a factorial completely randomized design. Treatments that have a significant effect were further tested by using honestly significant different (HSD) test at $\alpha$ = 5%. Data were analyzed by using the software program of SAS version Windows 9 in terms of analysis of variance.

## RESULTS AND DISCUSSION

### Thickness

The average thickness of the edible film was in the range of
0.133 to 0.210 mm. Treatment of 40% concentration gambier filtrate (v/v) and 3% concentration red palm oil (v/v) had the highest thickness with a magnitude of 0.210 mm, whereas the lowest thickness was found on treatment of 20% concentration gambier filtrate (v/v) and 1% concentration red palm oil (v/v) with magnitude of 0.133 mm. The thickness of the edible film was higher than the edible film based on the starch of durian and jackfruit seeds, which were 0.032 – 0.041 mm and 0.035 – 0.043 mm, respectively **(Wahidin et al., 2021)**. Chitosan edible film incorporated with essential oil from *Chrysanthemum morifolium* and tumeric incorporated alginate edible film has a lower thickness than the resulting edible film, which were 0.05 – 0.15 mm and 0.096 mm, respectively **(Tan et al., 2021; Bojorges et al., 2020)**. The thickness of the

edible film is the same as the edible film thickness of canna starch which was incorporated with the gambier extract, which is 0.143 mm **(Santoso et al., 2019)** and is lower than the edible based on taro tuber starch combined with galangal essential oil, which is 0.3 mm **(Handayani and Nurzanah, 2018)**. The average thickness value of the edible film was shown in Figure 1.

Analysis of variance results showed that treatments of gambier filtrate and red palm oil had a significant effect on the thickness of the functional edible film, whereas interaction of both factors had no significant effect. Results of the HSD test at $\alpha$ = 5% for gambier filtrate treatment were presented in Table 1and Table 2.

HSD test (Table 1) showed that the higher the gambier filtrate concentration, the thicker is the functional edible film. This is due to the fact that gambier filtrate contains catechin compounds which have crystalline form at dry conditions. This crystalline form is a solid that affect edible film thickness, i.e. the higher the total solid content within the edible film matrix, the higher is the edible film thickness. **Santoso et al (2016)** reported that the addition of gambier extract had a significant effect on increasing the thickness of the canna starch-based edible film.

Table 2 (HSD test) showed that the higher the red palm oil concentration, the higher the thickness of the edible film. This is due to the fact that red palm oil contains 44% of palmitic acid which is in solid form at room temperature and has white color. Therefore, the higher the red palm oil concentration, the higher is the thickness of edible film because palmitic acid has an effect on the increase of total solid content within the edible film matrix. **Praseptiangga et al. (2017)** revealed that the incorporation of palmitic acid in the composite semi-refined iota carrageenan-based edible film caused a significant effect increase in thickness.

The role of gambier filtrate and red palm oil significantly increased the edible film thickness, but no synergistic or antagonistic role in interaction treatment of gambier filtrate and red palm oil on the increase of edible film thickness. This can be described by the fact that complex bonds will develop amongst starch-glycerol-gambier filtrate-CMC-red palm oil within an edible film matrix. Catechin compound within gambier filtrate binds to glycerol molecules, starch, and CMC resulting in a low concentration of free catechin compound. In a similar fashion, red palm oil which binds to CMC also decreases the numbers of free palmitic acid within the edible film matrix. The existence of complex bonds which occurred within the edible film matrix causes the effect of catechin compound and red palm oil on edible film thickness to cancel each other out.

**Elongation Percentage**

The elongation percentage value of the functional edible film was in the range of 58.67 to 136%. The treatment of 20% (v/v) gambier filtrate and 3% (v/v) red palm oil had the lowest elongation percentage, whereas treatment of 40% (v/v) gambier filtrate and 1% (v/v) red palm oil had the highest elongation percentage. The results of this study were higher than the edible films based on alginate, teff-starch, and pumpkin, namely 27.67 – 43.57%, 33.12%, and 13.13-14.47%, respectively **(Lim, Tan and Pui, 2021; Prabhu et al., 2021; Lalnunthari, Devi, and Badwaik, 2020)**. The elongation percentage of this edible film is similar to the results of research by **Kim, Seo and Kim (2018)** and lower than that reported by **Sancakli et al. (2021)**, namely 132% and 258.41%, respectively. AThe average value of elongation percentage for the edible film was shown in Figure 2.

The average value of elongation percentage for the edible film had met the **JIS (1975)** standard with a minimum value of 70%. This was found on treatments of $A_2B_1$, $A_2B_2$, $A_2B_3$, $A_3B_1$, $A_3B_2$ and $A_3B_3$. The elongation percentage of canna starch-based-edible film was better than that of corn starch-based-edible film in which both were incorporated with gambier filtrate. **Santoso et al. (2018)** had described that corn starch containing high amylose as a raw material of edible film incorporated with gambier filtrate had produced low elongation percentage values in the range of 13.33 to 16.67%. This is primarily related to the composition of amylose and amylopectin within corn starch. It is known that amylopectin molecules had an effect on elasticity increment of edible film and the opposite happened to amylose molecules.

Analysis of variance results showed that treatments interaction of red palm oil and gambier filtrate had no significant effect on elongation percentage of the edible film, whereas gambier filtrate treatments had a significant effect on elongation percentage of edible film. Results of HSD test at $\alpha$ = 5% for gambier filtrate treatment related to elongation percentage of edible film was presented in Table 1.

HSD test (Table 1) showed that the higher the gambier filtrate concentration, the higher is the elongation percentage of functional edible film. It is known that gambier filtrate contains a catechin compound with a hydroxyl group (OH) as its active group. The higher the gambier filtrate concentration, the higher is the numbers of OH group that is capable to bind free water within matrix of edible film. According to **Santoso et al. (2017)**, the use of extract gambier in edible film formulation has a function to increase the elasticity of edible film because the catechin compound has a hydroxyl group that is capable to bind free water within the matrix of edible film.

**Water Vapor Transmission Rate**

The water vapor transmission rate of the edible film was in the range of 26.03 to 26.8 $g.m^2.d^{-1}$ and it did not fulfill the **JIS (1975)** standard (maximum of 10 $g.m^2.d^{-1}$). The edible film with the highest water vapor transmission rate was found on the $A_3B_1$ treatment (26.03 $g.m^2.d^{-1}$) and the lowest one was found on the $A_1B_3$ treatment (26.80 $g.m^2.d^{-1}$). The value of the vapor transmission rate of edible film produced was higher than the carrageenan-based edible film with added sorbitol of 6.83 $g.m^2.d^{-1}$ **(Rahmawati, Arief and Satyantini, 2019)**, gelatin edible films incorporated with casein phosphopeptides were 4.5 – 7.9 $g.m^2.d^{-1}$ **(Khedri et al., 2021)**, and carboxymethyl chitosan-pullulan edible films enriched with galangal essential oil of 0.185 – 0.290 $g.m^2.d^{-1}$ **(Zhou et al., 2021)**. The results of this study were lower than those reported by **Li et al. (2021)**, namely in the amount of 31.10 – 38.02 $g.m^2.d^{-1}$. Average value of water vapor transmission rate for the edible film was presented in Figure 3.

Analysis of variance results showed that gambier filtrate treatment had a significant effect on water vapor transmission rate, whereas red palm oil treatment and treatment interaction of gambier filtrate and red palm oil had no significant effect. Results of the HSD test related to gambier filtrate treatment toward water vapor transmission rate were shown in Table 1.

**Table 1** HSD test ($\alpha$ = 5%) related to concentration of gambier filtrate treatment on thickness, elongation percentage, water vapor transmission rate, antioxidant activity and antibacterial activity of functional edible film.

| Treatment | Thickness (mm) | Elongation percentage (%) | Water vapor transmission rate ($g.m^2.d^{-1}$) | Antioxidant activity ($IC_{50}$ ppm) | Antibacterial activity (inhibition diameter, mm) |
|---|---|---|---|---|---|
| $A_1$ (20%) | 0.154 ±0.025a | 65.11 ±6.05a | 26.19 ±0.21a | 196.60 ±3.33a | 2.19 ±0.20a |
| $A_2$ (30%) | 0.172 ±0.023b | 91.56 ±7.67b | 26.52 ±0.20b | 190.06 ±2.40b | 2.69 ±0.49b |
| $A_3$ (40%) | 0.199 ±0.015c | 127.33 ±8.35c | 26.66 ±0.17c | 182.04 ±6.69c | 3.18 ±0.47c |

Note: Numbers followed by the letters in the same columns are not significantly different.

**Table 2** HSD test ($\alpha$ = 5%) related to concentration of red palm oil treatment on antioxidant activity and antibacterial activity of functional edible film.

| Concentration of red palm oil (%) | Antioxidant activity ($IC_{50}$ ppm) | Antibacterial activity (inhibition diameter, mm) |
|---|---|---|
| $B_1$ (1%) | 194.13 ±5.64a | 3.08 ±0.62a |
| $B_2$ (2%) | 188.49 ±7.45b | 2.67 ±0.52b |
| $B_3$ (3%) | 186.09 ±9.09c | 2.31 ±0.35c |

Note: Numbers followed by the letters in the same columns are not significantly different.

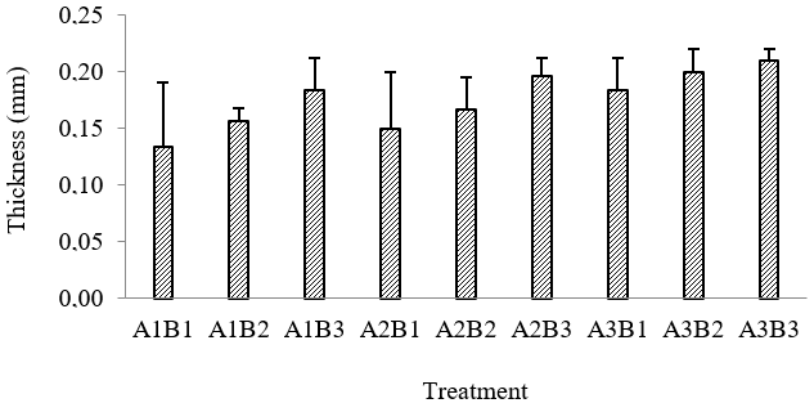


**Figure 1** Average thickness value of functional edible film.

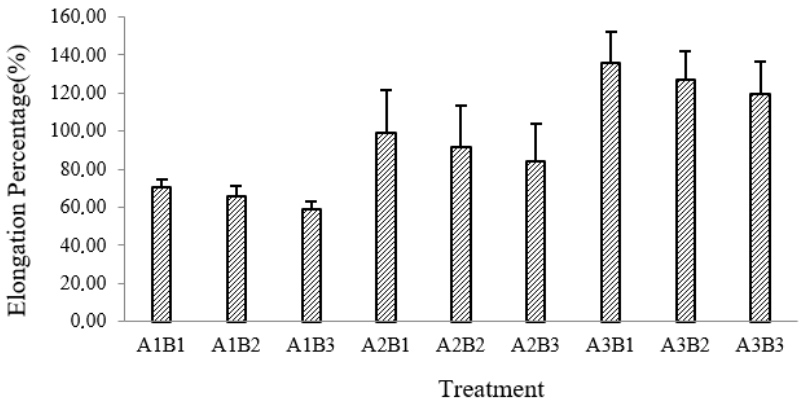


**Figure 2** Average value of elongation percentage of functional edible film.

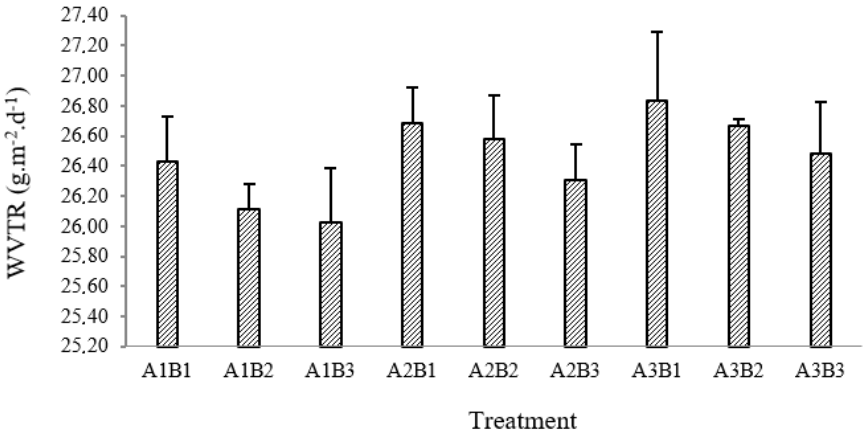


**Figure 3** Average value of water vapor transmission rate of functional edible film.

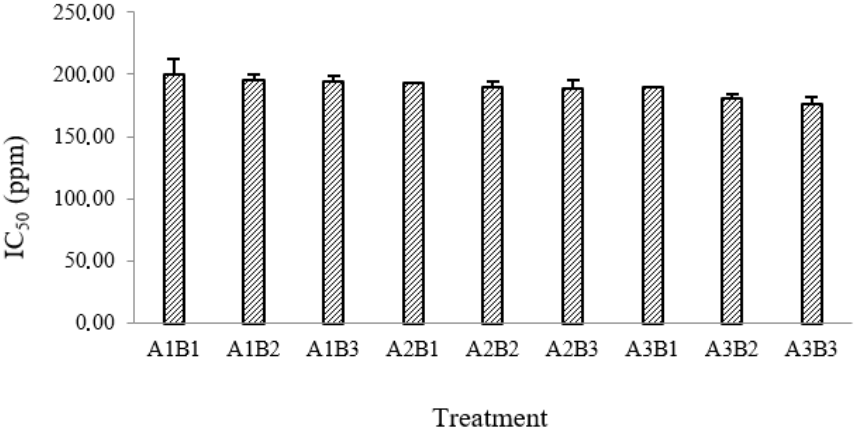


**Figure 4** Average value of $IC_{50}$ of functional edible film.

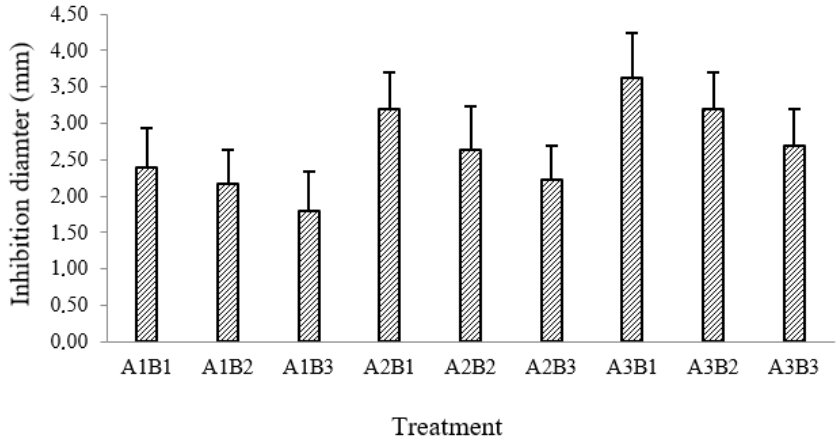


**Figure 5** Average value of inhibition diameter of functional edible film functional.

Results of the HSD test at $\alpha$ = 5% (Table 1) showed that the higher the concentration of gambier filtrate, the higher is the water vapor transmission rate. This is due to the fact that gambier filtrate decreases inter-molecular and trans-molecular interactions of starch, increases free space amongst starch chains, and increases polymer mobility. In addition, the high value of water vapor transmission rate is due to a hydrophilic group within gambier filtrate that will decrease matrix density of edible film resulting in free space within film matrix which facilitates water vapor diffusion. **Santoso et al. (2018)** had described that gambier filtrate addition to the formulation of corn starch-based-edible film had increased water vapor transmission rate. This is related to catechin compound content within gambier filtrate. It is known that the catechin compound contains a hydroxyl group (OH) having the hydrophilic characteristic.

**Antioxidant Activity**

The average value of $IC_{50}$ for the edible film was in the range of 176.11 to 200.34 ppm. $A_1B_1$ treatment had produced the highest $IC_{50}$ and the lowest one was found on $A_3B_3$ treatment. These antioxidant activity values were classified as weak because the $IC_{50}$ value was higher than 50 ppm. The $IC_{50}$ values in this research were lower than the study results by **Santoso et al. (2018)** using canna starch-based-edible film with $IC_{50}$ of 258.14 – 469.32 ppm. These research results were higher than the study results by **Yerramathi et al. (2021)** with $IC_{50}$ of 50.42 – 77.41 ppm and **Jiang et al. (2021)** with $IC_{50}$ of 87.41 ppm. The average value of $IC_{50}$ for the edible film was presented in Figure 4.

Analysis of variance results showed that treatments of gambier filtrate and red palm oil had a significant effect on antioxidant activity of functional edible film, whereas interaction of both factors had no significant effect on antioxidant activity of functional edible film. Results of HSD test at $\alpha$ = 5% for gambier filtrate and red palm oil treatments were presented in Table 1 and Table 2, respectively.

Results of the HSD test (Table 1) showed that the higher the gambier filtrate concentration, the lower is the $IC_{50}$ value.

It means that the antioxidant property of functional edible film will increase with the increase of gambier filtrate concentration. It is known that gambier filtrate contains epigallocatechin gallate compound and the addition of this compound in the sodium alginate and CMC-based edible films increases antioxidant activity **(Ruan et al., 2019)**. Table 2 (HSD test) showed that the higher the red palm oil concentration, the lower is the $IC_{50}$ value, or the higher the red palm oil concentration, the higher is the antioxidant property of the edible film. According to **Goon et al. (2019)**, red palm oil contains such as tocotrienol, tocopherol, and carotenes.

**Antibacterial Activity**

Results of antibacterial activity on functional edible film produced a diameter of inhibition in the range of 2 to 3.6 mm. $A_3B_1$ treatment had the highest value of inhibition diameter with a magnitude of 3.6 mm and $A_1B_3$ treatment had the lowest value of inhibition diameter with a magnitude of 2 mm. The inhibition diameter value of functional edible film produced in this research was classified as a low antibacterial activity because it was less than 5 mm. The results of this study are lower than that reported by **Çakmak et al. (2020)** and **Sharma, Dhanjal, and Mittal (2017)** in a research on whey protein-based edible film which was incorporated with essential oil and cinnamon oil, namely 5.5 mm and 13.37 mm, respectively. Compared to the research of **Dinika et al (2020)** on starch-based edible film added with papaya leaf extract, it was also lower. The antibacterial activity of this edible film was similar to that produced by **Zerihun et al. (2016)** in a study of banana peel edible film incorporated with ginger. The average value of inhibition diameter for the functional edible film was presented in Figure 5.

Analysis of variance results showed that treatments of gambier filtrate and red palm oil had a significant effect on antibacterial of functional edible film, whereas interaction of both factors had no significant effect on antibacterial activity of functional edible film. Results of HSD test at $\alpha$ = 5% for gambier filtrate and red palm oil treatments toward inhibitory power diameter of the edible film were presented in Table 1 and Table 2, respectively.

Table 1 (HSD test) showed that the higher the gambier filtrate concentration, the higher is the inhibition diameter. It means that the antibacterial property of functional edible film will increase with the increase of gambier filtrate concentration. It is described previously that gambier filtrate contains catechin compound. According to **Ma et al. (2019)**, catechin compound not only has antioxidant property but also has aan ntibacterial property. Results of the HSD test (Table 2) showed that the higher the red palm oil concentration, the higher is the antibacterial property of the edible film. It is known that red palm oil has hydrophobic or non-polar properties so that bacteria are incapable to grow and develop because food-destroying bacteria usually have hydrophilic characteristics.

## CONCLUSION

The addition of the gambier filtrate and red palm oil can form an edible film with antibacterial and antioxidant properties, the inhibition diameter value was 2,0 – 3.6 mm and the $IC_{50}$ value was 176.11 – 200.34 ppm, respectively. They have a role in increasing the elongation percentage, thickness, and water vapor transmission rate of edible film, but the water vapor transmission rate of the edible film has not yet been capable to fulfill the JIS (1975) standard.

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This article does not contain any studies that would require an ethical statement.

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# AN IMPROVED METHOD FOR DETERMINING THE MASS FRACTION OF CALCIUM CARBONATE IN THE CARBONATE BEDROCK

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## ABSTRACT

In the article it is offered to enter in the technological audit of the lime department of sugar factory the adjusted technique of the definition of the maintenance of calcium carbonate in carbonate breed. For this purpose, a complete chemical analysis of limestone was performed, which includes determination of moisture content, impurities insoluble in hydrochloric acid, the amount of one and a half oxides of aluminum and iron, calcium carbonate (advanced method), and magnesium carbonate, calcium sulfate, alkali metal oxides, potassium, and sodium. The obtained experimental data are summarized in one table and the material balance of all components of carbonate bedrock is summarized. The proposed method made it possible to obtain objective data on the component composition of the carbonate material. This, in turn, avoids many technological problems, namely to reduce the formation of melts in the lime kiln, improve the filtration of juices, increase the ability of lime to chemically interact with water, reduce the volume of water on the juicer etc. Thus, the use of the recommended method for determining calcium carbonate ($CaCO_3$), as part of the technological audit, will allow early adjustment of the process, which will give maximum energy and resource savings, as well as increase the level of environmental friendliness of the enterprise.

**Keywords:** limestone; carbonate bedrock; calcium carbonate; lime separation; sugar industry

## INTRODUCTION

In the sugar industry of Ukraine, calcium hydroxide [$Ca(OH)_2$] and carbon dioxide ($CO_2$) are mainly used to purify diffusion juice from non-sugars **(Zheplinska et al., 2020)**. These components can be obtained by firing carbonate rocks and subsequent quenching of lime (CaO), obtained by dissociation of calcium carbonate ($CaCO_3$). Limestone is a sedimentary rock consisting mainly of calcite and impurities **(Zheplinska et al., 2019)**. The chemical composition of pure limestone is close to calcite, where CaO – 56% and $CO_2$ – 44%. As is known, limestone is an exhaustible resource, its deposits are limited and make up 19 – 22% of the total mass of sedimentary rocks **(Sheiko et al., 2019)**.

In Ukraine, limestones with a $CaCO_3$ content of 93.00% and impurities with a mass fraction are allowed for use in sugar production:

- substances that are insoluble in hydrochloric acid not more than 3.00%;
- oxides of aluminum and iron in the amount not exceeding 1.50%;
- magnesium carbonate not more than 2.50%;
- calcium sulfate not more than 0.40%;
- alkali metal oxides of potassium and sodium in the amount not exceeding 0.25%;
- impurities (clay, etc.) not more than 3.00% **DSTU 1451-96 (1997)**.

Despite the seemingly high condition of limestone, many problems often arise in practice: the formation of melts in the lime kiln, which is the irreversible loss of lime; formation of sediments on the heating surface of the evaporator; saturating juices are poorly filtered; the stability of the furnace lining is reduced; the rate of chemical interaction of CaO with water decreases, so the quenching process is significantly slowed down; the volume of molasses increases; the volume of water on the juicer increases, etc **(Alves et al., 2017)**. All the above technological problems lead to an increase in the cost of lime on the machine juicer and its irreversible losses in the lime department **(Palamarchuk et al., 2019)**. To provide the required amount of CaO, it is necessary to increase the use of limestone, which will increase the amount of fuel used for its firing and increase carbon dioxide emissions into the atmosphere, which worsens the ecological situation of the planet.

One of the primary causes of the above problems is the lack of a correct technological audit of the lime department, which includes the use of correct methods of control of technological parameters **(Mushtruk et al., 2020)**. One of these methods, which is part of the audit of the limestone department, namely the method of determining the mass

fraction of calcium carbonate in the carbonate rock, will be presented in this article **(Bhatia et al., 2016)**.

Each technique currently used in practice in the technological flow implies that it should be as accurate as possible, easy to use, and affordable. Often researchers omit some of the points of the above requirements and to date, there is very little research in this area. In particular, there is a method of determining the mass fraction of calcium carbonate in the carbonate rock **DSTU 1451-96 (1997)**, which is based on the acidimetric determination of the mass fraction of calcium carbonate in the carbonate rock by calculating the percentage of Trilon B solution, which went to titrate the calcium content. Other authors presented a method **(Zheplinska et al., 2021)**, which involves analysis by a complexometric method. At the same time, the proposed methods do not provide the high accuracy of the results of determinations.

There are modern techniques that can be used to simultaneously determine the isotopic ratios of calcium and magnesium from a single aliquot of biological and geological samples **(Somaratne et al., 2019)**. However, for the needs of a sugar factory, it is necessary to know the concentration of $CaCO_3$ in the carbonate rock, and not the isotopes of calcium and magnesium separately. As in the carbonate rock, there are impurities of calcium sulfate, which negatively affect the technological process, the isotopes of calcium from this component will give an error in estimating the content of $CaCO_3$. Also, this method is highly costly for ongoing control.

Therefore, the audit of the lime department, which includes the use of an adjusted method for the determination of calcium carbonate in limestone, will lead to the fact that the technologist will be able to choose in advance the most effective way to conduct the process. In turn, this will not only ensure energy and resource savings in sugar production but also reduce emissions.

The task of our research was to improve the method of determining the mass fraction of calcium carbonate in the carbonate rock, which will increase the accuracy of the results and reduce its cost.

## Scientific hypothesis

To increase the accuracy of experimental data, the improved method should be based on the material balance of all components of the carbonate rock. This will make it possible to self-check the accuracy of the results obtained, as having summed up the balance of all components of limestone, approximately we should get a result of 100%. Given this fact and improving the method of determining the content of calcium carbonate in the carbonate rock and entering the results into the material balance, we hope to get the sum of all components approximately 100%, which will indicate the accuracy of the data.

## MATERIAL AND METHODOLOGY

### Samples

Carbonate rocks of ten different deposits were used for experiments, namely with (Bogdanivsky, Hlynsko-Rozbyshivsky, Malodivytsky, Milkivsky, Kybyntsivsky, Pryluky, Rybalsky, Pivdenno-Panasivsky, Velykobubnivsky, Sukhodolivsky).

### Chemicals

Distilled water (producer «Inter-Synthesis» Limited Liability Company, Ukraine).

Hydrochloric acid (HCl, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Sulfuric acid ($H_2SO_4$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Nitric acid ($HNO_3$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Ammonia ($NH_3$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Sodium (Na, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Ethyl alcohol ($C_2H_5OH$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Indicator acid chromium dark blue ($C_{16}H_9ClN_2Na_2O_9S_2$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Dry murexide indicator ($C_8H_8N_6O_6$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Trilon B ($C_{10}H_{14}N_2Na_2O_8$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Ammonium carbonate ($(NH_4)_2CO_3$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

### Instruments

Analytical scales (BTHE-6-H1K-1, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Drying cabinet (DC-300, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Desiccator (EximLab 150, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Chemical cups (CC-100, CC-150, CC-200, CC-250, CC-500, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Petri dish (producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Measuring flasks (MF-100, MF-150, MF-200, MF -250, MF-500, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Muffle furnace (SNOL 8,2/1100, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Measuring pipettes (MP-0,001, MP-0,002, MP-0,005, MP-0,01, MP-0,015, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Conical flask (CF-100, CF-150, CF-200, CF-250, CF -500, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Burette for titration (producer "Laboratory equipment" Limited Liability Company, Ukraine).

Filters (producer "Laboratory equipment" Limited Liability Company, Ukraine).

Photometer (eXact® Micro 20, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

### Laboratory Methods

For technological evaluation of carbonate rocks, their complete chemical analysis was performed, which includes the determination of mass fractions of calcium carbonate ($CaCO_3$); impurities insoluble in hydrochloric acid ($SiO_2$);

sums of one and a half oxides of aluminum and iron ($Fe_2O_3 + AI_2O_3$); magnesium carbonate ($MgCO_3$); calcium sulfate ($CaSO_4$); alkali metal oxides of potassium and sodium ($K_2O + Na_2O$); moisture content.

The content of impurities insoluble in hydrochloric acid, the amount of one and a half oxides of aluminum and iron, and calcium sulfate were determined by weight. Calcium and magnesium carbonates – by the method of complexometric titration with Trilon B. The content of alkali metal oxides of potassium and sodium – by the photometric method.

Determination of moisture content was performed as follows: 5 g of limestone from the sample preparation for analysis was weighed on analytical balances in a dry pre-weighed beaker. Then the beaker with the sample, without closing the lid, was placed in an oven and kept for 3 hours at a temperature of 105 – 110 °C. The beaker was then capped, removed from the oven, and placed in a desiccator for 20 minutes to cool, then weighed. The portion was dried to constant weight until the difference between the results of the two weighings was not more than 0.001 g.

The ability of silicates to dissolve under the action of strong concentrated acids was used to determine the content of impurities insoluble in hydrochloric acid. The obtained precipitate of silicic acids was calcined at a temperature of 1000 °C to constant weight and weighed. At high temperatures, silicic acids lose water and turn into silicon dioxide.

One and a half oxides of aluminum and iron after separation of silicic acids were precipitated in the form of hydroxides with concentrated ammonia solution at pH = 5.5, which corresponds to the isoelectric point of colloidal solutions of $Al(OH)_3$ and $Fe(OH)_3$. Next, the resulting precipitate was calcined at a temperature of 900 °C and weighed. In the process of calcination, hydroxides lose water and turn into oxides.

After the separation of silicic acids, the content of calcium sulfate was determined by adding barium chloride to the solution. The precipitate was washed with cold water until a negative reaction to the chlorine ion. The resulting precipitate was burned to constant weight in a muffle furnace at a temperature of 850 – 900 °C and weighed.

The complexometric method for the determination of magnesium ions was carried out using the disodium salt of ethylenediaminetetraacetic acid (Trilon B), which forms with this ion strong colorless complex compounds. The content of magnesium ions was determined in the presence of an indicator of dark blue acid chromium in ammonia buffer.

**Description of the Experiment**

All measurements of instrument readings were performed 5 times. The number of repetitions of each experiment to determine one value was also 5 times.

## Statistical 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 2019 values were estimated using mean and standard deviations.

## RESULTS AND DISCUSSION

The results (Figure 1) obtained by repeating the experiment twice show that the moisture content in all samples was not significant. Only in sample no. 6 a jump in moisture, concentration was observed, which is explained by the fact that chalk-like limestone was analyzed. Cretaceous limestones are characterized by increased hygroscopicity. The authors of scientific works **(Buniowska et al., 2017; Verma et al., 2018; Shmyrin, Kanyugina and Kuznetsov, 2017)** engaged in similar research but obtained other scientific results. The presence of moisture in limestone has almost no effect on the firing process. However, if the moisture concentration significantly exceeds the norm, it will increase fuel consumption and reduce the concentration of $CO_2$ in the furnace gas. The last component ($CO_2$) is very important in the technological process of sugar production. The authors of scientific works **(Nadeem et al., 2018; Avalos-Llano, Molina and Sgroppo, 2020; Rahimi et al., 2020)** do not believe that the concentration ($CO_2$) is not an important factor in the technological process of sugar production.

Therefore, all samples of the studied limestones contained the normative concentration of moisture, which indicates that this indicator will not adversely affect the firing process of carbonate rocks. The next test component was impurities that are insoluble in hydrochloric acid. The standard concentration of these impurities in the carbonate rock for Ukrainian plants is not more than 3%. The authors of the following scientific papers **(Dalfré Filho, Assis and Genovez, 2015; Luo et al., 2019; Dhar et al., 2015)** other methods for determining impurities were used for similar scientific research. According to the results of experiments (Figure 2) we observe that samples no. 2, 3, and 7 have an inflated content of the investigated component of the carbonate rock.

Given that during the firing of limestone $SiO_2$ can form silicates of calcium and magnesium, which is an irreversible loss of limestone, the use of these samples in the process is not recommended. Besides, silicates contribute to the formation of sediment on the heating surface of the evaporator. The authors of scientific works **(Guo et al., 2018; Ozerov and Sapronov, 1985; Jiang, 2015; Almohammed et al., 2015)** recommend not to use such samples in the production process.

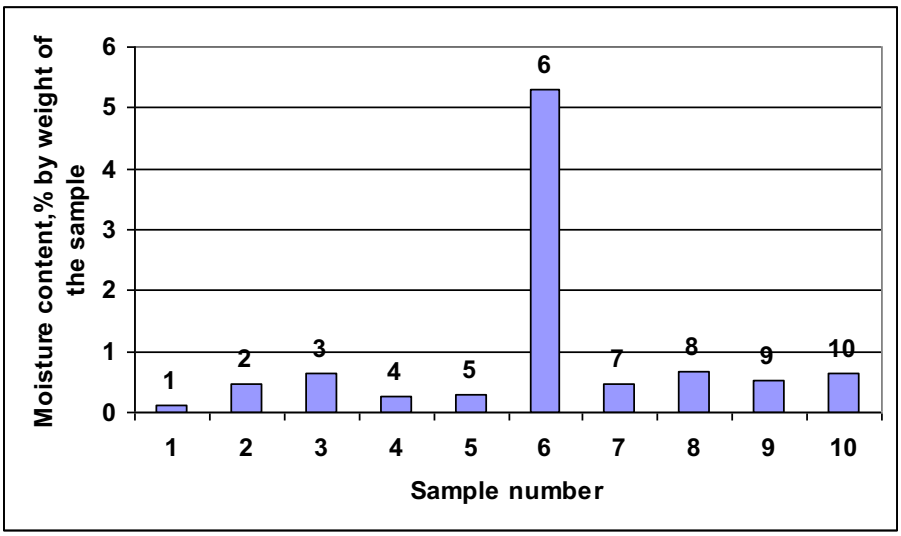


**Figure 1** Moisture content in the studied samples of carbonate bedrocks.

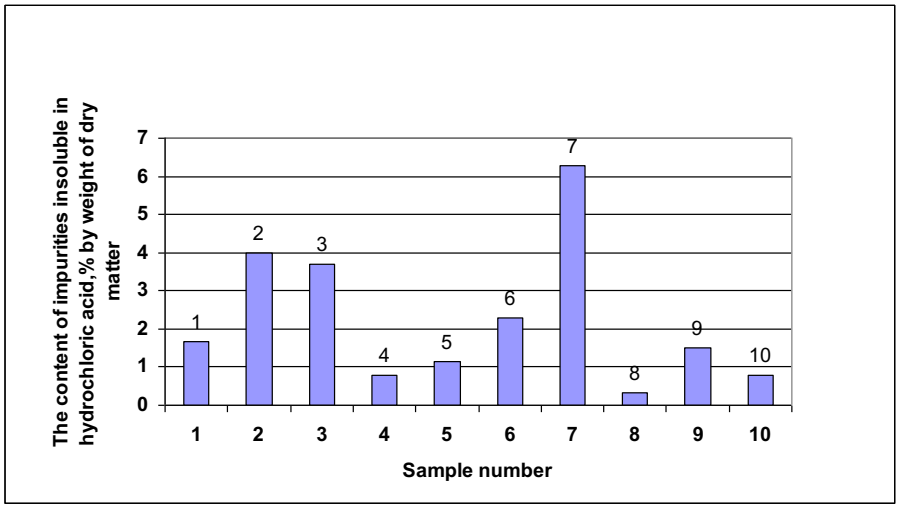


**Figure 2** The content of impurities insoluble in hydrochloric acid in the studied samples of carbonate rocks.

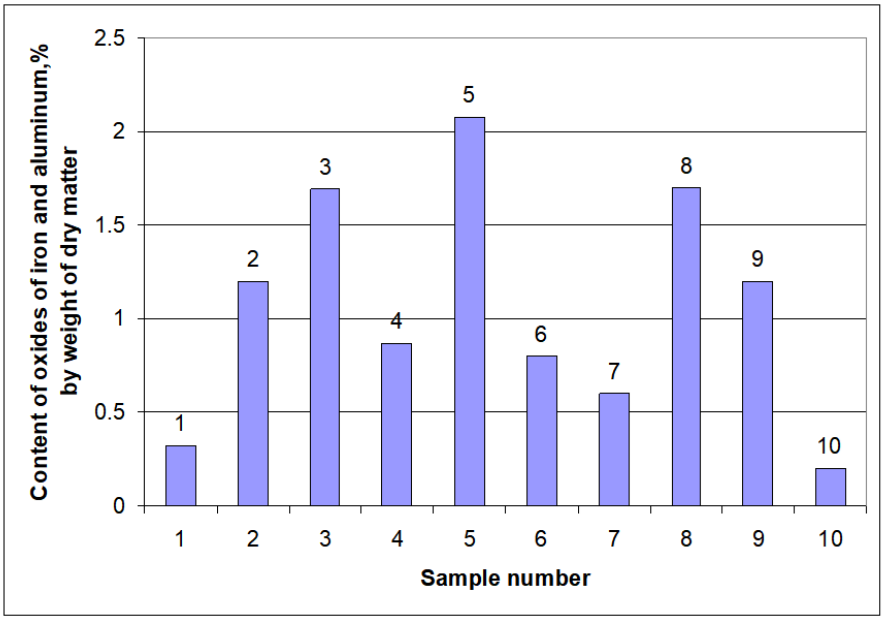


**Figure 3** The content of iron oxide and aluminum oxide in the studied samples of carbonate rocks.

Carbonate rocks were analyzed for their content of iron and aluminum oxides. Since this component (both $AI_2O_3$ and $Fe_2O_3$) harms the filtration rates of saturating juices, and iron compounds reduce the stability of the lining and slow down the process of slaking lime, control over its content is necessary. The content of the sum of oxides of aluminum and iron in the carbonate rock according to **DSTU 1451-96 (1997)** should not exceed 1.5%. As the results of research (Figure 3) show, samples no. 3, 5, and 8 exceed the recommended norms, so their use in the technological process will lead to a number of the above negative consequences. The authors of the following scientific works **(Johnson, Zhou, and Wangersky, 1986; Lee et al., 2012; Lebovka et al., 2007)** recommend not to use such samples in the production process.

Control of the content of calcium sulfate in the carbonate rock should be carried out to prevent the formation of scale on the surface of the evaporator and slow down the hydration of lime. The mass fraction of calcium sulfate according to the recommendations **DSTU 1451-96 (1997)** should not exceed 0.4%. Figure 4 shows that samples no. 2, 7 and 8 cannot be recommended for use in the sugar industry. In subsequent scientific works **(Katariya, Arya and Pandit, 2020; Kim et al., 2016; Kozelová et al., 2011; Krasulya et al., 2016)** such studies were not conducted.

Determination of calcium carbonate content in the carbonate rock was carried out as follows. Pipette 0.15 $m^3$ of solution into a 2.50 $m^3$ porcelain cup or conical flask, add 1 $m^3$ of distilled water and 0.25 $m^3$ of sodium hydroxide solution with a mass fraction of 20% from a volumetric flask containing the filtrate obtained after precipitation of iron and aluminum oxides. After 1 – 2 minutes on the tip of a spatula made from 0.1 to 0.2 kg of murexide in a mixture with potassium chloride. The solution was stirred and titrated with 0.1 M solution of Trilon B until the transition from red to purple color, observed on a black background. Water was monitored by titration of the blank. The authors of scientific works **(Dehghannya et al., 2018; Moser et al., 2017; Zhu et al., 2016; Zdziennicka et al., 2017)** used other standardized methods to conduct similar studies.

Our proposed method **(Noguiera Felix et al., 2019)** differs from the method presented in **DSTU 1451-96 (1997)** in that the filtrate after precipitation of iron and aluminum oxides was additionally introduced murexide in a mixture with potassium chloride and then titrated with 0.1 M solution of Trilon B to the transition from red to violet color, and the mass fraction of calcium carbonate in the carbonate rock was calculated with a factor of 0.005008.

Thus, the mass fraction of calcium carbonate ($CaCO_3$) in the carbonate rock (based on the dry matter) (X) in percent was calculated by the formula (1):

$$X = \left( \frac{B \cdot K \cdot 0.005008 \cdot 100}{m} - 0.735 \cdot a \right) \cdot \frac{100}{100 - W} \quad (1)$$

Where:

B – is the volume of 0.1 M solution of Trilon B, which went to the titration of calcium, $m^3$;

K – is the correction factor for the solution of Trilon B;

0.005008 – the mass of calcium carbonate corresponding to 0.01 $m^3$ of a solution of Trilon B with a concentration of 0.1 $mol.m^{-3}$, kg;

a – is the mass fraction of calcium sulfate in limestone,%;

0.735 – conversion factor of calcium sulfate to calcium carbonate;

m – is a sample of limestone in the amount of 0.15 kg;

W – it is the mass fraction of moisture in percent.

The results of determining the mass fraction of calcium carbonate in the carbonate rock are shown in Table 1 and Figure 5.

Based on Table 1 and Table 2, all analyzed samples, which were carried out according to the method **DSTU 1451-96 (1997)**, have slightly inflated results. Therefore, an adjusted technique was proposed that gives more accurate results.

According to Figure 5, we see that the concentration of calcium carbonate in samples no. 2, 3, 5, 6, and 7 does not reach the recommended **DSTU 1451-96 (1997)**. Therefore, the use of such limestones is economically and technologically unprofitable.

The share of magnesium carbonate in carbonate rocks should not exceed 2.5%. Since the excess of this impurity contributes to the deterioration of the filtration rates of juices. From Figure 6 we observe that samples no. 2, 3, 5, 6, and 8 do not meet the recommended standards. It is also observed that the indicators obtained by the method **DSTU 1451-96 (1997)** are slightly inflated (Table 2). Therefore, this method of determining magnesium carbonate in carbonate rocks requires clarification, which will be discussed in the next article. The author of **(Sasikumar, Chutia and Deka, 2019)** used other standardized methods to conduct similar studies.

Alkali metal oxides of potassium and sodium also attract a lot of attention and should not exceed 0.25% **DSTU 1451-96 (1997)**. K + and Na + ions are active molasses-forming agents. Therefore, control over their concentration must be carried out. From Figure 7 it is seen that all samples corresponded to the normative concentration.

To provide an objective assessment of limestones, in terms of their technological condition, it is not enough to consider their individual components. It is necessary to consider a comprehensive complete chemical composition of carbonate rocks and only then provide certain technological characteristics of limestone. For this purpose, all the obtained data were summarized (Table 2) and the balance of all components of carbonate rocks was summed up. When determining the chemical composition of the carbonate rock, the sum of the mass fractions of impurities and calcium carbonate in the rock should be approximately 100%. According to the results of the final calculations, the total sum of the components of carbonate rocks in all samples according to the method **DSTU 1451-96 (1997)** gives slightly inflated results, the data obtained by the proposed method are almost close to 100%. Therefore, our proposed method for determining the mass fraction of calcium carbonate in the carbonate rock **(Cervantes-Elizarrarás et al., 2017)** which is based on the material balance makes it possible to most accurately assess the chemical composition of the carbonate rock.

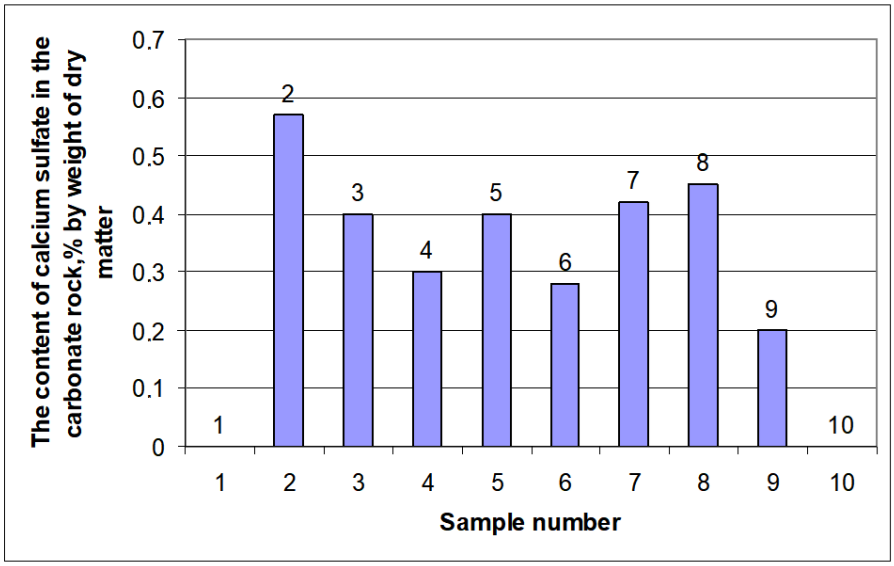


**Figure 4** The content of calcium sulfate in the studied samples of carbonate rocks.

**Table 1** The content of calcium sulfate in samples.

| Sample number | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | |
|---|---|---|---|---|
| | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| Sample no. 1 | 193.22 | 193.41 | 96.70 | 96.78 |
| Sample no. 2 | 179.16 | 180.06 | 89.42 | 89.87 |
| Sample no. 3 | 178.18 | 179.25 | 89.02 | 89.56 |
| Sample no. 4 | 193.80 | 194.29 | 96.88 | 97.12 |
| Sample no. 5 | 183.99 | 184.54 | 91.92 | 91.20 |
| Sample no. 6 | 173.4 | 183.11 | 86.66 | 91.51 |
| Sample no. 7 | 179.37 | 180.27 | 89.60 | 90.05 |
| Sample no. 8 | 186.12 | 187.36 | 92.95 | 93.57 |
| Sample no. 9 | 190.0 | 190.25 | 94.83 | 95.30 |
| Sample no. 10 | 195.16 | 196.33 | 97.61 | 98.20 |

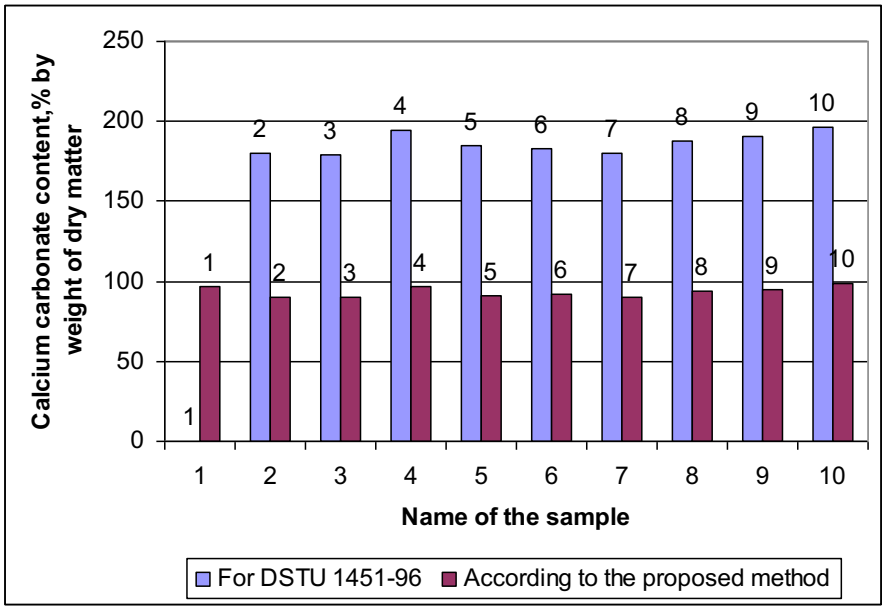


**Figure 5** The content of calcium carbonate in the studied samples of carbonate rocks.

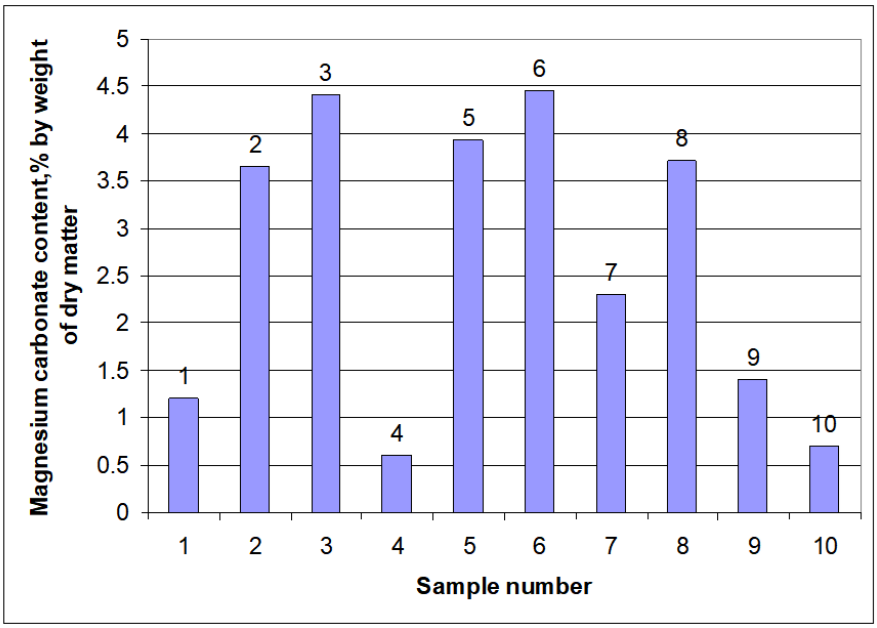


**Figure 6** The content of magnesium carbonate in the studied samples of carbonate rocks.

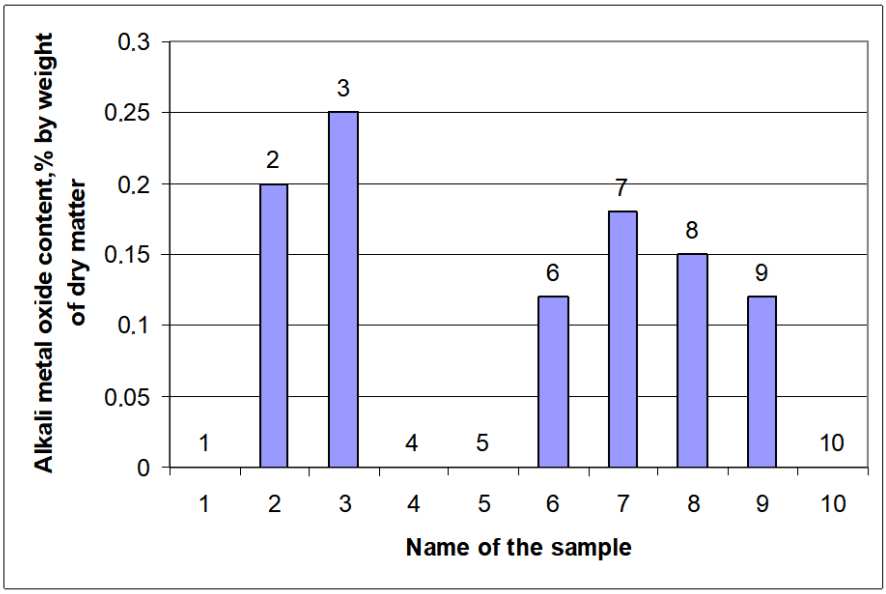


**Figure 7** The content of alkali metal potassium oxide and sodium in the studied samples of carbonate rocks.

**Table 2** Chemical composition of carbon bedrock.

| | Carbon bedrock component | Sample no. 1 | | | | Sample no. 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.13 | | 0.13 | | 0.46 | | 0.46 | |
| | | 0.13 | | 0.13 | | 0.46 | | 0.46 | |
| | Moderate | 0.13 | - | 0.13 | - | 0.46 | - | 0.46 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 1.66 | 1.66 | 1.66 | 1.66 | 3.96 | 3.98 | 3.96 | 3.98 |
| | | 1.66 | 1.66 | 1.66 | 1.66 | 3.95 | 3.97 | 3.95 | 3.97 |
| | Moderate | 1.66 | 1.66 | 1.66 | 1.66 | 3.96 | 3.98 | 3.96 | 3.98 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 0.32 | 0.32 | 0.32 | 0.32 | 1.20 | 1.20 | 1.20 | 1.20 |
| | | 0.32 | 0.32 | 0.32 | 0.32 | 1.20 | 1.20 | 1.20 | 1.20 |
| | Moderate | 0.32 | 0.32 | 0.32 | 0.32 | 1.20 | 1.20 | 1.20 | 1.20 |
| 4. | Calcium carbonate ($CaCO_3$) | 193.21 | 193.40 | 96.69 | 96.77 | 179.17 | 180.07 | 89.43 | 89.88 |
| | | 193.23 | 193.42 | 96.71 | 96.79 | 179.16 | 180.06 | 89.42 | 89.87 |
| | Moderate | 193.22 | 193.41 | 96.70 | 96.78 | 179.16 | 180.06 | 89.42 | 89.87 |
| 5. | Magnesium carbonate ($MgCO_3$) | 2.25 | 2.25 | 1.2 | 1.2 | 6.79 | 6.82 | 3.63 | 3.65 |
| | | 2.25 | 2.25 | 1.2 | 1.2 | 6.81 | 6.84 | 3.64 | 3.66 |
| | Moderate | 2.25 | 2.25 | 1.2 | 1.2 | 6.80 | 6.83 | 3.63 | 3.65 |
| 6. | Calcium sulfate ($CaSO_4$) | - | - | - | - | 0.57 | 0.57 | 0.57 | 0.57 |
| | | - | - | - | - | 0.57 | 0.57 | 0.57 | 0.57 |
| | Moderate | - | - | - | - | 0.57 | 0.57 | 0.57 | 0.57 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | - | - | - | - | | 0.20 | 0.20 | 0.20 |
| | | - | - | - | - | 0.20 | 0.20 | 0.20 | 0.20 |
| | Moderate | - | - | - | - | 0.20 | 0.20 | 0.20 | 0.20 |
| | Summary of the carbon bedrock component | | 197.64 | | 99.96 | | 192.84 | | 99.47 |

Continuation of **Table 2**.

| | Carbon bedrock component | Sample no. 1 | | | | Sample no. 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | | Following method DSTU 1451-96 (1997) | | Following method DSTU 1451-96 (1997) | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 |
| 1. | Moisture | 0.63 | | 0.63 | | 0.25 | | 0.25 | |
| | | 0.63 | | 0.63 | | 0.25 | | 0.25 | |
| | Moderate | 0.63 | - | 0.63 | - | 0.25 | - | 0.25 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 3.65 | 3.68 | 3.65 | 3.68 | 0.77 | 0.77 | 0.77 | 0.77 |
| | | 3.64 | 3.67 | 3.64 | 3.67 | 0.77 | 0.77 | 0.77 | 0.77 |
| | Moderate | 3.65 | 3.68 | 3.65 | 3.68 | 0.77 | 0.77 | 0.77 | 0.77 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 1.68 | 1.69 | 1.68 | 1.69 | 0.87 | 0.87 | 0.87 | 0.87 |
| | | 1.68 | 1.69 | 1.68 | 1.69 | 0.87 | 0.87 | 0.87 | 0.87 |
| | Moderate | 1.68 | 1.69 | 1.68 | 1.69 | 0.87 | 0.87 | 0.87 | 0.87 |
| 4. | Calcium carbonate ($CaCO_3$) | 178.19 | 179.26 | 89.03 | 89.57 | 193.80 | 194.29 | 96.88 | 97.12 |
| | | 178.17 | 179.24 | 89.02 | 89.56 | 193.79 | 194.28 | 96.88 | 97.12 |
| | Moderate | 178.18 | 179.25 | 89.02 | 89.56 | 193.80 | 194.29 | 96.88 | 97.12 |
| 5. | Magnesium carbonate ($MgCO_3$) | 8.2 | 8.2 | 4.37 | 4.40 | 1.12 | 1.13 | 0.6 | 0.6 |
| | | 8.2 | 8.2 | 4.39 | 4.42 | 1.12 | 1.13 | 0.6 | 0.6 |
| | Moderate | 8.2 | 8.2 | 4.38 | 4.41 | 1.12 | 1.13 | 0.6 | 0.6 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.40 | 0.40 | 0.40 | 0.40 | 0.3 | 0.3 | 0.3 | 0.3 |
| | | 0.40 | 0.40 | 0.40 | 0.40 | 0.3 | 0.3 | 0.3 | 0.3 |
| | Moderate | 0.40 | 0.40 | 0.40 | 0.40 | 0.3 | 0.3 | 0.3 | 0.3 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.25 | 0.25 | 0.25 | 0.25 | - | - | - | - |
| | | 0.25 | 0.25 | 0.25 | 0.25 | - | - | - | - |
| | Moderate | 0.25 | 0.25 | 0.25 | 0.25 | - | - | - | - |
| Summary of the carbon bedrock component | | | 193.47 | | 99.99 | | 197.36 | | 99.66 |

Continuation of **Table 2**.

| | Carbon bedrock component | Sample no. 1 | | | | Sample no. 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | | Following the newly introduced method | | Following method DSTU 1451-96 (1997) | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.29 | | 0.29 | | 5.31 | | 5.31 | |
| | | 0.29 | | 0.29 | | 5.31 | | 5.31 | |
| | Moderate | 0.29 | - | 0.29 | - | 5.31 | - | 5.31 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 1.13 | 1.13 | 1.13 | 1.13 | 2.16 | 2.28 | 2.16 | 2.28 |
| | | 1.13 | 1.13 | 1.13 | 1.13 | 2.16 | 2.28 | 2.16 | 2.28 |
| | Moderate | 1.13 | 1.13 | 1.13 | 1.13 | 2.16 | 2.28 | 2.16 | 2.28 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 2.07 | 2.08 | 2.07 | 2.08 | 0.76 | 0.80 | 0.76 | 0.80 |
| | | 2.07 | 2.08 | 2.07 | 2.08 | 0.76 | 0.80 | 0.76 | 0.80 |
| | Moderate | 2.07 | 2.08 | 2.07 | 2.08 | 0.76 | 0.80 | 0.76 | 0.80 |
| 4. | Calcium carbonate ($CaCO_3$) | 183.99 | 184.54 | 91.92 | 91.20 | 173.4 | 183.11 | 86.65 | 91.50 |
| | | 184.00 | 184.55 | 91.92 | 91.20 | 173.4 | 183.11 | 86.67 | 91.52 |
| | Moderate | 183.99 | 184.54 | 91.92 | 91.20 | 173.4 | 183.11 | 86.66 | 91.51 |
| 5. | Magnesium carbonate ($MgCO_3$) | 7.37 | 7.40 | 3.93 | 3.94 | 7.92 | 8.36 | 4.21 | 4.45 |
| | | 7.35 | 7.38 | 3.94 | 3.95 | 7.94 | 8.38 | 4.23 | 4.47 |
| | Moderate | 7.36 | 7.39 | 3.93 | 3.94 | 7.93 | 8.37 | 4.22 | 4.46 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.4 | 0.4 | 0.4 | 0.4 | 0.27 | 0.28 | 0.27 | 0.28 |
| | | 0.4 | 0.4 | 0.4 | 0.4 | 0.27 | 0.28 | 0.27 | 0.28 |
| | Moderate | 0.4 | 0.4 | 0.4 | 0.4 | 0.27 | 0.28 | 0.27 | 0.28 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | - | - | - | - | 0.11 | 0.12 | 0.11 | 0.12 |
| | | - | - | - | - | 0.11 | 0.12 | 0.11 | 0.12 |
| | Moderate | - | - | - | - | 0.11 | 0.12 | 0.11 | 0.12 |
| Summary of the carbon bedrock component | | | 195.54 | | 99.75 | | 194.96 | | 99.45 |

Continuation of **Table 2**.

| | Carbon bedrock component | Sample no. 1 | | | | Sample no. 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 |
| 1. | Moisture | 0.47<br>0.47 | | 0.47<br>0.47 | | 0.67<br>0.67 | | 0.67<br>0.67 | |
| | Moderate | 0.47 | - | 0.47 | - | 0.67 | - | 0.67 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 6.24<br>6.24 | 6.27<br>6.27 | 6.24<br>6.23 | 6.27<br>6.26 | 0.32<br>0.32 | 0.32<br>0.32 | 0.32<br>0.32 | 0.32<br>0.32 |
| | Moderate | 6.24 | 6.27 | 6.24 | 6.27 | 0.32 | 0.32 | 0.32 | 0.32 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 0.60<br>0.60 | 0.60<br>0.60 | 0.60<br>0.60 | 0.60<br>0.60 | 1.7<br>1.7 | 1.7<br>1.7 | 1.7<br>1.7 | 1.7<br>1.7 |
| | Moderate | 0.60 | 0.60 | 0.60 | 0.60 | 1.7 | 1.7 | 1.7 | 1.7 |
| 4. | Calcium carbonate ($CaCO_3$) | 179.36<br>179.38 | 180.26<br>180.28 | 89.60<br>89.60 | 90.05<br>90.05 | 186.13<br>186.11 | 187.37<br>187.35 | 92.95<br>92.95 | 93.57<br>93.57 |
| | Moderate | 179.37 | 180.27 | 89.60 | 90.05 | 186.12 | 187.36 | 92.95 | 93.57 |
| 5. | Magnesium carbonate ($MgCO_3$) | 4.26<br>4.27 | 4.28<br>4.29 | 2.3<br>2.3 | 2.3<br>2.3 | 6.91<br>6.91 | 6.95<br>6.96 | 3.69<br>3.69 | 3.72<br>3.72 |
| | Moderate | 4.27 | 4.29 | 2.3 | 2.3 | 6.91 | 6.96 | 3.69 | 3.72 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.42<br>0.42 | 0.42<br>0.42 | 0.42<br>0.42 | 0.42<br>0.42 | 0.45<br>0.45 | 0.45<br>0.45 | 0.45<br>0.45 | 0.45<br>0.45 |
| | Moderate | 0.42 | 0.42 | 0.42 | 0.42 | 0.45 | 0.45 | 0.45 | 0.45 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.18<br>0.18 | 0.18<br>0.18 | 0.18<br>0.18 | 0.18<br>0.18 | 0.15<br>0.15 | 0.15<br>0.15 | 0.15<br>0.15 | 0.15<br>0.15 |
| | Moderate | 0.18 | 0.18 | 0.18 | 0.18 | 0.15 | 0.15 | 0.15 | 0.15 |
| Summary of the carbon bedrock component | | | 192.03 | | 99.82 | | 196.94 | | 99.91 |

Continuation of **Table 2**.

| | Carbon bedrock component | Sample no. 1 | | | | Sample no. 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | | Following method DSTU 1451-96 (1997) | | Following the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.53 | | 0.53 | | 0.63 | | 0.63 | |
| | | 0.53 | | 0.53 | | 0.63 | | 0.63 | |
| | Moderate | 0.53 | - | 0.53 | - | 0.63 | - | 0.63 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 1.49 | 1.5 | 1.49 | 1.5 | 0.79 | 0.8 | 0.79 | 0.8 |
| | | 1.49 | 1.5 | 1.49 | 1.5 | 0.79 | 0.8 | 0.79 | 0.8 |
| | Moderate | 1.49 | 1.5 | 1.49 | 1.5 | 0.79 | 0.8 | 0.79 | 0.8 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 1.19 | 1.20 | 1.19 | 1.20 | 0.19 | 0.20 | 0.19 | 0.20 |
| | | 1.19 | 1.20 | 1.19 | 1.20 | 0.19 | 0.20 | 0.19 | 0.20 |
| | Moderate | 1.19 | 1.20 | 1.19 | 1.20 | 0.19 | 0.20 | 0.19 | 0.20 |
| 4. | Calcium carbonate ($CaCO_3$) | 190.0 | 190.25 | 94.83 | 95.30 | 195.15 | 196.32 | 97.61 | 98.20 |
| | | 190.0 | 190.25 | 94.83 | 95.30 | 195.17 | 196.34 | 97.62 | 98.21 |
| | Moderate | 190.0 | 190.25 | 94.83 | 95.30 | 195.16 | 196.33 | 97.61 | 98.20 |
| 5. | Magnesium carbonate ($MgCO_3$) | 2.81 | 2.82 | 1.39 | 1.4 | 4.22 | 4.24 | 0.69 | 0.7 |
| | | 2.81 | 2.82 | 1.39 | 1.4 | 4.22 | 4.24 | 0.69 | 0.7 |
| | Moderate | 2.81 | 2.82 | 1.39 | 1.4 | 4.22 | 4.24 | 0.69 | 0.7 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.2 | 0.2 | 0.2 | 0.2 | - | - | - | - |
| | | 0.2 | 0.2 | 0.2 | 0.2 | - | - | - | - |
| | Moderate | 0.2 | 0.2 | 0.2 | 0.2 | - | - | - | - |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.12 | 0.12 | 0.12 | 0.12 | - | - | - | - |
| | | 0.12 | 0.12 | 0.12 | 0.12 | - | - | - | - |
| | Moderate | 0.12 | 0.12 | 0.12 | 0.12 | - | - | - | - |
| Summary of the carbon bedrock component | | | 196.72 | | 99.72 | | 201.57 | | 99.9 |

## CONCLUSION

The obtained data indicate that the use of the proposed method for determining the mass fraction of calcium carbonate in the carbonate rock makes it possible to obtain more accurate indicators of its concentration and, accordingly, the chemical composition of limestone.

Thus, the inclusion of the proposed improved method for determining the content of calcium carbonate in the carbonate rock, as one of the elements of the technological audit of the lime department will allow to give an objective assessment of the processes of the sugar plant and their early correction, which will lead to economic and environmental efficiency.

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# THE INFLUENCE OF VARIETAL CHARACTERISTICS ON THE QUALITY INDICATORS OF CANDIED FRUITS FROM PLUM FRUITS

*Tatiana Prichko, Natalia Drofícheva*

## ABSTRACT

The influence of varietal characteristics of plum fruits on the physicochemical parameters of the drying process has been investigated. Revealed significant differences in the content of biologically active compounds on the example of varieties: Stanley, Big Stanley, Grossa di Felisio, President, Blue moon, August Delish, Kabardinskaya early. The consumption rates of raw materials for the production of candied fruits have been determined. The results of laboratory studies on the variation of the content of vitamins (*C, P*), anthocyanins in plum fruits, taking into account varietal characteristics, are presented. The drying process on a dryer with infrared rays led to a slight decrease in polyphenolic and mineral substances, and higher losses of vitamins in the finished candied fruit. The mathematical processing of the experimental data was carried out by the method of analysis of variance and descriptive statistics using the Microsoft Excel software package. In the production of candied plum fruits, optimal technological modes have been selected that contribute to a high yield of high-quality finished products, taking into account the varietal characteristics of the feedstock. According to biochemical and organoleptic indicators of the quality of candied fruits, Stanley and President varieties were distinguished, the energy value of which is 147.0 kcal with an organoleptic assessment of 4.9 points. The results of experimental biochemical and technological analyzes of fruits at individual technological stages of production made it possible to assess the intensity of complex processes that cause a qualitative transformation of raw materials.

**Keywords:** plum; technological process; candied fruits; physical methods; organoleptic indicators

## INTRODUCTION

Plum is a high-yielding stone fruit plant, the origin of which is the result of a natural hybridization of cherry plum and blackthorn, which historically occurred in the Caucasian and Asia Minor regions. Plum cultivation continued in the Mediterranean and Central Asia. From Mediterranean Italy, plum has penetrated European countries and has firmly taken the position of one of the most popular fruit trees in the world of fruit growing **(Doroshenko, Ryazanova and Maksimtsov, 2015; Eremin et al., 2000; Popova et al., 2020)**. The scientific interest in studying the medicinal properties of plums is explained by the high content of phenols in its fruits, mainly anthocyanins, which are natural antioxidants **(Prichko, 2009; Kim, Jeong and Lee, 2003; Gościnna et al., 2021)**. Many world scientists have substantiated the connection between the use of plums and the improvement of cognitive function (in particular, a positive effect on memory and related processes) **(Igwe and Charlton, 2016; Turaeva, 2017)**. In addition to the aforementioned and antioxidant properties, the interrelation of the inclusion of plum fruits in the diet and the reduction of risk factors for cardiovascular diseases is emphasized.The anti-inflammatory, analgesic, antibacterial and anti-cancer properties of home plum gum have been analyzed in dozens of studies **(Islam et al., 2017; Milskaya, 1998)**. Several scientific works emphasize the following: the chemical composition of plum fruits allows the use of this fruit as a source of raw materials for the production of candied fruits and dried fruits. As a rule, fresh fruits and vegetables have a short shelf life, since various microbes and enzymes affect them and quickly spoil. Therefore, fresh fruits and vegetables must be eaten immediately, and the surplus must be skillfully preserved for the autumn-winter and spring periods. In this regard, the development and implementation of modern processing technologies for perishable raw materials in the form of fruits, fruits, and vegetables with full preservation of the nutritional and taste qualities of the final product is relevant in the modern world **(Prichko, Machneva and Drofícheva, 2020; Amin et al., 2019, Svanes and Johnsen, 2019)**. Candied fruit – fruit processing products made by single or multiple cooking in sugar syrup, dried to a mass fraction of dry matter of at least 80%, sprinkled with sugar, powdered sugar, or glazed **(GOST 28322, 2014)**. American scientists in the course of a retrospective study found that dried fruits are no less beneficial for health than fresh ones, and in winter they can be successfully replaced **(Sullivan et al., 2020)**. The analysis made it possible to conclude the relevance of research in the field of processing plum fruits

into dried fruits and candied fruits. In this regard, it is of interest to study the influence of varietal characteristics on the quality indicators of candied fruits.

### Scientific hypothesis

As a result of the analysis of the dynamics of changes in the biochemical indicators of the quality of plum fruits, a working hypothesis was put forward about the influence of varietal characteristics of fruits on the quality indicators of processed products, in particular candied fruits. This served as the basis for experimental studies of the systemic influence of the varietal characteristics of plum fruits on the physicochemical parameters of the technological process of drying and finished products.

## MATERIAL AND METHODOLOGY

### Samples

Fresh fruits of domestic plum (*Prunus domestica*) varieties: Stanley, Big Stanley, Grossa di Felisio, President, Blue moon, August Delish, Kabardinskaya early. The material for the research was obtained in the industrial plantings of domestic plum, JSC "Plodovod" (Krasnodar), planted in 2006;

Candied plum fruit varieties: Stanley, Big Stanley, Grossa di Felisio, President, Blue moon, August Delish, Kabardin early. Candied fruits were obtained in the laboratory for storage and processing of fruits and berries in 2020.

### Chemicals

To assess the chemical composition and quality indicators of fresh plum and candied fruits in a varietal section, reagents of the following qualifications were used:

- 2,6-dichlorophenolindophenolate sodium, mass concentration 0.250 $g.^{dm-3}$; JSC "Vekton", chemically pure;
- ascorbic acid – 1.0 and 0.1 $g.^{dm-3}$; JSC "Vekton", chemically pure;
- nitric acid according to GOST 4461 with a density of 1.41 $g.^{cm-3}$; JSC "Vekton", chemically pure;
- metaphosphoric acid according to GOST 841, solutions with a mass fraction of 3 and 6%; JSC "LenReaktiv", reagent grade hydrochloric acid according to GOST 3118 with a density of 1.19 $g.^{cm-3}$; JSC "Vekton", chemically pure;
- glacial acetic acid according to GOST 61 and a solution with a mass fraction of 3%; JSC "Vekton", chemically pure;
- sulfuric acid according to GOST 4204, chemically pure grade, with a density of 1836 $kg.m^{-3}$; JSC "Vekton", chemically pure;
- oxalic acid according to GOST 22180, chemically pure grade; JSC "Uralkhiminvest", chemically pure;
- perchloric acid, solution of molar concentration 0.1 $mol.dm^{-3}$; JSC "Vekton", chemically pure;
- ethylenediaminetetraacetic acid with a mass fraction of 5%; JSC "Vekton", chemically pure;
- orthophosphoric acid according to GOST 6552-80; JSC "Vekton", chemically pure;
- potassium iodide according to GOST 4232, a solution with a mass fraction of 1% in a solution of acetic acid with a mass fraction of 3%; JSC "LenReaktiv", reagent grade;
- formaldehyde with a mass fraction of 36 – 40%; JSC "Vekton", chemically pure;
- buffer solutions with pH 4.01 and 9.18; JSC "Uralkhiminvest", chemically pure;
- sodium hydroxide in accordance with GOST 4328, phenolphthalein with a mass concentration of 10 $g.dm^{-3}$; JSC "Uralkhiminvest", chemically pure;
- acetone in accordance with GOST 2603; JSC "Vekton", chemically pure;
- sodium phosphate disubstituted according to GOST 4172 with a mass concentration of 200 $g.dm^{-3}$; JSC "Vekton", chemically pure;
- sodium sulfate 10-water in accordance with GOST 4171; JSC "Vekton", chemically pure;
- potassium ferrous-cyanide 3-water in accordance with GOST 4207; JSC "Vekton", chemically pur;
- zinc acetate 2-water in accordance with GOST 5823 with a mass concentration of 230 $g.dm^{-3}$; JSC "LenReaktiv", reagent grade;
- zinc sulfate 7-water in accordance with GOST 4174; JSC "LenReaktiv", reagent grade;
- acetic acid lead according to GOST 1027; JSC "LenReaktiv", reagent grade;
- copper (II) sulfate pentahydrate according to GOST 4165; JSC "Vekton", chemically pure;
- sodium hydroxide following GOST 4328, with a mass concentration of 10 $g.dm^{-3}$; JSC "Vekton", chemically pure;
- iron ammonium alum according to NTD; JSC "Vekton", chemically pure;
- potassium permanganate according to GOST 20490; JSC "Uralkhiminvest", chemically pure;
- methyl orange with a mass concentration of 1 $g.dm^{-3}$; JSC "Vekton", chemically pure;
- distilled water according to GOST 6709 or water of equivalent purity, etc.

### Tools

- Refractometer – PAL-H Digital refractometer for sauces and purees 0.0 93.0% for high-temperature samples; "ATAGO"; Japan;
- pH Meter – ST2100-E Laboratory pH / ORP; "Ohaus";
- Electronic laboratory scales AF-R220CE, "VIBRA", LLC "Eykom Msk";
- Water jet pump in accordance with GOST 25336, JSC "LenReaktiv";
- Hourglass clock for 2, 3, 5 minutes or stopwatch; LLC "First Thermometer Plant";
- Homogenizer – rotary, up to 10,000 rpm, Ultra-Turrax T 50 digital; manufacturer "IKA";
- Gas chromatograph "Crystal – 2000M" – reg. No. 14516-12; manufacturer "GRANATE";
- DROP-103RT; manufacturer – Tech Equipment LLC, St. Petersburg;
- "KAPEL®-105M", data collection and processing using specialized software "Elforan"; manufacturer – LLC "Lumex-marketing";
- Water bath – six-seater UT-4300E ULAB, manufacturer – ULAB;
- Laboratory centrifuge Armed LC-04B; manufacturer – NV-LAB;

- Liquid chromatograph (operating range of absorption wavelengths from 200 to 600 nm); "LYUMAKHROM";
- HPLC column; brand – Phenomenex Luna; "LYUMAKHROM;
- water bath – six-seater UT-4300E; ULAB;
- laboratory centrifuge Armed LC-04B; NV-LAB;
- utensils (ISO 835-1-81, ISO 1042-83, ISO 4788-80);
- flasks in accordance with GOST 1770, 1-250-2 or 2-250-2; LLC "MiniMedProm";
- pipettes according to NTD, 2-2-25 or 2-1-25, 2-2-50 or 2-1-50, 2-2-100 or 2-1-100; LLC "MiniMedProm";
- burettes according to NTD, 1-2-25-0.1 or 2-2-25-0.1 or 3-2-25-0.1; LLC "MiniMedProm";
- conical flasks in accordance with GOST 25336, Kn-2-250-34 THS; LLC "MiniMedProm";
- glasses following GOST 25336, V-1-50 TS, N-1-100 TS, V-1-250 TS; LLC "MiniMedProm";
- laboratory funnels following GOST 25336, B-56-80 XC or B-75-140 XC or B-100-150 XC; LLC "MiniMedProm";
- a stick made of chemical laboratory glass following GOST 21400; LLC "MiniMedProm";
- laboratory porcelain mortar and pestle following GOST 9147, respectively, with an outer diameter of 70 or 90 mm and a height of 90 mm;
- laboratory glass measuring cylinders following GOST 1770 with a capacity of 100, 250 $cm^3$; LLC "MiniMedProm";
- laboratory thermometer with a measurement range of 0 °C – 100 °C, with a graduation value of not more than 0.5 °C following GOST 28498; LLC "First Thermometer Plant";
- laboratory glass dropper following GOST 25336; LLC "MiniMedProm";
- chromatographic micro tweezers with a capacity of 10 $mm^3$, a graduation rate of 0.1 $mm^3$ and a capacity of 20 $mm^3$; membrane filters with pore diameters of 0.20 and 0.45 microns for filtering the mobile phase and samples; "LYUMAKHROM".

**Laboratory methods**

In the process of studying the influence of varietal characteristics of plum fruits for use in the food processing industry in the laboratory for storage and processing of fruits and berries of FSBSI NCFSCHVW, technical and biochemical quality indicators were determined using modern methods and GOSTs **(GOST 26313; GOST 26671, 2014; GOST 25555.0, 1982; GOST ISO 2173, 2014; GOST 24556, 2014; GOST 32709, 2014; Yakuba, Kuznetsova and Lozhnikova, 2011; Methods of analysis, 2002; Vigorov, 1972; GOST 31745, 2012; GOST 8756.1, 1987)**. Sampling – following **GOST 26313 (2013)**, sample preparation – following **GOST 26671 (2014)**; determination of soluble dry substances (RSV) in fruits and candied fruits was carried out by refractometric method according to **GOST ISO 2173 (2014)**; the content of titratable acids by the potentiometric method following **GOST 25555.0-82 (1982)**; the content of vitamin C by a titrometric method according to **GOST 24556 (2014)**; common sugars by photocolorimetric method on a photoelectric colorimeter; the content of total polyphenols and P-active substances was determined by the method of L. I. Vigorov on a KFK-2 photoelectric colorimeter; the content of water-soluble plant pigments (anthocyanins), by the method of pH differential spectrophotometry following **GOST 32709 (2014)**. Flesh hardness using FT – 372 penetrometer with 8 mm plunger after skin removal. The amino acid composition in plums and candied fruits was determined by the method of highly efficient capillary electrophoresis on a KAPEL-103RT device. Determination of the content of polycyclic aromatic hydrocarbons (aromatic substances) in the test samples was determined by the method of high-performance liquid chromatography on a gas chromatograph "Crystal - 2000M" following **GOST 31745 (2012)**. The organoleptic indices of the tasting evaluation of candied fruits from plum fruits in the varietal section were determined following **GOST 8756.13-79 (1987)**.**Experiment Description**

Sample preparation following **GOST 26313 (2014), GOST 26671 (2014)**.

The number of analyzed samples – 8 varieties of fresh fruits and candied fruits: Stanley, Big Stanley, Grossa di Felisio, President, Blue moon, August Delish, Kabardinskaya early.

**Number of replicates:** Assays were performed in duplicate.

**Number of repetitions of the experiment:** The experiment was carried out in triplicate.

## Statistical Analysis

When processing experimental data for a significance level of $p = 0.05$, the following statistical criteria were used: Cochran's criterion – to assess the homogeneity of the variance; Student's test – to assess the significance of the calculated coefficients; Fisher's criterion – to assess the adequacy of the obtained regression equations. Calculations were performed using the Microsoft Excel 2020 software package (Microsoft XLSTAT).

## RESULTS AND DISCUSSION

The taste and technological qualities of plums are largely determined by their chemical composition **(Eremin et al., 2009)**. On the example of 7 plum varieties investigated in the laboratory for storage and processing of fruits and berries of the FSBSI NCFSCHVW, technical indicators of fruits are presented (Table 1).

The studied varieties vary significantly in the content of dry substances, sugars, acids, vitamins, and polyphenolic substances (Table 2).

There is a wide range of variation in the acid content in plum fruits 0.72 – 1.31%. The varieties such as Blue Moon, Big Stanley, and August Delish stand out for their high acidity. The Blue Moon variety (sugar-acid index 8.4) has the sourest taste.

The plum culture is characterized by a low content of vitamin *C* (a natural antioxidant), on average 5 – 6 $mg.100g^{-1}$. The varieties Blue Moon and Grossa di Felisio with an increased level of vitamin *C* accumulation 7.0 – 7.5 $mg.100g^{-1}$ were identified. In terms of the amount of vitamin *P*, plum varieties differ 2 times: Big Stanley fruits accumulate up to 40 $mg.100g^{-1}$, and Stanley varieties

more than 70 mg.100g$^{-1}$. The total content of polyphenolic substances varies slightly, taking into account the variety, from 166.4 mg.100g$^{-1}$ (Big Stanley variety) to 189.0 mg.100g$^{-1}$ (Grossa de Felisio variety), which also form the antioxidant activity of fruits **(Sheikh, Saini and Sharma, 2021; Demidov, Popov and Savelyev, 2015; Qin et al., 2020; Li et al., 2021; Makarova and Eremeeva, 2015)**. Large varietal differences can be traced in the content of anthocyanins: 60.2 mg.100g$^{-1}$ for the Blue Moon variety, 32.9 mg.100g$^{-1}$ for the Big Stanley variety (Figure 1).

The composition of free amino acids is presented in detail in two studied varieties: Stanley and President. In the fruits of the Stanley variety, 12 amino acids were identified, which is 71.3 mg.100g$^{-1}$, including arginine 2.1, phenylalanine 0.1, histidine 0.37, leucine 0.18, methionine 2.6, valine 0.18, proline 19.0, threonine 42.5, tryptophan 2.8, serine 0.42, alanine 0.96 , glycine 0.02 mg.100g$^{-1}$.

It has been established that the amino acid composition of the fruit of the President variety is represented by 13 amino acids, including five essential ones (methionine, phenylalanine, leucine, valine, and threonine). Among the essential amino acids, methionine was found in the maximum amount (3.8 mg.100g$^{-1}$), the content of phenylalanine is 0.27 mg.100g$^{-1}$, leucine – 0.24 mg.100g$^{-1}$, valine – 0.45 mg.100g$^{-1}$. The amount of threonine is less than 0.1 mg.100g$^{-1}$. Among the nonessential amino acids, the maximum amount contains arginine (0.62 mg.100g$^{-1}$), proline (2.4 mg.100g$^{-1}$), and α-alanine.

The aroma-forming substances of the fruits of the President variety are represented by 3 aldehydes (acetaldehyde, furfural, 5-methyl furfural), the total amount of which is 2.2 mg.100g$^{-1}$. In addition to them, 2 saturated aliphatic carboxylic acids were noted – propionic and acetic in the amount of 8.2 mg.100g$^{-1}$, as well as a small amount of aromatic and aliphatic alcohols and esters.

In the new varieties August Delish, Big Stanley, Grossa di Felisio, President in 2020, a complex of macronutrients was investigated, which makes it possible to establish that plum is a valuable raw material for the production of canned food enriched with minerals. The fruits contain a significant amount of potassium, sodium, and, which is very important for the processed raw materials, calcium, which can maintain the structure of the fruit tissue during processing (Table 3).

The data given in the tables allow us to conclude that each variety is characterized by its own set of biologically active substances and is of interest to food processing enterprises. According to the results of evaluating the technical indicators of plum fruits for 2019 – 2020, the most interesting for the production of candied fruits is the variety: President, Grossa de Felisio, August Delish, the fruits of which were processed in comparison with the control, which has wide production plantings in the territory of the Russian Federation (variety Stanley) **(Kaufmane et al., 2007; Prichko and Drofichеva, 2019)**.

Candied fruits are complex drying objects, where it is necessary to take into account such indicators as the initial and final moisture content, chemical composition, structural and mechanical changes, and mass transfer characteristics. Candied fruit drying is a process that, in essence, is not only thermophysical but also technological **(Meretukova, 2012)**. As a technological process, drying of fruits and other moist materials is a combination of heat and mass transfer processes, accompanied by biochemical, technological, and structural-mechanical changes. When dried in candied fruits, not only the native properties of the product should be preserved, but also be significantly improved. The main feature of the process of sorption of raw materials is that the mechanical, thermophysical, diffusion properties of the raw materials change significantly upon saturation, which has a significant effect on all subsequent technological operations **(Deiana et al., 2019; Gonzalez-Cebrino et al., 2013; Gonzalez et al., 2016)**.

The fruits, after pitting and blanching, were kept in sugar syrup three times for 4 to 6 hours. For the production of candied fruits from plum fruits, a high initial concentration of syrup is undesirable, because this increases the stickiness of the fruit, which significantly reduces the attractiveness of the appearance of the finished product **(Alekseeva et al. 2018; Cerri et al., 2019; Reichel et al., 2017; Richmond, Bowyer and Vuong, 2019; Sommano et al., 2013)**. The change in the concentration of the syrup in the first period of exposure between the fruit and the syrup is achieved mainly due to their dehydration and due to this dilution of the concentration of the syrup. After 4 hours of exposure, the concentration of the syrup decreases to 45 – 50% and insignificant phase changes are already taking place in the system. The third filling is carried out with part of the released syrup, brought to 60% concentration. During this period, the phenomenon of dehydration of fruits was also noted with a simultaneous, but already less saturation of them with syrup.

At this technological stage of candied fruit production, the following varieties proved to be unsuitable for this type of processing of plum fruits: Blue moon and August Delish. The fruits of the Blue Moon variety did not hold their shape after the first pouring with syrup, and the fruits of the August Delish variety lost a significant amount of coloring pigment and acquired a pale appearance.

Optimization of drying processes in a drying oven with IR rays has been carried out under conditions that prevent sugar caramelization, protein denaturation, which worsen the presentation and quality of candied fruits **(Nunes et al., 2009a; Nunes et al., 2009b)**. Plum fruits were dried in 2 stages: in the first two hours at a temperature of 40 – 45 °C, then the temperature was increased to 60 – 65 °C for 4 – 5 hours. If necessary, especially for large fruits, the raw material was dried at 40 °C to a moisture content of 20 – 23%. During the drying process, the raw materials were periodically turned over and the temperature in the drying oven was controlled. The drying time is determined by the intensity of the technological process, the type and size of raw materials, and on average is 28 – 36 hours for plum fruits **(Cabrera-Bañegil et al., 2020; Prichko, 2002; Pisano et al., 2019; Ionica et al., 2013; Michalska et al., 2016)**. The drying process in an oven with infrared rays led to a decrease in polyphenolic substances by 10%, and vitamins from 13% (*P*) to 29% (*C*) in finished candied fruits (Table 4).

The content of macroelements in candied fruits concerning the original fresh raw materials decreased by 10 – 13%, according to the accumulation of potassium and sodium, the following varieties were distinguished: President, Stanley, and Big Stanley (Table 5).

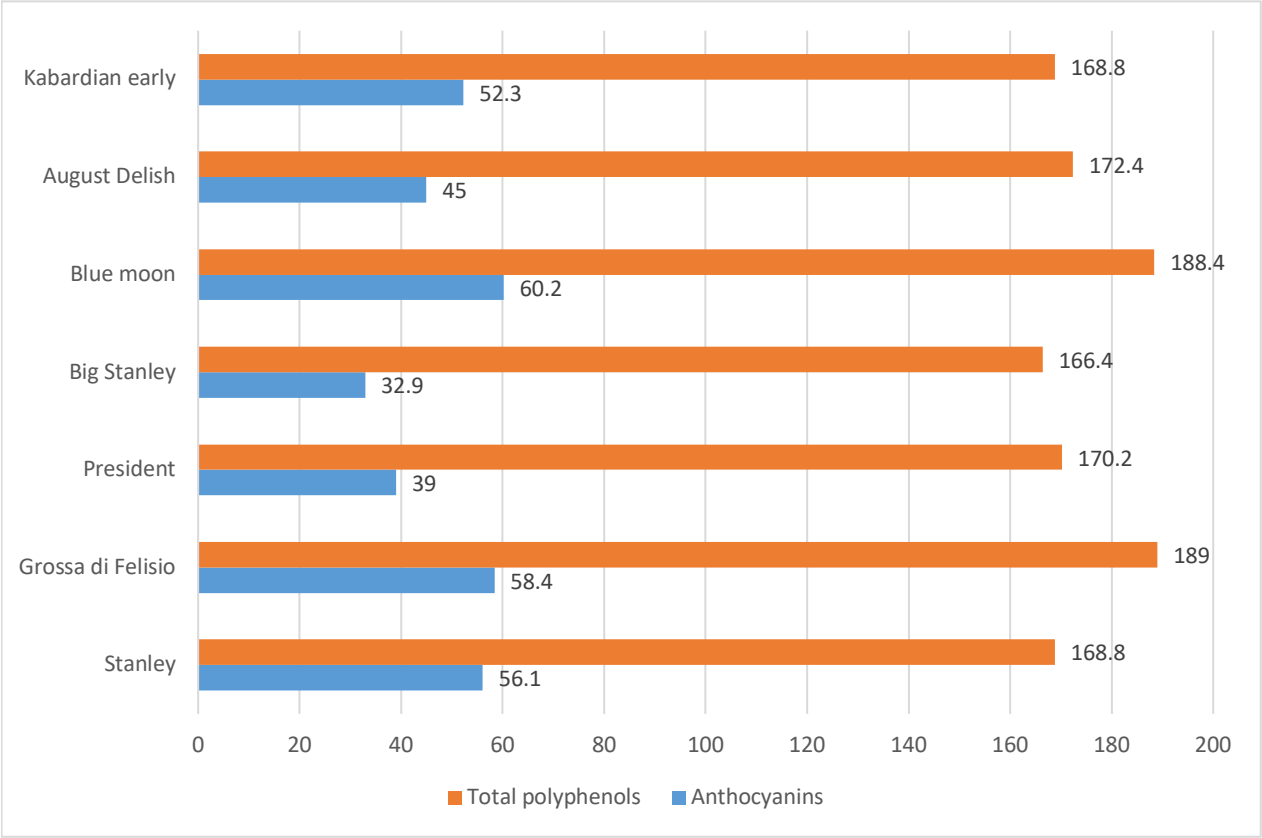


**Figure 1** content of total polyphenols and anthocyanins in plum fruits depending on varietal characteristics, mg.100g$^{-1}$.

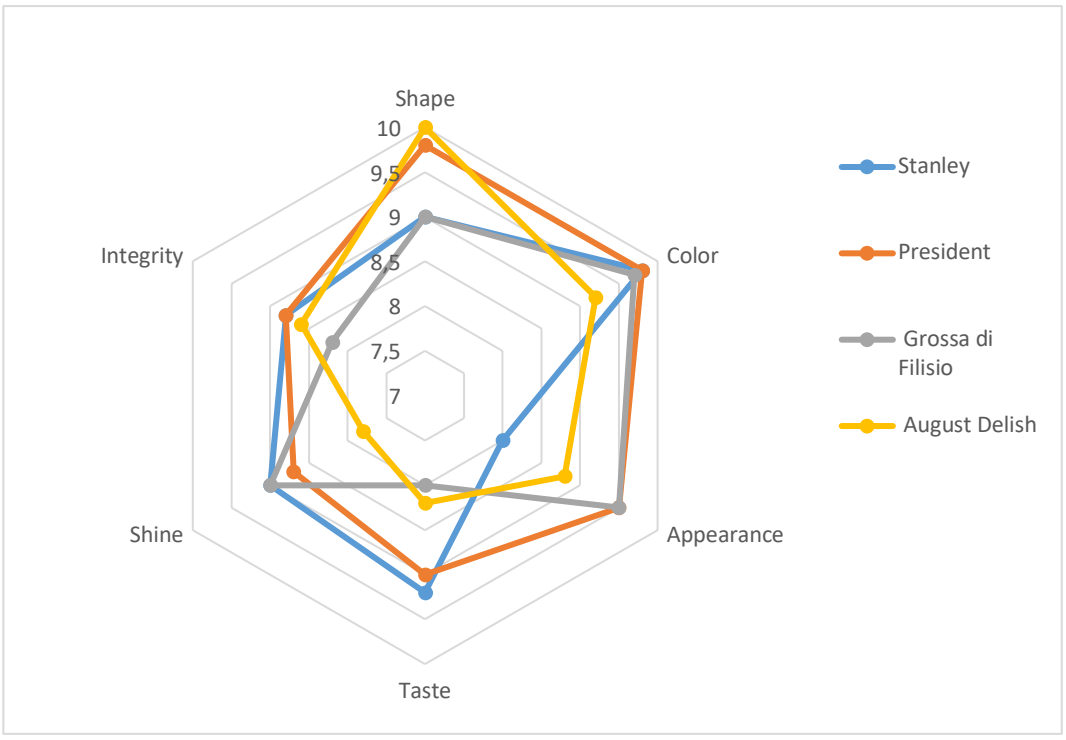


**Figure 2** Organoleptic characteristics of candied fruits, taking into account varietal characteristics



Variety President



Candied fruit, variety President



Variety Stanley



Candied fruit, Stanley variety

**Figure 3** Technical indicators of the quality of candied fruits and plum fruits.

**Table 1** Technical indicators of plum fruits, 2019 – 2020.

| Mycotoxins | Amet dolor sit consectetur | | | | |
|---|---|---|---|---|---|
| | Pulp hardness ($kg.cm^{-3}$ ± SD) | Weight (g ± SD) | H (mm ± SD) | D (mm ± SD) | Shape index (o.e ± SD) |
| Kabardian early | 3.9 ±0.5 | 85.0 ±7.5 | 60.4 ±3.5 | 49.2 ±2.5 | 1.2 ±0.2 |
| August Delish | 4.4 ±0.4 | 140.0 ±5.0 | 69.1 ±4.5 | 68.2 ±3.5 | 0.9 ±0.3 |
| Blue moon | 4.3 ±0.4 | 132.0 ±4.5 | 65.0 ±3.0 | 64.8 ±2.5 | 1.0 ±0.2 |
| President | 4.8 ±0.3 | 122.0 ±8.5 | 65.4 ±4.5 | 58.8 ±3.0 | 1.2 ±0.5 |
| Grossa di Felisio | 4.8 ±0.5 | 138.0 ±7.5 | 68.9 ±3.0 | 66.9 ±2.5 | 1.1 ±0.3 |
| Big Stanley | 4.4 ±0.5 | 117.0 ±6.5 | 70.3 ±2.5 | 59.6 ±3.0 | 1.3 ±0.2 |
| Stanley | 4.0 ±0.5 | 58.0 ±8.5 | 64.0 ±3.0 | 44.0 ±3.5 | 1.4 ±0.3 |

**Table 2** Chemical composition of plum fruits, taking into account the varietal characteristics, 2019 – 2020.

| Variety | Soluble Solids (% ± SD) | Total sugar (% ± SD) | Acidity (% ± SD) | Sugar acid Index | Vitamins ($mg.100g^{-1}$ ± SD) | |
|---|---|---|---|---|---|---|
| | | | | | C | P |
| Kabardian early | 14.2 ±0.2 | 10.4 ±0.1 | 0.72 ±0.02 | 14.4 | 5.0 ±0.5 | 55.3 ±0.8 |
| August Delish | 16.6 ±0.3 | 12.1 ±0.2 | 1.04 ±0.03 | 11.6 | 6.6 ±0.4 | 51.6 ±0.9 |
| Blue moon | 15.4 ±0.3 | 11.0 ±0.2 | 1.31 ±0.03 | 8.4 | 7.0 ±0.5 | 39.1 ±0.5 |
| President | 16.2 ±0.1 | 11.8 ±0.1 | 0.99 ±0.03 | 10.9 | 6.1 ±0.3 | 53.3 ±0.4 |
| Grossa di Felisio | 15.8 ±0.2 | 11.2 ±0.1 | 1.26 ±0.03 | 8.9 | 7.5 ±0.4 | 70.2 ±0.5 |
| Big Stanley | 14.2 ±0.2 | 10.4 ±0.2 | 1.15 ±0.02 | 9.0 | 5.7 ±0.3 | 36.6 ±0.4 |
| Stanley | 16.2 ±0.2 | 11.8 ±0.2 | 0.75 ±0.02 | 15.8 | 6.0 ±0.2 | 72.1 ±0.6 |

**Table 3** Content of macronutrients in plums, 2020.

| Variety | Content (mg.100g$^{-1}$) | | | |
|---|---|---|---|---|
| | Potassium | Sodium | Magnesium esium | Calcium |
| Big Stanley | 122.4 | 29.8 | 10.5 | 13.5 |
| President | 165.0 | 23.0 | 11.1 | 17.4 |
| August Delish | 105.2 | 37.6 | 11.1 | 19.3 |
| Grossa di Felisio | 107.6 | 9.3 | 9.5 | 16.2 |

**Table 4** Content of biologically active compounds in candied fruits from plum fruits.

| Variety | Total polyphenols (mg.100g$^{-1}$) | Anthocyanins (mg.100g$^{-1}$) | Vitamins (mg.100g$^{-1}$) | |
|---|---|---|---|---|
| | | | C | P |
| August Delish | 166.4 | 39.9 | 3.8 | 42.0 |
| President | 164.8 | 30.6 | 4.0 | 42.8 |
| Grossa di Felisio | 185.4 | 43.7 | 5.2 | 59.6 |
| Big Stanley | 152.6 | 28.8 | 4.0 | 29.8 |
| Stanley | 162.8 | 40.6 | 3.8 | 60.2 |
| Kabardian early | 135.4 | 18.2 | 3.2 | 42.2 |
| Blue moon | 178.0 | 49.8 | 5.4 | 30.2 |

**Table 5** Content of macronutrients in candied plum fruits, 2020.

| Variety | Content (mg.100g$^{-1}$) | | | |
|---|---|---|---|---|
| | Potassium | Sodium | Magnesium esium | Calcium |
| Stanley | 118.6 | 24.7 | 9.4 | 13.1 |
| Big Stanley | 110.2 | 25.5 | 8.9 | 12.2 |
| President | 148.4 | 21.2 | 10.0 | 15.6 |
| August Delish | 96.0 | 33.8 | 10.2 | 17.1 |
| Grossa di Felisio | 98.4 | 8.8 | 9.0 | 14.8 |

**Table 6** Chemical indicators of the quality of candied fruits from plum fruits.

| Variety | Ash (% ± SD) | Carbohydrates (% ± SD) | Protein (% ± SD) | Vitamins (mg.100g$^{-1}$) | | Energy Value (kcal) |
|---|---|---|---|---|---|---|
| | | | | C | P | |
| President | 0.8 | 45.0 | 0.5 | 4.0 | 42.8 | 147.0 |
| Stanley | 0.7 | 42.0 | 0.4 | 3.8 | 60.2 | 125.0 |

When processing experimental data for a significance level of $p = 0.05$, the following statistical criteria were used: Cochran's criterion – to assess the homogenity of the variance; Student's test – to assess the significance of the calculated coefficients; Fisher's criterion – to assess the adequacy of the obtained regression equations **(Moscattello et al., 2019; Michalska et al., 2016; Xiang et al., 2020)**.

To assess the quality of plum fruits and to highlight the best ones in terms of a set of signs, a regression, correlation analysis of technical, biochemical indicators was carried out **(Reichel et al., 2017; Prichko, Machneva and Drofícheva 2020)**. A model of the relationship between the quality of fruits (*Y*-sensory assessment) and complex chemical indicators has been obtained. The regression equation for plum varieties looks like this at ($R = 0.914$):

$$Y = 8.2 - 0.3X_1 - 0.004X_2 + 0.11X_3 - 0.16X_4 + 0.66X_5 + 0.7X_6 + 0.001X_7 - 0.0002X_8;$$

Where:

-$X_1$ – is the shape index; $X_2$ – average weight, $X_3$ – dry matter; $X_4$ – acidity, $X_5$ – sugar; $X_6$ – vitamin *C*, $X_7$ – vitamin *P*, $X_8$ – anthocyanins.

Good agreement between the experimental and calculated data indicates the adequacy of the found regression equations.

Organoleptic evaluation of candied plum fruits of the variety: Stanley, President, Grossa di Felisio, August Delish was carried out (Figure 2).

The candied fruits of the plum variety Grossa di Felisio were distinguished by their pronounced sour tastes, due to the high acidity of fresh fruits.

Based on the organoleptic assessment carried out, it can be concluded that there is a prospect of using such varieties as Stanley and President for the production of candied fruits, which have a pleasant taste and appearance, with a high tasting score – 4.9 points. The technical characteristics of the quality of plum and candied fruits of the following varieties have been established: President and Stanley (Figure 3).

Taking into account the peculiarities of the chemical composition of candied fruits from the fruits of plum varieties: Stanley and the President, a comparative mathematical calculation of the energy value of the finished product was made (Table 6).

The varieties that carry high valuable technical and biochemical characteristics have been identified. According to the content of biologically active substances of the 7 studied varieties, the following are distinguished: Grossa di Felisio, Blue moon, Stanley. The most valuable varieties for processing in terms of accumulation of total polyphenols, anthocyanins, vitamin C, P are Grossa di Felisio and Blue moon. According to the content of minerals (potassium, sodium, calcium), capable of maintaining the structure of fruit tissue during processing, the President variety stands out. Theoretically and experimentally confirmed the possibility of forming the quality of candied fruit based on the complex influence of the factors of the technological process, taking into account their variation within the specified limits and characteristics of varieties. According to the technical indicators that appear during processing, the fruits of the plum variety proved to be unsuitable for the production of candied fruits: variety Blue moon after the first filling with syrup, the structure of the fruit tissue changed and the finished candied fruits did not look attractive to the consumer.

According to the results of the organoleptic evaluation, candied fruits from plum fruits of the Grossa di Felisio variety were distinguished by pronounced sour tastes, due to the high acidity of fresh fruits, for the production of which it was recommended to increase the sugar consumption rate. Candied fruits from plum fruits of the August Delish variety had a pale, slightly colored appearance with a slightly harmonious taste.

## CONCLUSION

Based on the studies carried out, experimental data were obtained on the influence of the varietal characteristics of plum fruits on the technological indicators of the quality of candied fruits and the yield of the finished product was determined. The results of experimental biochemical and technological analyzes of fruits at individual technological stages of production made it possible to judge the influence of varietal characteristics on the quality indicators of the finished product. For the industrial production of candied fruits, plum varieties with high biochemical and technical indicators are recommended: Stanley, Big Stanley, President, Kabardinskaya early, Grossa di Felisio.

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# IMPROVING THE METHOD OF DETERMINING THE MASS FRACTION OF MAGNESIUM CARBONATE AND THE STUDY OF THE CHEMICAL COMPOSITION OF CARBONATE ROCKS FOR THE EFFECTIVE CONDUCT OF THE TECHNOLOGICAL PROCESS OF SUGAR PRODUCTION

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## ABSTRACT
The article considers an improved method for determining the content of magnesium carbonate in the carbonate rock. An adjusted method for determining the content of magnesium carbonate was included in the complete establishment of chemical analysis of limestone, which includes the determination of moisture, impurities insoluble in hydrochloric acid, the amount of one and a half oxides of aluminum and iron, calcium carbonate and magnesium carbonate (advanced method), calcium sulfate, alkali metal oxides of potassium and sodium. The obtained experimental data were entered into a single table and summed up the material balance of all components of the carbonate rock. As a result, it was found that this technique includes the following criteria: it is the most accurate, most accessible, and cheap. The use of adjusted methods for determining the content of calcium and magnesium carbonate in limestone will make it possible to establish the objective chemical composition of the carbonate rock and avoid several technological problems. Namely, the excess of uncontrolled magnesium carbonate contributes to the formation of the liquid phase, which in turn reduces the concentration of chemically active lime and promotes the formation of melts in the lime kiln, deteriorating filtration rates, clogging the evaporating station, and so on. Therefore, having information about the real component composition of limestone, the technologist will be able to adjust the technological process in advance, which will lead to the preservation of natural resources while the quality of finished products will not decrease.

**Keywords:** carbonate rock; material balance; sugar industry; dolomite; lime department

## INTRODUCTION
In Ukraine, limestone is used in the technological process of sugar production to purify diffusion juice from non-sugars. Initially, limestone is fired at high temperatures to obtain lime and carbon dioxide. Then the lime is quenched with water to obtain lime milk. Lime milk ($Ca(OH)_2$) and carbon dioxide ($CO_2$) are used to effectively remove non-sugars from diffusion juice.

The quality of these components ($Ca(OH)_2$ and $CO_2$) depends on the degree of the condition of the limestone. According to Ukrainian requirements, the content of calcium carbonate ($CaCO_3$) in the carbonate rock must be at least 93.00%. The mass fraction of impurities in limestone should not exceed:
- substances that are insoluble in hydrochloric acid – not more than 3.00%;
- oxides of aluminum and iron – in the amount not exceeding 1.50%;
- magnesium carbonate – not more than 2.50%;
- calcium sulfate – not more than 0.40%;
- alkali metal oxides of potassium and sodium – in the amount not exceeding 0.25%;
- impurities (clay, etc.) – not more than 3.00%.

Only by using conditioned components for the purification of diffusion juice, it is possible to receive carbonized juices of high purity. After the non-sugars are removed from the diffusion juice, the sucrose content accumulates in the system.

The yield and quality of the final product – sugar – will depend on the concentration of sucrose and the purity of the juice. Sugar is used as a finished product for human consumption and as an intermediate in the production of other foods (Figure 1).

For the needs of the sugar factory, only calcium carbonate is considered a useful component of the carbonate rock, all other components are harmful impurities **(Zheplinska et al., 2020)**. In nature, very rare limestones whose chemical composition would be close to calcite. Magnesium is often a component of limestone, if the content of magnesium oxide (MgO) in it is 4 – 17%, then such limestone is called dolomitized **(Zheplinska et al., 2019)**.

Magnesium carbonate ($MgCO_3$) contained in the carbonate rock can lead to several subsequent adverse events during its (carbonate rock) firing. Magnesium carbonate, which is part of dolomite ($CaMg(CO_2)_2$) promotes the formation of a liquid phase during its thermal dissociation. The liquid phase, in turn, envelops the grains of active calcium oxide with a non-reactive film, which leads to the fact that part of the potentially chemically active lime becomes inactive. The formed melt enters the pores of active lime, as a result of which the dissociation of a significant part of the carbonate rock will not be able to pass in full. In addition, the liquid phase in significant quantities can enter the lower zone of the furnace where it meets the colder gas solidifies with the formation of sintered depths. This phenomenon can lead to a "hang" of the furnace, and in less optimistic forecasts, and to the shutdown of the plant.

During the firing of limestone, the proportion of $MgCO_3$ contained in it decomposes into MgO and carbon dioxide ($CO_2$). Once on the plant machine during the quenching of magnesium oxide, we obtain magnesium hydroxide ($Mg(OH)_2$), and later during the saturation of magnesium carbonate ($MgCO_3$). The latter ($MgCO_3$) significantly impairs the filtration rates of saturated juice precipitates. In the face of such a situation, when there is a significant content of magnesium in lime milk, the technologist tries to maintain a high pH value (not less than 11) on the first saturation, and before the second saturation not to give lime at all **(Sukhenko et al., 2019)**. Such measures are carried out because $Mg(OH)_2$ begins to precipitate at pH 11 and above. It is in this state that this component ($Mg(OH)_2$) has a crystalline structure and is most easily removed from the system in contrast to magnesium carbonate, which has an amorphous structure.

Also, in the case of saturation of juices to a slightly alkaline or neutral reaction, Ca and Mg go into solution in the form of acidic carbon salts and at vigorous boiling of the juice precipitates at the evaporator station.

On the other hand, scientists **(Mushtruk et al., 2020a)** have studied the use of magnesium oxide for the purification of diffusion juice in the laboratory. As a result of research comparing the classical method of purification and the one proposed by the authors, it was found that the quality of purified juices and syrup was much better, namely the content of colloidal substances and color decreased, but the filtration performance deteriorated.

To date, Ukrainian scientists **(Sheiko et al., 2019)** have studied the effect of the use of dolomitized limestone on the process of purification of diffusion juice. As a result, recommendations for the use of dolomitized limestone in a sugar, factory have been developed.

Therefore, despite both the positive and negative effects of magnesium on the technological process of the sugar plant, it is very important to monitor the concentration of this component.

The current method of determining the mass fraction of magnesium carbonate in carbonate rocks **DSTU 1451-96 (1996)**, is based on the acidimetric determination of the mass fraction of magnesium carbonate in limestone by calculating the percentage of trilon B solution, which went to titrate the magnesium content to the mass of limestone. The disadvantage of this method is the high error in determining the mass fraction of magnesium carbonate in limestone.

Other authors presented a method **(Palamarchuk et al., 2019)**, which involves analysis by the complexometric method. The disadvantage of this method is its high cost because a large amount of sample is taken for titration, as well as significantly inflated test results.

It is impossible to give a complete objective chemical analysis of limestone without the use of correct methods of analysis of all components of the carbonate rock. Therefore, the inclusion of the proposed method for the determination of magnesium carbonate in the carbonate rock (**Mushtruk et al., 2020b)** and the method for the determination of calcium carbonate in the carbonate rock to **(Somaratne et al., 2019)** comprehensive determination of the chemical composition of limestone will provide objective indicators of $CaCO_3$ and $MgCO_3$. In turn, this will allow the technologist to take the necessary measures in advance to conduct the technological process, which will give the maximum economic effect and save natural resources (limestone, fuel).

Our research aimed to improve the method of determining the mass fraction of magnesium carbonate in the carbonate rock to increase the objectivity of the results and the introduction of an adjusted method to establish the full chemical composition of limestone.

## Scientific hypothesis

To increase the accuracy of experimental data, the improved method should be based on the material balance of all components of the carbonate rock. This will make it possible to self-check the accuracy of the results obtained, as having summed up the balance of all components of limestone, approximately we should get a result of 100%. Given this fact and improving the method of determining the content of calcium carbonate and magnesium carbonate in the carbonate rock and entering the results into the material balance, we hope to get the sum of all components approximately 100%, which will indicate the accuracy of the data.

## MATERIAL AND METHODOLOGY

### Samples

Carbonate rocks of 10 different deposits were used for experiments, namely with (Bogdanivsky, Hlynsko-Rozbyshivsky, Malodivytsky, Milkivsky, Kybyntsivsky, Pryluky, Rybalsky, Pivdenno-Panasivsky, Velykobubnivsky, Sukhodolivsky). To determine the concentration of magnesium carbonate, the following utensils and devices were used.

### Chemicals

Distilled water (producer «Inter-Synthesis» Limited Liability Company, Ukraine).

Hydrochloric acid (HCl, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Sulfuric acid ($H_2SO_4$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Hydrofluoric acid (HF, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Ammonia ($NH_3$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Sodium hydroxide (NaOH, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Ethyl alcohol ($C_2H_5OH$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Indicator acid chromium dark blue ($C_{16}H_9ClN_2Na_2O_9S_2$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Dry murexide indicator ($C_8H_8N_6O_6$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Trilon B ($C_{10}H_{14}N_2Na_2O_8$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

Ammonium carbonate ($(NH_4)_2CO_3$, producer «Inter-Synthesis» Limited Liability Company, Ukraine, chemically pure for analysis).

**Instruments**

Analytical scales (BTHE-6-H1K-1, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Drying cabinet (DC-300, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Desiccator (EximLab 150, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Chemical cups (CC-100, CC-150, CC-200, CC-250, CC-500, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Petri dish (producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Measuring flasks (MF-100, MF-150, MF-200, MF -250, MF-500, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Muffle furnace (SNOL 8.2/1100, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Measuring pipettes (MP-0.001, MP-0.002, MP-0.005, MP-0.01, MP-0.015, producer "Inter-Synthesis" Limited Liability Company, Ukraine).

Conical flask (CF-100, CF-150, CF-200, CF-250, CF - 500, producer "Laboratory equipment" Limited Liability Company, Ukraine).

Burette for titration (producer "Laboratory equipment" Limited Liability Company, Ukraine).

Filters (producer "Laboratory equipment" Limited Liability Company, Ukraine).

Photometer (eXact® Micro 20, producer “Inter-Synthesis” Limited Liability Company, Ukraine).

**Laboratory Methods**

For technological evaluation of carbonate rocks, their complete chemical analysis was performed, which includes the determination of mass fractions of calcium carbonate ($CaCO_3$) (according to an improved method); impurities insoluble in hydrochloric acid ($SiO_2$); the sum of one and a half oxides of aluminum and iron ($Fe_2O_3$ + $AI_2O_3$); magnesium carbonate ($MgCO_3$) (according to an improved method); calcium sulfate ($CaSO_4$); alkali metal oxides of potassium and sodium ($K_2O$ + $Na_2O$); moisture content.

Determination of moisture content was carried out by the method of which the essence is that the weight loss of a sample of limestone after drying at a temperature of 105 – 110 ºC.

The method of determining the content of the mass fraction of impurities insoluble in hydrochloric acid, which was used was that a portion of the carbonate rock was treated with hydrochloric acid. Silicates that are part of limestone decompose to form metal chlorides and silicic acids. The resulting solution was evaporated and dried to convert the silicic acid into an insoluble form. The precipitate was then washed with hydrochloric acid to convert to a solution of salts of iron, aluminum, and magnesium, which are poorly soluble in water. The washed silicic acids were filtered, calcined, and weighed.

The method of determining the mass fraction of iron and aluminum was based on the treatment of the filtrate obtained after precipitation of impurities insoluble in hydrochloric acid, aqueous ammonia solution. As a result of such actions, iron, and aluminum ions in a slightly alkaline medium precipitate in the form of iron and aluminum hydroxides. The precipitate obtained was filtered off and calcined, after which iron and aluminum oxides were obtained and weighed.

The mass fraction of calcium sulfate was determined by a weight method based on precipitated anion $SO^4_2$-barium chloride, which forms insoluble barium sulfate in an acidic environment. The resulting solution was then filtered, calcined, and weighed.

The content of calcium carbonate and magnesium carbonate was determined by a complexometric method, which is based on the fact that calcium and magnesium with indicators (murexide and acid chromium dark blue) in an alkaline environment form complexes with a certain color. When titrated with Trilon B, these complexes form colorless compounds. The titration is considered complete when the first stable color change of the solution takes place.

To determine the mass fraction of alkali metal oxides of potassium and sodium in the carbonate rock, 1 g of crushed carbonate rock was taken from the prepared sample and placed in a crucible, the carbonate rock was moistened with distilled water; 8 – 10 $cm^3$ of the hydrofluoric acid solution was added. in a ratio of 1:2, then evaporated on an electric stove, without bringing to a boil, until almost complete cessation of sulfuric anhydride vapor. A dry cooled precipitate was obtained in which 8 – 10 $cm^3$ of hydrochloric acid diluted in a ratio of 1:3 was added and heated to dissolve the salts. The container was transferred to a 100 $cm^3$ volumetric flask, cooled, neutralized with ammonia solution until the complete separation of alkali metal oxides of potassium and sodium, added 10 $cm^3$ of saturated ammonium carbonate solution, and adjusted the contents of the flask with distilled water to the mark, mixed and filtered. alkali metals of potassium and sodium in the carbonate rock.

**Description of the Experiment**

All measurements of instrument readings were performed 5 times. The number of repetitions of each experiment to determine one value was also 5 times.

## Statistical 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 2019 values were estimated using mean and standard deviations.

## RESULTS AND DISCUSSION

To increase the accuracy of experimental data, the improved method should be based on the material balance of all components of the carbonate rock. This will make it possible to self-check the accuracy of the results obtained, as having summed up the balance of all components of limestone, approximately we should get a result of 100%. The authors of scientific works **(Buniowska et al., 2017; Verma et al., 2018; Shmyrin, Kanyugina and Kuznetsov, 2017)** engaged in similar research but obtained other scientific results. Given this fact and improving the method of determining the content of calcium carbonate and magnesium carbonate in the carbonate rock and entering the results into the material balance, we hope to get the sum of all components approximately 100%, which will indicate the accuracy of the data.

The establishment of the component composition of limestone began with the determination of its moisture content. According to the results of the research (Figure 2), it is seen that all samples contained a low moisture content. The authors of scientific works **(Nadeem et al., 2018; Avalos-Llano, Molina and Sgroppo, 2020; Rahimi et al., 2020)** do not believe that the concentration ($CO_2$) is not an important factor in the technological process of sugar production.

Therefore, the concentration of moisture contained in the experimental samples of limestone will not affect the dissociation process, and consequently the technological process as a whole.

According to the recommendations **DSTU 1451-96 (1996)**, which are put forward to carbonate rocks in Ukraine, to further use them in the sugar industry, the mass fraction of impurities insoluble in hydrochloric acid should not exceed 3%. The obtained data (Figure 3) indicate that samples No. 3, 6, 9, 10 contain an increased concentration of silicon dioxide. The authors of the following scientific papers **(Bober et al., 2020; Luo et al., 2019; Dhar et al., 2015)** other methods for determining impurities were used for similar scientific research.

During the firing of such limestones, refractory compounds such as calcium and magnesium silicates can be formed. It is these compounds (calcium silicates) that cause irreversible loss of lime. Once on the plant machine, silicates can settle on the heating surface of the evaporator. Given all the above negative consequences, the use of carbonate rocks under No. 3, 6, 9, and 10 in the sugar industry is not possible.

Limestone's were further analyzed for the content of one and a half oxides of aluminum and iron ($AI_2O_3 + Fe_2O_3$). The authors of scientific works **(Guo et al., 2018; Zheplinska, Mushtruk and Salavor, 2020; Jiang, 2015; Almohammed et al., 2015)** recommends not to use such samples in the production process.

Super-important components, as they form low-melting aluminates and ferrites from CaO during firing, which are the main component of the liquid phase in the furnace. It (liquid phase) reduces the mass fraction of free lime in quicklime because a significant part of bound lime is formed (in the form of aluminates and calcium ferrites), and also envelops chemically active lime with non-reactive film, thereby increasing the concentration of inactive lime. Melting leads to soldering of pieces of limestone between themselves and the lining, which in turn increases the amount of overheating and the stability of the lining The authors of the following scientific works **(Ivanova et al., 2021; Lee et al., 2012; Lebovka et al., 2007)** recommend not to use such samples in the production process. The approximate composition of quicklime is given in **(Noguiera Felix et al., 2019)**.

Impurities $AI_2O_3 + Fe_2O_3$ passing into lime milk and further on the machine of the plant get into juice promoting formation in the saturated juices of a deposit which is badly filtered.

According to the recommendations of **DSTU 1451-96 (1996)** $AI_2O3 + Fe_2O_3$, the content of one and a half oxides in limestones, which are suitable for firing in lime-fired kilns at the sugar factory, should be in the amount of not more than 1.5% by weight of dry matter. In subsequent scientific works **(Katariya, Arya and Pandit, 2020; Kim et al., 2016; Kozelová et al., 2011; Krasulya et al., 2016)** such studies were not conducted.

Observing Figure 3, we see that samples No. 1, 3, 5, 6, 7, 9, 10 contain an increased concentration of iron and aluminum oxides.

Therefore, given the above dangers, the use of limestone with a high content of $AI_2O3 + Fe_2O_3$ can not be recommended for the needs of the sugar factory.

The next test component was calcium sulfate ($CaSO_4$). The recommended normative value **DSTU 1451-96 (1996)** of $CaSO_4$ content in carbonate rock is not more than 0.4% by weight of dry matter. The greatest danger that this impurity can cause is that it easily precipitates on the surface of the evaporator. As well as the process of hydration of lime in the presence of $CaCO_4$ and $CaCO_3$ is greatly slowed down.

As the results of research (Figure 4) show, samples No. 1, 3, 6, 10 exceed the recommended norms (calcium sulfate content), so their use will lead to the above negative consequences.

Determination of calcium and magnesium carbonate content was carried out as follows:

Pipette 15 $cm^3$ of solution into a 250 $cm^3$ conical flask, add 100 $cm^3$ of distilled water and 25 $cm^3$ of 20% sodium hydroxide solution from a volumetric flask containing the filtrate after precipitation of iron and aluminum oxides. After 1 – 2 min on the tip of a spatula made 0.1 – 0.2 g of murexide in a mixture with potassium chloride. The solution was stirred and titrated with 0.1 M solution of Trilon B until the transition from red to purple, which was observed on a black background. The authors of scientific works **(Dehghannya et al., 2018; Zheplinska et al., 2021; Zhu et al., 2016; Zdziennicka et al., 2017)** used other standardized methods to conduct similar studies.

In parallel with the breakdown, distilled water was monitored for calcium carbonate content. To control the distilled water for the content of calcium carbonate, 25 $cm^3$ of 20% sodium hydroxide solution was added to the distilled water. After 1 – 2 min on the tip of a spatula made 0.1 – 0.2 g of murexide in a mixture with potassium chloride. The solution was stirred and titrated with 0.1 M solution of Trilon B until a transition from red to purple, which was observed on a black background.



**Figure 1** Photo of the final product (beet sugar).

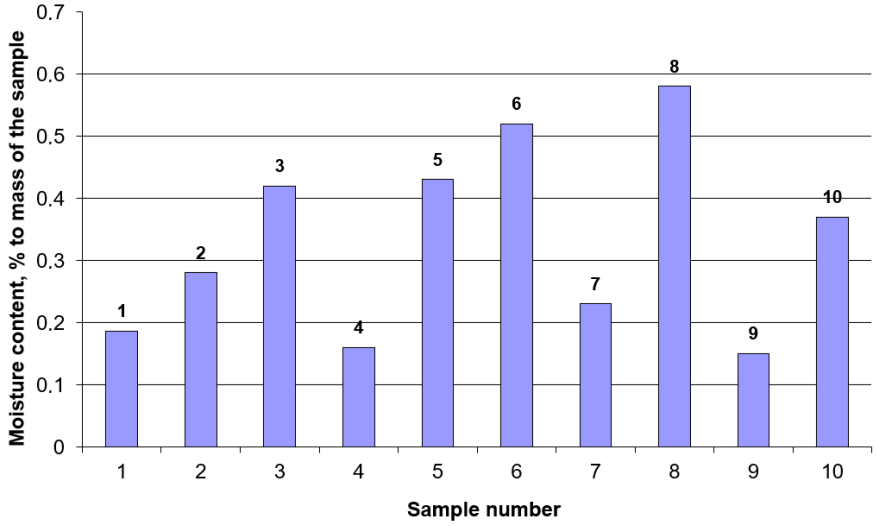


**Figure 2** Moisture content in the studied samples of carbonate rocks.

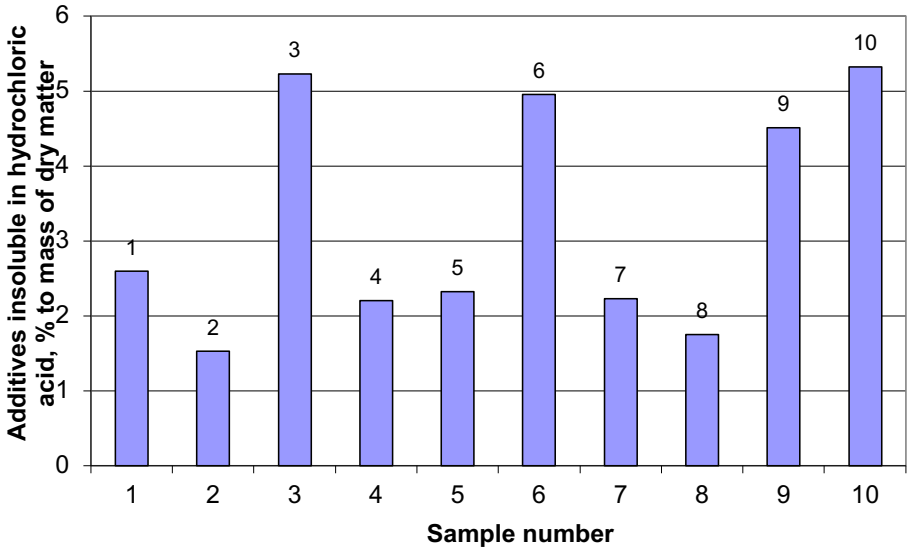


**Figure 3** The content of additives insoluble in hydrochloric acid in the studied samples of carbonate rocks.

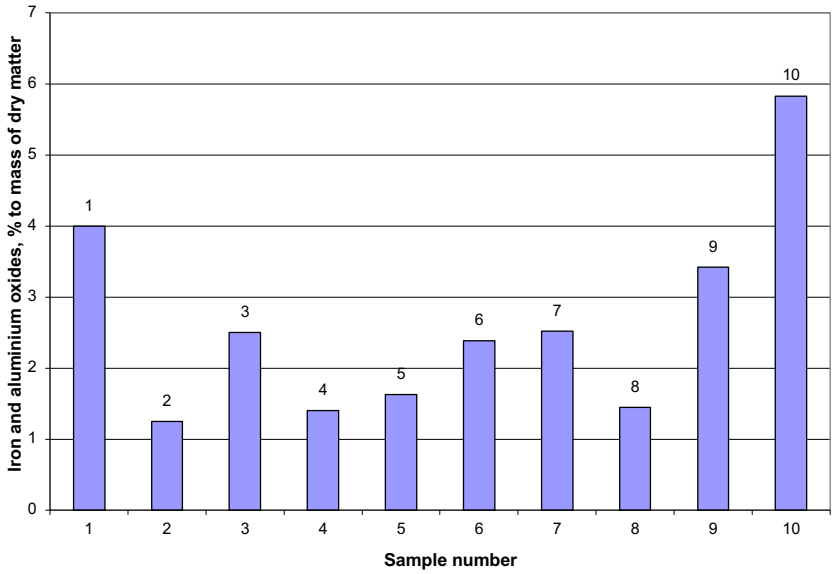


**Figure 4** The content of iron and aluminum oxides in the studied samples of carbonate rocks.

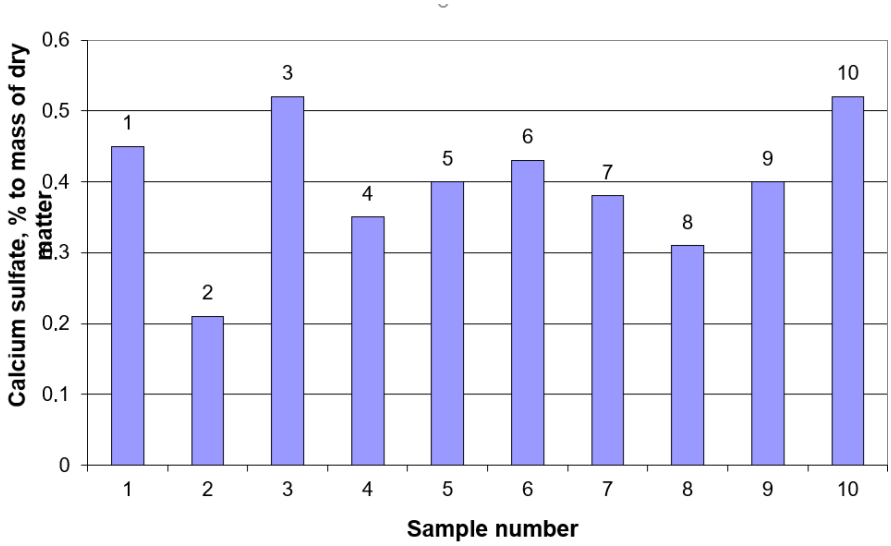


**Figure 5** The content of calcium sulfate in the studied samples of carbonate rocks.

**Table 1** The content of calcium carbonate in the studied samples of carbonate rocks.

| Sample | According to DSTU 1451-96 (1997) | | According to the newly introduced method | |
|---|---|---|---|---|
| | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| No. 1 | 180.68 | 181.02 | 90.23 | 90.4 |
| No. 2 | 190.33 | 190.86 | 95.03 | 95.3 |
| No. 3 | 176.09 | 176.83 | 87.82 | 88.20 |
| No. 4 | 186.89 | 187.19 | 93.35 | 93.5 |
| No. 5 | 185.19 | 185.98 | 92.4 | 92.8 |
| No. 6 | 177.83 | 178.75 | 88.86 | 89.32 |
| No. 7 | 182.53 | 182.99 | 91.12 | 91.35 |
| No. 8 | 187.25 | 188.34 | 93.66 | 94.2 |
| No. 9 | 174.85 | 175.11 | 87.37 | 87.5 |
| No. 10 | 167.25 | 167.87 | 83.49 | 83.8 |

The difference between the volume of 0.1 M solution of Trilon B, which went to the titration of the sample, which used the filtrate after precipitation of iron and aluminum oxides, and the volume of 0.1 M solution of Trilon B, which went to the titration, which controlled distilled water, makes it possible to set the volume of 0.1 M solution of Trilon B, which went to the titration of calcium – b, $cm^3$. After that, the mass fraction of calcium carbonate in the carbonate rock was calculated **(Smetanska et al., 2021)**.

In Ukraine, only high-calcium carbonate rocks are used for the sugar industry, in which the $CaCO_3$ content is at least 93% by weight of dry matter. The results of determining the content of the mass fraction of calcium carbonate in the carbonate rock are given in Table 1 and Figure 5.

Observing Figure 5 we see that samples No. 1, 3, 5, 6, 7, 9, 10 are not recommended for use in the technological process of sugar production due to the low content of calcium carbonate.

Control over the content of magnesium carbonate in the carbonate rock was carried out as follows:

The volume of Trilon B spent on titration of magnesium in the carbonate rock was calculated by the difference between the volumes of Trilon B spent on titration of calcium and magnesium – a, and separately calcium – b.

To determine the volume of Trilon B, which went to the titration of calcium and magnesium, from the filtrate after precipitation of iron and aluminum oxides, took a sample of 15 $cm^3$, which corresponds to 0.15 g of carbonate rock and is a sample of carbonate rock – m, in grams, which was taken into account during the calculation of the mass fraction of magnesium carbonate in the carbonate rock according to formula 1. To the sample was added 100 $cm^3$ of distilled water, 10 $cm^3$ of ammonia buffer solution and on the tip of a spatula made 0.1 – 0.2 g of dark chromium acid indicator mixture, stirred vigorously with a glass rod, and titrated with 0.1 M solution of Trilon B until the transition of red-purple to blue. The author of **(Sasikumar, Chutia and Deka, 2019)** used other standardized methods to conduct similar studies.

In parallel with the breakdown, distilled water was monitored for magnesium and calcium carbonate content. Control of distilled water for the content of magnesium and calcium carbonate involves the introduction into the distilled water of 10 $cm^3$ of ammonia-buffer solution and dark blue acid chromium indicator, followed by titration of water with 0.1 M solution of Trilon B. The difference between the volume of 0.1 M solution of Trilon B, spent on the titration of the sample, which uses the filtrate after precipitation of iron and aluminum oxides, and a volume of 0.1 M solution of Trilon B, spent on titration, where the control of distilled water, made it possible to establish the volume of 0.1 M solution of Trilon B spent on titration of calcium and magnesium – a, $cm^3$.

The mass fraction of magnesium carbonate in the carbonate rock was calculated by the formula:

$$X = \left[\frac{(a-b)\cdot K\cdot 0.004498\cdot 100}{m}\right]\cdot\frac{100}{100-W}, \qquad (1)$$

Where:

$X$ – mass fraction of magnesium carbonate in the carbonate rock,%;

a is the volume of 0.1 M solution of Trilon B, which went to the titration of calcium and magnesium, $cm^3$;

b is the volume of 0.1 M solution of Trilon B, which went to the titration of calcium, $cm^3$;

0.004498 – coefficient indicating the mass of magnesium carbonate, which titrates 1 $cm^3$ of Trilon B solution with a concentration of 0.1 $mol.dm^{-3}$, g;

K is the correction factor for the solution of Trilon B;

m – portion of carbonate rock, corresponding to 0.15 g;

W – a mass fraction of moisture,%.

The results of determining the mass fraction of magnesium carbonate in the carbonate rock are shown in Table 2 and Figure 7.

From Table 2 and Table 3, and Figure 6 we see that the results of research obtained by the method **DSTU 1451-96 (1996)** have slightly inflated indicators. Therefore, the above-mentioned improved method for determining $MgCO_3$ was proposed, which gives more accurate results (Table 3).

Given that the share of magnesium carbonate in carbonate rocks should not exceed 2.5% **DSTU 1451-96 (1996)**, we see that samples No. 3, 5, 6, 9, 10 do not meet the stated standards. Therefore, in those samples where there is an inflated content of $MgCO_3$ technologist, it is necessary to correct the process following the recommendations given at the beginning of the article.

Control of alkali metal oxides of potassium and sodium ($K_2O$ + $Na_2O$) is carried out to reduce the concentration of molasses in the system, which they are. $K_2O$ and $Na_2O$ also form fusible compounds during firing. The mass fraction of potassium and sodium oxides in the carbonate rock according to the recommendations **DSTU 1451-96 (1996)** should not exceed the amount of not more than 0.25% by weight of dry matter. Figure 8 shows that samples No. 1 and 3 cannot be recommended for use in the sugar industry.

To objectively conduct a technological audit of the lime department of the sugar factory, it is necessary to use the correct methods of control of the parameters of technological indicators. One component of the technological audit of the lime department is the chemical composition of the carbonate rock, which is fed for firing. The quality of finished products and their cost will mainly depend on the condition of raw materials. Therefore, to objectively assess the quality of the studied limestones, the concentration of all components included in them was summarized in one table (Table 3) and the balance of all impurities and calcium carbonate was adjusted for each sample. When determining the chemical composition of the carbonate rock, the sum of the mass fractions of $CaCO_3$ and impurities should be approximately 100%. From the Table 3 we see that all analyzed samples according to the method **DSTU 1451-96 (1996)** have inflated results. In turn, the lined material balance of all components of the carbonate rock and in all samples according to the proposed method **(Cervantes-Elizarrarás et al., 2017)** approached 100%. This indicates the high accuracy of the method and the possibility of its use in the production process.

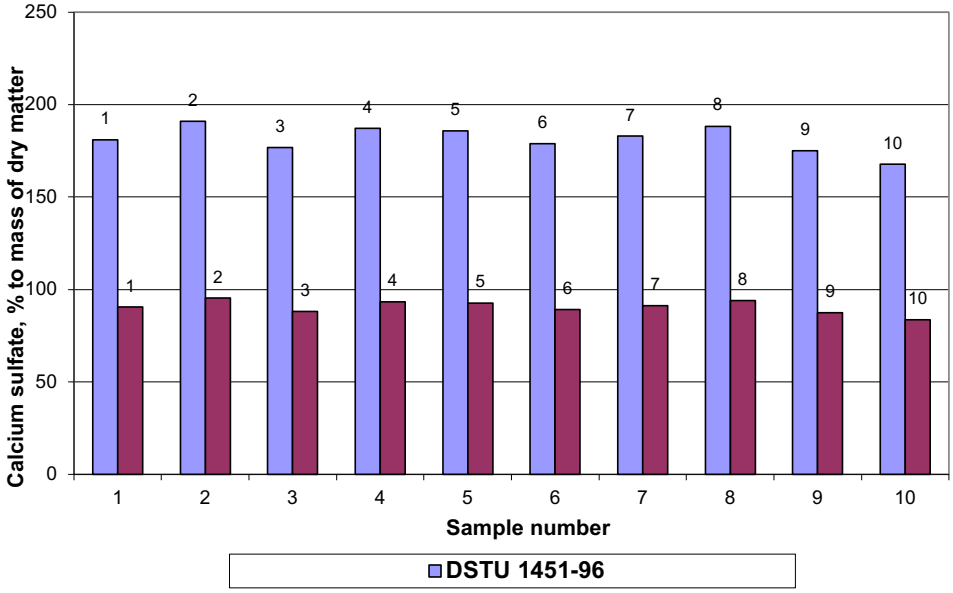


**Figure 6** The content of calcium carbonate in the studied samples of carbonate.

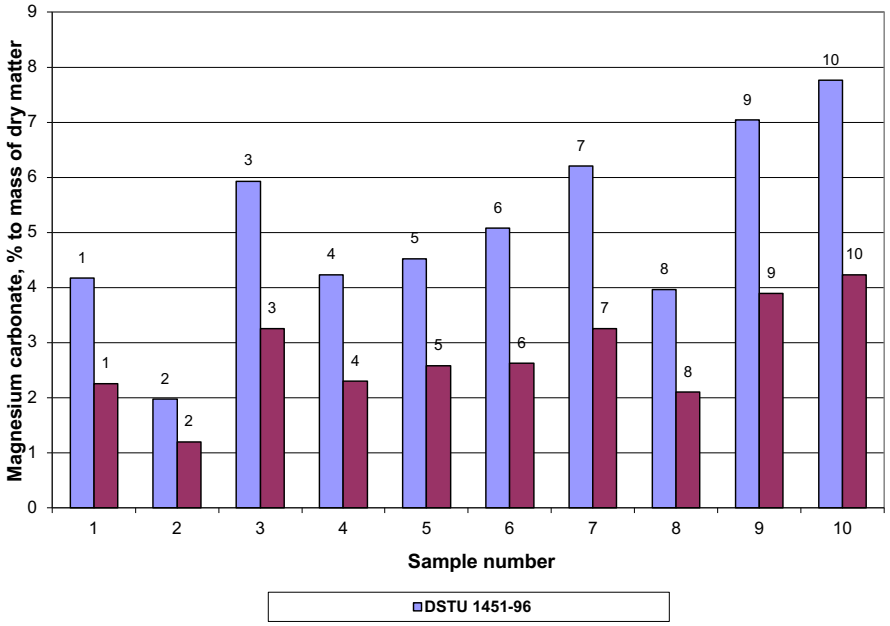


**Figure 7** The content of magnesium carbonate in the studied samples of carbonate rocks.

**Table 2** The content of magnesium carbonate in the studied samples of carbonate rocks.

| Sample | According to DSTU 1451-96 (1997) | | According to the newly introduced method | |
|---|---|---|---|---|
| | % to probe mass | % to dry matter mass | % to probe mass | % to probe mass |
| No. 1 | 4.16 | 4.17 | 2.25 | 2.25 |
| No. 2 | 1.97 | 1.97 | 1.2 | 1.2 |
| No. 3 | 5.90 | 5.93 | 3.24 | 3.25 |
| No. 4 | 4.22 | 4.23 | 2.15 | 2.3 |
| No. 5 | 4.50 | 4.52 | 2.57 | 2.58 |
| No. 6 | 5.06 | 5.08 | 2.61 | 2.62 |
| No. 7 | 6.18 | 6.2 | 3.24 | 3.25 |
| No. 8 | 3.94 | 3.96 | 2.08 | 2.10 |
| No. 9 | 7.03 | 7.04 | 3.88 | 3.89 |
| No. 10 | 7.73 | 7.76 | 4.21 | 4.23 |

**Table 3** Chemical composition of the studied samples of carbonate rocks.

| | Carbon bedrock component | Sample 1 | | | | Sample 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.186 | | 0.186 | | 0.28 | | 0.28 | |
| | Moderate | 0.186 | - | 0.186 | - | 0.28 | - | 0.28 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 2.6 | 2.6 | 2.6 | 2.6 | 1.52 | 1.53 | 1.52 | 1.53 |
| | | 2.6 | 2.6 | 2.6 | 2.6 | 1.52 | 1.53 | 1.52 | 1.53 |
| | Moderate | 2.6 | 2.6 | 2.6 | 2.6 | 1.52 | 1.53 | 1.52 | 1.53 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 3.96 | 3.99 | 3.96 | 3.99 | 1.25 | 1.25 | 1.25 | 1.25 |
| | | 3.98 | 4.10 | 3.98 | 4.10 | 1.25 | 1.25 | 1.25 | 1.25 |
| | Moderate | 3.97 | 4.0 | 3.97 | 4.0 | 1.25 | 1.25 | 1.25 | 1.25 |
| 4. | Calcium carbonate ($CaCO_3$) | 180.67 | 181.01 | 90.24 | 90.5 | 190.32 | 190.85 | 95.03 | 95.3 |
| | | 180.69 | 181.03 | 90.22 | 90.3 | 190.34 | 190.87 | 95.03 | 95.3 |
| | Moderate | 180.68 | 181.02 | 90.23 | 90.4 | 190.33 | 190.86 | 95.03 | 95.3 |
| 5. | Magnesium carbonate ($MgCO_3$) | 4.16 | 4.16 | 2.24 | 2.24 | 1.97 | 1.97 | 1.2 | 1.2 |
| | | 4.16 | 4.18 | 2.26 | 2.26 | 1.97 | 1.97 | 1.2 | 1.2 |
| | Moderate | 4.16 | 4.17 | 2.25 | 2.25 | 1.97 | 1.97 | 1.2 | 1.2 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.45 | 0.45 | 0.45 | 0.45 | 0.21 | 0.21 | 0.21 | 0.21 |
| | | 0.45 | 0.45 | 0.45 | 0.45 | 0.21 | 0.21 | 0.21 | 0.21 |
| | Moderate | 0.45 | 0.45 | 0.45 | 0.45 | 0.21 | 0.21 | 0.21 | 0.21 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.30 | 0.30 | 0.30 | 0.30 | 0.15 | 0.15 | 0.15 | 0.15 |
| | | 0.30 | 0.30 | 0.30 | 0.30 | 0.15 | 0.15 | 0.15 | 0.15 |
| | Moderate | 0.30 | 0.30 | 0.30 | 0.30 | 0.15 | 0.15 | 0.15 | 0.15 |
| | Summary of the carbon bedrock component | | 192.54 | | 100 | | 195.97 | | 99.64 |

Continuation of **Table 3**.

| | Carbon bedrock component | Sample 3 | | | | Sample 4 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.42 | | 0.42 | | 0.16 | | 0.16 | |
| | | 0.42 | | 0.42 | | 0.16 | | 0.16 | |
| | Moderate | 0.42 | - | 0.42 | - | 0.16 | - | 0.16 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 5.21 | 5.23 | 5.21 | 5.23 | 2.20 | 2.20 | 2.20 | 2.20 |
| | | 5.21 | 5.23 | 5.21 | 5.23 | 2.20 | 2.20 | 2.20 | 2.20 |
| | Moderate | 5.21 | 5.23 | 5.21 | 5.23 | 2.20 | 2.20 | 2.20 | 2.20 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 2.49 | 2.50 | 2.49 | 2.50 | 1.4 | 1.4 | 1.4 | 1.4 |
| | | 2.49 | 2.50 | 2.49 | 2.50 | 1.4 | 1.4 | 1.4 | 1.4 |
| | Moderate | 2.49 | 2.50 | 2.49 | 2.50 | 1.4 | 1.4 | 1.4 | 1.4 |
| 4. | Calcium carbonate ($CaCO_3$) | 176.08 | 176.82 | 87.82 | 88.20 | 186.90 | 187.20 | 93.35 | 93.5 |
| | | 176.10 | 176.84 | 87.82 | 88.20 | 186.88 | 187.18 | 93.35 | 93.5 |
| | Moderate | 176.09 | 176.83 | 87.82 | 88.20 | 186.89 | 187.19 | 93.35 | 93.5 |
| 5. | Magnesium carbonate ($MgCO_3$) | 5.90 | 5.93 | 3.24 | 3.25 | 4.22 | 4.23 | 2.15 | 2.3 |
| | | 5.90 | 5.93 | 3.24 | 3.25 | 4.22 | 4.23 | 2.15 | 2.3 |
| | Moderate | 5.90 | 5.93 | 3.24 | 3.25 | 4.22 | 4.23 | 2.15 | 2.3 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.52 | 0.52 | 0.52 | 0.52 | 0.35 | 0.35 | 0.35 | 0.35 |
| | | 0.52 | 0.52 | 0.52 | 0.52 | 0.35 | 0.35 | 0.35 | 0.35 |
| | Moderate | 0.52 | 0.52 | 0.52 | 0.52 | 0.35 | 0.35 | 0.35 | 0.35 |
| 7. | Alkali metal oxides (K O+$Na_2O$) | 0.28 | 0.28 | 0.28 | 0.28 | 0.20 | 0.20 | 0.20 | 0.20 |
| | | 0.28 | 0.28 | 0.28 | 0.28 | 0.20 | 0.20 | 0.20 | 0.20 |
| | Moderate | 0.28 | 0.28 | 0.28 | 0.28 | 0.20 | 0.20 | 0.20 | 0.20 |
| | Summary of the carbon bedrock component | | 191.29 | | 99.98 | | 195.57 | | 99.95 |

Continuation of **Table 3**.

| | Carbon bedrock component | Sample 5 | | | | Sample 6 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.43<br>0.43 | | 0.43<br>0.43 | | 0.52<br>0.52 | | 0.52<br>0.52 | |
| | Moderate | 0.43 | - | 0.43 | - | 0.52 | - | 0.52 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 2.31<br>2.31 | 2.32<br>2.32 | 2.31<br>2.31 | 2.32<br>2.32 | 4.92<br>4.92 | 4.95<br>4.95 | 4.92<br>4.92 | 4.95<br>4.95 |
| | Moderate | 2.31 | 2.32 | 2.31 | 2.32 | 4.92 | 4.95 | 4.92 | 4.95 |
| 3. | Oxides ($Al_2O_3$+$Fe_2O_3$) | 1.62<br>1.62 | 1.63<br>1.63 | 1.62<br>1.62 | 1.63<br>1.63 | 2.37<br>2.37 | 2.38<br>2.38 | 2.37<br>2.37 | 2.38<br>2.38 |
| | Moderate | 1.62 | 1.63 | 1.62 | 1.63 | 2.37 | 2.38 | 2.37 | 2.38 |
| 4. | Calcium carbonate ($CaCO_3$) | 185.18<br>185.20 | 185.97<br>185.99 | 92.4<br>92.4 | 92.8<br>92.8 | 177.83<br>177.83 | 178.75<br>178.75 | 88.86<br>88.86 | 89.32<br>89.32 |
| | Moderate | 185.19 | 185.98 | 92.4 | 92.8 | 177.83 | 178.75 | 88.86 | 89.32 |
| 5. | Magnesium carbonate ($MgCO_3$) | 4.50<br>4.50 | 4.52<br>4.52 | 2.57<br>2.57 | 2.58<br>2.58 | 5.06<br>5.06 | 5.08<br>5.08 | 2.61<br>2.61 | 2.62<br>2.62 |
| | Moderate | 4.50 | 4.52 | 2.57 | 2.58 | 5.06 | 5.08 | 2.61 | 2.62 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.4<br>0.4 | 0.4<br>0.4 | 0.4<br>0.4 | 0.4<br>0.4 | 0.43<br>0.43 | 0.43<br>0.43 | 0.43<br>0.43 | 0.43<br>0.43 |
| | Moderate | 0.4 | 0.4 | 0.4 | 0.4 | 0.43 | 0.43 | 0.43 | 0.43 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.23<br>0.23 | 0.23<br>0.23 | 0.23<br>0.23 | 0.23<br>0.23 | 0.21<br>0.21 | 0.21<br>0.21 | 0.21<br>0.21 | 0.21<br>0.21 |
| | Moderate | 0.23 | 0.23 | 0.23 | 0.23 | 0.21 | 0.21 | 0.21 | 0.21 |
| Summary of the carbon bedrock component | | | 195.08 | | 99.96 | | 191.8 | | 99.91 |

Continuation of **Table 3**.

| | Carbon bedrock component | Sample 7 | | | | Sample 8 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.23 | | 0.23 | | 0.58 | | 0.58 | |
| | | 0.23 | | 0.23 | | 0.58 | | 0.58 | |
| | Moderate | 0.23 | - | 0.23 | - | 0.58 | - | 0.58 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 2.22 | 2.23 | 2.22 | 2.23 | 1.74 | 1.75 | 1.74 | 1.75 |
| | | 2.22 | 2.23 | 2.22 | 2.23 | 1.74 | 1.75 | 1.74 | 1.75 |
| | Moderate | 2.22 | 2.23 | 2.22 | 2.23 | 1.74 | 1.75 | 1.74 | 1.75 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 2.51 | 2.52 | 2.51 | 2.52 | 1.44 | 1.45 | 1.44 | 1.45 |
| | | 2.51 | 2.52 | 2.51 | 2.52 | 1.44 | 1.45 | 1.44 | 1.45 |
| | Moderate | 2.51 | 2.52 | 2.51 | 2.52 | 1.44 | 1.45 | 1.44 | 1.45 |
| 4. | Calcium carbonate ($CaCO_3$) | 182.53 | 182.99 | 91.12 | 91.35 | 187.26 | 188.35 | 93.66 | 94.2 |
| | | 182.53 | 182.99 | 91.12 | 91.35 | 187.24 | 188.33 | 93.66 | 94.2 |
| | Moderate | 182.53 | 182.99 | 91.12 | 91.35 | 187.25 | 188.34 | 93.66 | 94.2 |
| 5. | Magnesium carbonate ($MgCO_3$) | 6.18 | 6.2 | 3.24 | 3.25 | 3.94 | 3.96 | 2.08 | 2.10 |
| | | 6.18 | 6.2 | 3.24 | 3.25 | 3.94 | 3.96 | 2.08 | 2.10 |
| | Moderate | 6.18 | 6.2 | 3.24 | 3.25 | 3.94 | 3.96 | 2.08 | 2.10 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.38 | 0.38 | 0.38 | 0.38 | 0.31 | 0.31 | 0.31 | 0.31 |
| | | 0.38 | 0.38 | 0.38 | 0.38 | 0.31 | 0.31 | 0.31 | 0.31 |
| | Moderate | 0.38 | 0.38 | 0.38 | 0.38 | 0.31 | 0.31 | 0.31 | 0.31 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.20 | 0.20 | 0.20 | 0.20 | 0.18 | 0.18 | 0.18 | 0.18 |
| | | 0.20 | 0.20 | 0.20 | 0.20 | 0.18 | 0.18 | 0.18 | 0.18 |
| | Moderate | 0.20 | 0.20 | 0.20 | 0.20 | 0.18 | 0.18 | 0.18 | 0.18 |
| Summary of the carbon bedrock component | | | 194.52 | | 99.93 | | 195.99 | | 99.99 |

Continuation of **Table 3**.

| | Carbon bedrock component | Sample 9 | | | | Sample 10 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | | Follwing method DSTU 1451-96 (1997) | | According to the newly introduced method | |
| | | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass | % to probe mass | % to dry matter mass |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. | Moisture | 0.15 | | 0.15 | | 0.37 | | 0.37 | |
| | | 0.15 | | 0.15 | | 0.37 | | 0.37 | |
| | Moderate | 0.15 | - | 0.15 | - | 0.37 | - | 0.37 | - |
| 2. | Impurities insoluble in hydrochloric acid, ($SiO_2$) | 4.50 | 4.51 | 4.50 | 4.51 | 5.30 | 5.32 | 5.30 | 5.32 |
| | | 4.50 | 4.51 | 4.50 | 4.51 | 5.30 | 5.32 | 5.30 | 5.32 |
| | Moderate | 4.50 | 4.51 | 4.50 | 4.51 | 5.30 | 5.32 | 5.30 | 5.32 |
| 3. | Oxides ($AI_2O_3$+$Fe_2O_3$) | 3.41 | 3.42 | 3.41 | 3.42 | 5.81 | 5.83 | 5.81 | 5.83 |
| | | 3.41 | 3.42 | 3.41 | 3.42 | 5.81 | 5.83 | 5.81 | 5.83 |
| | Moderate | 3.41 | 3.42 | 3.41 | 3.42 | 5.81 | 5.83 | 5.81 | 5.83 |
| 4. | Calcium carbonate ($CaCO_3$) | 174.85 | 175.11 | 87.37 | 87.5 | 167.26 | 167.88 | 83.49 | 83.8 |
| | | 174.85 | 175.11 | 87.37 | 87.5 | 167.24 | 167.86 | 83.49 | 83.8 |
| | Moderate | 174.85 | 175.11 | 87.37 | 87.5 | 167.25 | 167.87 | 83.49 | 83.8 |
| 5. | Magnesium carbonate ($MgCO_3$) | 7.03 | 7.04 | 3.88 | 3.89 | 7.73 | 7.76 | 4.21 | 4.23 |
| | | 7.03 | 7.04 | 3.88 | 3.89 | 7.73 | 7.76 | 4.21 | 4.23 |
| | Moderate | 7.03 | 7.04 | 3.88 | 3.89 | 7.73 | 7.76 | 4.21 | 4.23 |
| 6. | Calcium sulfate ($CaSO_4$) | 0.4 | 0.4 | 0.4 | 0.4 | 0.52 | 0.52 | 0.52 | 0.52 |
| | | 0.4 | 0.4 | 0.4 | 0.4 | 0.52 | 0.52 | 0.52 | 0.52 |
| | Moderate | 0.4 | 0.4 | 0.4 | 0.4 | 0.52 | 0.52 | 0.52 | 0.52 |
| 7. | Alkali metal oxides ($K_2O$+$Na_2O$) | 0.23 | 0.23 | 0.23 | 0.23 | 0.20 | 0.20 | 0.20 | 0.20 |
| | | 0.23 | 0.23 | 0.23 | 0.23 | 0.20 | 0.20 | 0.20 | 0.20 |
| | Moderate | 0.23 | 0.23 | 0.23 | 0.23 | 0.20 | 0.20 | 0.20 | 0.20 |
| | Summary of the carbon bedrock component | | 190.71 | | 99.95 | | 187.5 | | 99.9 |

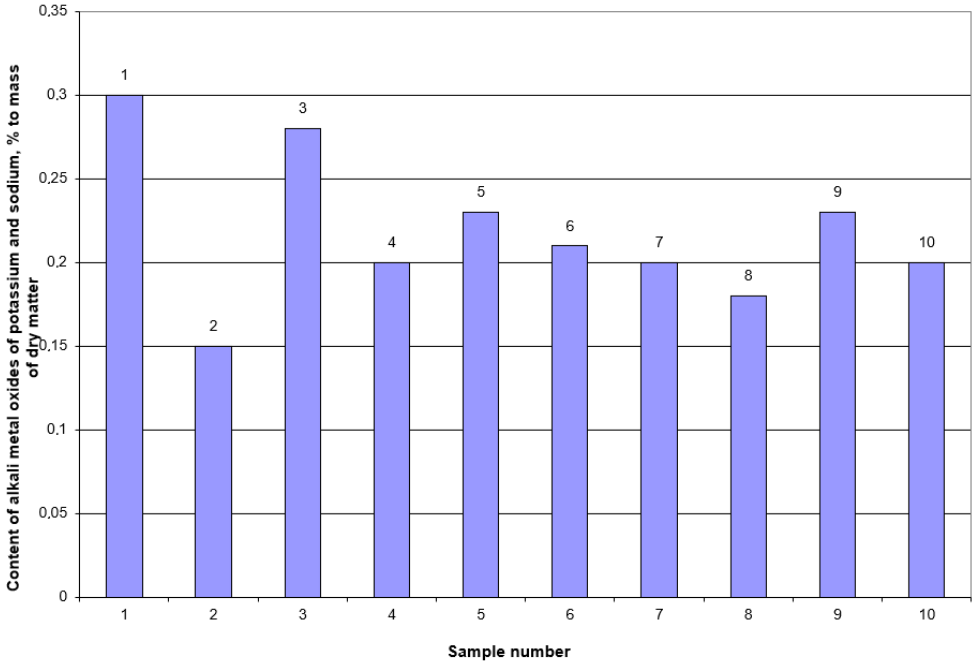


**Figure 8** The content of alkali metal oxides of potassium and sodium in the studied samples of carbonate rocks.

## CONCLUSION

Therefore, the use of the proposed method for determining the content of magnesium and calcium carbonate in limestone will allow the technologist to obtain timely objective information on the component composition of the carbonate rock. Knowing the exact concentration of $MgCO_3$, you can use its positive effect on the technological process, eliminating the negative factors of technological methods. Thus, as a result, we get finished products of good quality, save natural resources (limestone, fuel) and improve the ecological condition of the planet (by reducing harmful emissions into the atmosphere).

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# CONSUMPTION DIETARY SOURCES OF LYCOPENE, LUTEIN, AND ZEAXANTHIN AMONG YOUNG ADULTS LIVING IN MOSCOW

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## ABSTRACT

Carotenoids are natural antioxidants, affecting apoptosis, absorbing active forms of oxygen, and improving visual performance through their blue light filtering capabilities. Lutein and zeaxanthin are carotenoids of the macular pigment that play a significant role in protecting against age-related macular degeneration (AMD), cataracts, and diabetic retinopathy. We analyzed the dietary sources of lycopene, lutein, and zeaxanthin of young adults living in a megapolis. We analyzed 431 food frequency questionnaires and used two different criteria for comparison: age (groups A1 and A2) and season (groups S1, spring, and S2, autumn). Raw red tomatoes and eggs are the main sources of carotenoids for the majority of respondents regardless of age and season. Significant differences between age groups were found for parsley, carrot, cheeseburger, and spring onion. Foods with high levels of carotenoids (tomato juice, pumpkin, spinach, and sprouts) were absent in the diets of most of the study participants. Watermelons and persimmons are seasonal sources of lycopene, lutein, and zeaxanthin. Dietary sources of carotenoids are present in insufficient quantities in the diets. Foods with high levels of lycopene, lutein, and zeaxanthin are absent or only occasionally included in the diet.

**Keywords:** lycopene; lutein; zeaxanthin; dietary source; diet

## INTRODUCTION

Carotenoids are the most common natural pigments. Lycopene, lutein, and zeaxanthin are tetra-terpenoid (and their derivatives) having eight isoprene units in their 40-carbon skeleton **(Britton, 1983)**. Carotenoids comprise two classes: carotenes (β-carotene, lycopene) and xanthophylls (lutein, zeaxanthin, and meso-zeaxanthin). Lycopene and xanthophylls cannot be converted into vitamin A and are called non-provitamin A carotenoids. These carotenoids form the macular pigment, the concentration of which peaks in the foveola. Meso-zeaxanthin is at the epicenter of the macula, zeaxanthin is in the mid-peripheral macula, and lutein is in the peripheral macula **(Lima, Rosen and Farah, 2016)**. Using resonance Raman spectroscopy, carotenoids species can be detected, quantified, and distinguished **(Arteni et al., 2015)**. Carotenoids are natural lipid-soluble antioxidants that quench oxygen free radicals and improve visual performance by filtering blue light. Lycopene affects apoptosis, growth factors, and expression of xenobiotic-metabolizing enzymes **(Dadali, Tutel'yan and Dadali, 2011)**.

Epidemiologic studies have revealed an inverse relationship between the risk of incidence and progression of age-related macular degeneration or cataract and xanthophyll levels in the diet **(Moeller, Jacques and Blumberg, 2000)**. The consumption of dietary sources of lutein, zeaxanthin, and meso-zeaxanthin increases macular pigment optical density, improves contrast sensitivity and visual function and prevents progression of diabetic retinopathy in patients with non-insulin-dependent type 2 diabetes **(Moschos et al., 2017)**.

Carotenoids obtained from food sources accumulate and modify in humans and animals. The possibility of biosynthesis of carotenoids in the human body is not currently proven. Lutein and zeaxanthin are found in relatively high levels in green leafy vegetables (kale, lettuce, broccoli, peas, and spinach), corn, and egg yolks **(Abdel-Aal et al., 2013)**. In addition, zeaxanthin is present in certain yellow or orange fruits and vegetables, for example, nectarines, oranges, papaya, and orange pepper. Carotenoids are found in guava (lutein) and lychee (zeaxanthin) **(Mozaffarieh, Sacu and Wedrich, 2003)**. Lycopene is found in red tomatoes and tomato-containing products (ketchup, tomato juice, tomato paste, pizza, and lasagna), watermelon, pink and red grapefruits, papaya, and persimmon **(USDA, 2018)**. Studies have shown that plasma lutein and zeaxanthin concentrations increase with the daily consumption of fruits and vegetables. Therefore, concentrations of lutein and zeaxanthin in plasma can be used as biomarkers of fruit and vegetable consumption. No positive relationship exists between plasma lycopene concentration and mixed fruits and vegetable intake. Thus, the concentration of lycopene in the plasma is the most

suitable biomarker of tomato (whole and processed) consumption **(Couillard et al., 2016)**.

The main carrier of lutein and zeaxanthin in the bloodstream is high-density lipoprotein. Lycopene is preferentially carried by low-density lipoprotein **(Mozaffarieh, Sacu and Wedrich, 2003)**. The bioavailability of carotenoids from food sources is associated with nutrient-rich sources, methods of preparation, and the presence of fats in the diet. The bioavailability of lutein and zeaxanthin from plant sources is low because these carotenoids are localized in the chloroplasts and chromoplasts of plants. Dietary fiber from plant sources reduces carotenoid absorption in the intestine. The bioavailability of lutein and zeaxanthin from egg yolks is higher than from vegetables due to the presence of fats **(Eisenhauer et al., 2017)**. Thermal and mechanical treatment of tomatoes increases the bioavailability of lycopene. The concentration of lycopene in plasma after consumption of tomato paste is much higher than after the intake of a similar amount of lycopene with fresh tomatoes **(Gärtner, Stahl and Sies, 1997)**. The official recommended daily carotenoid intake in Russia is 5 mg per day for lycopene and 6 mg per day for lutein and zeaxanthin (in total) **(Recommended consumption levels of food and biologically active substances, 2004)**. A study showed that 6 mg of lutein and zeaxanthin per day took with the diet reduces the risk of age-related degeneration **(Seddon et al., 1994)**. The use of enriched products, mainly wheat and milk products, is positively evaluated in ensuring daily recommended levels of lutein and zeaxanthin are consumed. The percentage of absorption of lutein and zeaxanthin does not depend on the dose of the consumed product, but increases depending on the content of carotenoids in the enriched product **(Granado-Lorencio et al., 2010)**. In this study, we analyzed the frequency of consumption and dietary sources of lycopene, lutein, and zeaxanthin in the diets of young adults living in a megapolis.

## Scientific hypothesis

There is no relationship between the age of the students, the season of the survey**,** and the leading sources of non-vitamin carotenoids.

## MATERIAL AND METHODOLOGY

### Samples

Our sample consisted of students from Federal State Autonomous Educational Institution of Higher Education I.M. Sechenov First Moscow State Medical University of the Ministry of Healthcare of the Russian Federation (Sechenov University). The structure of the respondents was:

- Total number of respondents: 431
- Man: 72.4%
- Woman: 27.6%

The age categories:

- 18.49 ±0.94: 55.7% (group A1)
- 23.42 ±1.35: 44.3% (group A2)

The season categories:

- spring (March and April): 50.5% (group S1)
- fall (September and October): 49.5% (group S2)

### Description of the Experiment

#### Questionnaire preparation

We used a food frequency questionnaire to assess dietary intake. The food frequency questionnaire contained 35 foods, including food sources of lycopene (tomato paste, pink and red grapefruit, and juice from them, and watermelon), lutein, and zeaxanthin (green peas, zucchini, corn, pumpkin, sprouts, spring onion, pistachios, spinach, parsley, basil, celery, egg yolks, carrot, blackberry, blueberry, and kiwi). The questionnaire included food sources with different levels of carotenoids: raw red tomatoes, tomato-containing products (pizza, lasagna, pasta, sandwiches from fast-food restaurants, hamburgers, cheeseburgers, sauces, and tomato juice), and persimmon **(USDA, 2018)**. The questionnaire considered the methods of food preparation: fresh consumption, pickling and salting, cooking, frying, and preservation. The questionnaire also included the frequency of consumption of carotenoids food sources: weekly, monthly (1 – 5 times per month), episodic consumption (once in 2 – 3 months), and absence in the diet. The weekly consumption was separated into several groups: 6 – 7, 3 – 5, and 1 – 2 times per week. The volume of one-time consumption was determined for every frequency group. The food frequency questionnaire included anthropometric data of respondents (height, body weight); their age and sex; data on additional intake of vitamin complexes containing lycopene, lutein, and zeaxanthin; and the presence of diseases of the organs of vision (glaucoma, myopia, etc.). All questions were mandatory.

#### Conducting a questionnaire survey:

A questionnaire survey was conducted at Sechenov University. Data collection was anonymous. Each participant could complete the questionnaire only once. The survey took place in the period: March – April 2019 and September – October 2019.

#### Number of questionnaires:

The total number of processed questionnaires was 143. A total of 19 questionnaires were rejected due to incorrect data: age and body weight were not specified and/or the frequency of inclusion in the diet and/or one-time consumption was not determined.

#### Creating a dataset:

We prepared the final data set for further processing in Microsoft Excell (Office 365). The structure of the dataset was adapted to further statistical processing.

#### Processing the answers:

We evaluated all questions and visualized the respondents' answers by figures.

### Statistical Analysis

We used Fisher's angular transformation criterion (φ) to determine the validity of differences between comparison groups. At $\varphi \leq 1.64$, the values are in the zone of statistical insignificance. Statistical significance was indicated according to the criterion $p < 0.05$. Statistical processing was performed in Microsoft Excel 365 in combination with XLSTAT (version 2019.3.2).

## RESULTS AND DISCUSSION

The weekly diet of more than half of the respondents (groups A1 and A2) contained sources of lycopene, lutein, and zeaxanthin. Around 30% of the students, in their weekly diet, included lettuce, ketchup, parsley, and orange juice. Significant differences between the comparison groups were found for parsley, carrot, cheeseburger, and spring onion (Table.1).

The frequency of the inclusion of non-vitamin carotenoids sources in the weekly diet varied: raw red tomatoes (14.10%), lettuce (8.97%), orange juice (7.69%), and eggs (6.41%) were consumed 6 – 7 times per week in group A1. In group A2, the same frequency is typical for red raw tomatoes (11.29%), parsley (9.68%), spring onion, and eggs (8.06% each). For the majority of respondents, regardless of age, the main source of lutein and zeaxanthin was eggs; the main source of lycopene was raw red tomatoes (Figure 1). With a frequency of consumption of one to five times a month in the diet, more than one-third of the respondents identified other sources of non-vitamin carotenoids. There was a smaller variety of sources of lutein and zeaxanthin and almost no lycopene sources in group A1 compared to group A2 (Table 2).

There were no reports of the consumption of foods with high levels of carotenoids in the diet of the majority of respondents in either group: spinach (75.7% in A1, 53.2% in A2); pumpkin (74.4% and 59.7%); sprouts (64.1% and 56.5%); celery (65.4% and 51.6%); basil (60.3% and 54.8%); fast food products with tomato" lasagna, hamburgers, sandwiches, paste (69.2% and 75.8%); and squashes including squash puree (55.1% and 56.5%, respectively). There are no spring onion (64.1%) or tomato juice (55.1%) reported in the diet of the majority of respondents in group A1. The frequency of consumption of these sources for respondents of group A2 was 38.75% for tomato juice and 16.3% for spring onion. Other products listed in the questionnaire were reported in the diet occasionally and in insufficient quantities. We found significant differences between red and pink grapefruit and canned red tomatoes (Table 3).

In the weekly diet in spring and fall, the main source of lycopene in the majority of students was red raw tomatoes; the main source of lutein and zeaxanthin was eggs (Figure 2). We found significant differences concerning parsley and eggs. These sources were present in the weekly diet in the spring, and we found no significant differences in the sources of non-vitamin carotenoids consumed 6 – 7 times per week between group S1 and group S2 students (Table 4).

Consumption of seasonal sources of lycopene (watermelon) and lutein and zeaxanthin (persimmon) corresponds to seasonality. In group S2 students, weekly consumption of watermelon was 10.4% and that of persimmon was 11.8%. Group S1 students reported no watermelon (100%) or persimmon (94.6%) in their weekly diet. The daily diet of students in groups S1 and S2 contained food sources with high levels of lycopene (red raw tomatoes) and sources with good bioavailability of lutein and zeaxanthin (eggs). The daily consumption of these foods does not ensure the intake of non-vitamin carotenoids at the recommended levels. Students of neither group included products with high levels of studied carotenoids in the diet that were presented in the questionnaire, among which were sources of lycopene: tomato juice (49.7% S1, 54.9% S2), pink and red grapefruit juice (49.0% and 58.3%), canned vegetables in tomato sauce (77.6% and 48.6%), fast food products with tomato (77.6% and 78.5%, respectively); and sources of lutein and zeaxanthin: pumpkin (68.0% S1, 72.2% S2), sprouts (64.6% and 73.6%), celery (64.0% and 72.2%), spinach (61.9% and 79.9%), blackberry (42.9% and 66.7%), and blueberry (48.3% and 70.8%, respectively). Among all respondents (431 students), 48.03% reported the presence of myopia, and 1.16%, the presence of retinopathy. The additional intake of vitamin complexes was at 6.03% of respondents, but only 1.62% took complexes containing lycopene or lutein and zeaxanthin.

Our results confirmed the data obtained in Spain: The major sources of lycopene are raw red tomatoes, dishes with tomato products and watermelon **(Estévez-Santiago, Beltrán-de-Miguel and Olmedilla-Alonso, 2016)**. These findings do not contradict the results of a study in Belgium, according to which the leading sources of lycopene were tomatoes, foods containing tomatoes and tomato sauces in ready-to-eat meals **(Vandevijvere et al., 2014)**. In accordance with the results of research conducted among the population of South Korea, the study found that there are insufficient quantities of these major sources of lycopene in people's diets **(Park, Kim and Shin, 2020)**. In Brazil, about 55% of people's daily intake of lycopene is due to the inclusion of vegetables (tomatoes and red lettuce) in their diet, and 45% is due to the inclusion of fruits (watermelon and papaya) **(Vargas-Murga et al., 2016)**.

Authors of scientific works **(Fukushima et al., 2021; Van Hoang et al., 2018)** have reported that in Japan and Vietnam, products that contain tomatoes are the main contributors to the consumption of lycopene in people's diets. These findings do not contradict the meta-analysis, according to which tomatoes, tomato sauces, ketchup, watermelon, pink grapefruit and rose hips are the main sources of lycopene **(Dias et al., 2021; Meléndez-Martínez et al., 2021)**.

According to **Böhm et al. (2021)**, consumption of tomato juice, products containing tomato sauce (such as pizza) and ketchup significantly increase levels of lycopene. Products containing tomatoes are also a major source of lycopene among the Polish population (50% of the incoming level came from processed tomatoes and 32% from fresh tomatoes) **(Hamułka, Wawrzyniak and Sulich, 2012)**. Tomatoes and bolognaise sauce were the major sources of lycopene in Australia **(Manzi et al., 2002)**. This confirms the data provided by **Stuetz et al. (2016)**, which indicated that the populations of Italy, Belgium, and Greece had high levels of lycopene intake from fruits and vegetables, as well as from the consumption of French fries with various tomato sauces and ketchup. In their assessment of seasonal intake, higher levels of lycopene were recorded in the summer and fall seasons.

**Table 1** Sources of non-vitamin carotenoids in the weekly diet.

| Food Sources | Group A1 % | Group A2 % | φ |
|---|---|---|---|
| Egg | 62.82 | 70.96 | 1.023 |
| Raw red tomato | 74.36 | 62.90 | 1.469 |
| Lettuce | 44.86 | 45.17 | 0.059 |
| Ketchup and tomato sauces | 43.59 | 37.09 | 0.764 |
| Parsley | 35.90 | 50.01 | 1.645* |
| Orange juice | 38.46 | 25.81 | 1.587 |
| Basil | 25.64 | 24.19 | 0.176 |
| Celery | 24.36 | 25.81 | 0.176 |
| Broccoli (boiled) | 23.08 | 20.97 | 0.294 |
| Raw carrot | 20.50 | 38.71 | 2.351* |
| Cheeseburger | 21.80 | 6.45 | 3.526* |
| Spring onion | 5.13 | 35.48 | 4.819* |

Note: * $p$ <0.05.

**Table 2** Sources of non-vitamin carotenoids in the monthly diet.

| Group A1 | Group A2 |
|---|---|
| Carrot, pizza, canned green peas, canned corn, orange juice | Canned green peas in dishes, canned corn in dishes, carrot, pizza, orange juice, squash puree, kiwi, grapefruit red and pink, raw red tomato, broccoli, spring onion |

**Table 3** Sources of non-vitamin carotenoids included occasionally in the diet.

| Food Sources | Group A1 % | Group A2 % | φ |
|---|---|---|---|
| Watermelon | 79.49 | 72.58 | 0.952 |
| Blueberry | 53.85 | 45.16 | 1.023 |
| Blackberry | 44.87 | 58.06 | 1.557 |
| Kiwi | 44.87 | 37.1 | 0.934 |
| Canned red tomatoes | 43.59 | 58.06 | 1.716* |
| Pistachios | 43.58 | 54.84 | 1.322 |
| Persimmon | 37.18 | 37.10 | 0.012 |
| Red and pink grapefruit | 46.16 | 30.65 | 1.887* |

Note: * $p$ <0.05.

**Table 4** Sources of non-vitamin carotenoids in the weekly diet, and consumed 6 – 7 times a week.

| Food Sources | Group S1 % weekly diet/ 6 – 7 times per week | Group S1 % weekly diet/ 6 – 7 times per week | φ |
|---|---|---|---|
| Eggs | 75.51/8.16 | 56.95/10.42 | 3.369*/0.648 |
| Red raw tomatoes | 69.39/10.88 | 72.22/16.67 | 0.529/1.441 |
| Ketchup | 40.14/2.72 | 34.42/3.47 | 0.997/0.392 |
| Parsley | 44.89/7.48 | 33.33/4.17 | 2.038*/1.211 |
| Lettuce | 40.82/3.40 | 31.95/4.17 | 1.560/0.358 |
| Spring onion | 34.01/6.80 | 31.25/4.86 | 0.495/0.699 |
| Carrot | 32.66/3.40 | 24.31/4.17 | 1.586/0.358 |
| Orange juice | 28.57/2.04 | 22.92/1.39 | 1.117/0.400 |
| Eggs | 75.51/8.16 | 56.95/10.42 | 3.369*/0.648 |
| Red raw tomatoes | 69.39/10.88 | 72.22/16.67 | 0.529/1.441 |
| Ketchup | 40.14/2.72 | 34.42/3.47 | 0.997/0.392 |
| Parsley | 44.89/7.48 | 33.33/4.17 | 2.038*/1.211 |
| Lettuce | 40.82/3.40 | 31.95/4.17 | 1.560/0.358 |

Note: * $p$ <0.05.

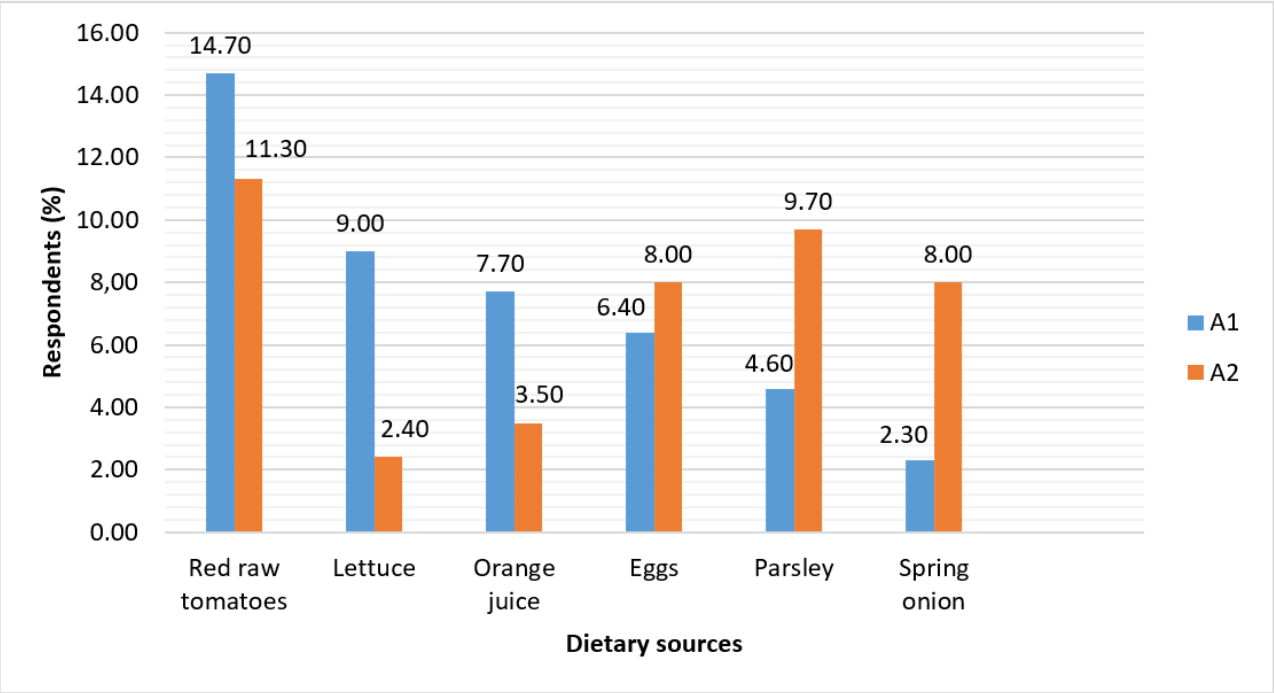


**Figure 1** Dietary sources of non-vitamin carotenoids in daily diet.

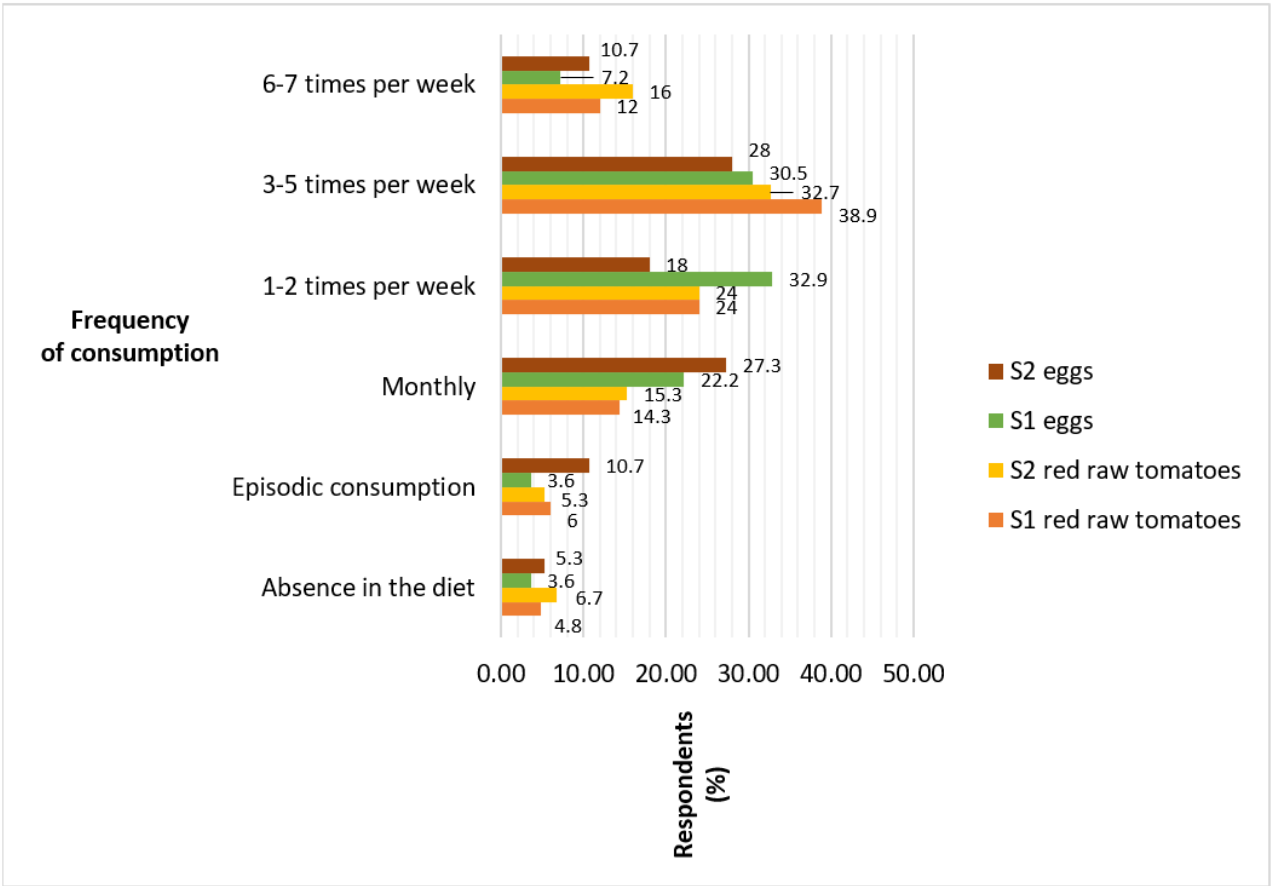


**Figure 2** The frequency of the inclusion of red raw tomatoes and eggs in the diet.

Raw tomatoes are the major source of lycopene for the respondents from Germany, regardless of age (the majority of respondents, regardless of gender and age, include them in their weekly and monthly diets), as well as tomato sauces and ketchup (most of the respondents include them in their diet two to three times per month) **(Weber et al., 2020)**. **Andarwulan et al. (2021)** also declared that rural Indonesians have a higher lycopene intake than urban residents. This is due to their more frequent consumption of vegetables and fruits. In the Hungarian population, the major sources of lycopene were found to be raw red tomatoes, sauces, ketchup, barbecue sauce, and instant foods containing tomato powder **(Lugasi et al., 2003)**.

Fast food products containing tomatoes provide different contributions to the total intake of lycopene in different countries. A five-country comparative study showed that the main source of lycopene in all countries studied, except the Republic of Ireland, was raw red tomatoes. In France, the U.K., and the Republic of Ireland, the main sources of lycopene were canned tomatoes and pizza. In the Netherlands, sources of lycopene included tomato soup and pizza; in Spain, the main source was tomato puree **(O'Neill et al., 2001)**. The data obtained in these studies agree with the results of our study and confirm that the main sources of lycopene are raw red tomatoes and sauces containing tomatoes.

Food sources of lycopene differed among ethnic groups. The major source of lycopene was cooked tomato products for Hispanics and pasta dishes for non-Hispanic whites **(Bermudez et al., 2005)**. According to **Zhou et al. (2016)**, pizza and pasta were the major sources of dietary lycopene among predominantly low-income middle-aged and older Blacks and Whites living in the southeastern U.S. **Mažeikienė et al. (2016)** also declared that in Lithuania, the major sources of lycopene were tomatoes and tomato products (78.8%), watermelons (11.9%), and pink grapefruits (9.3%). Ethnolinguistic region has a statistically significant impact on the lycopene intake in the Lithuanian population.

Authors of scientific works **(Sulich, Hamulka and Nogal 2015; Hamułka, Wawrzyniak and Sulich, 2012)** reported that the main contributors to the intake of lutein are fresh and cooked vegetables, including green leafy vegetables. **Manzi et al. (2002)** also declared that for people in Australia who is over the age of 55, the major sources of lutein and zeaxanthin were green peas, broccoli, and oranges. Meanwhile, the results of a University of Panama study reported that raw tomatoes, egg yolks, and corn tortillas were the main contributors to the intake of lutein and zeaxanthin in the daily diets of the majority of respondents **(Alvarado-Ramos et al., 2018)**.

There are differences in the dietary sources of xanthophylls, according to a survey in five European countries (U.K., Ireland, Spain, France, and the Netherlands). The highest levels of daily intake of lutein and zeaxanthin were found to be from lettuce and spinach in Spain and France; in the Netherlands, the average daily intake of lutein and zeaxanthin was achieved through the consumption of spinach and broccoli. The lowest intakes of lutein and zeaxanthin were found in the U.K. and Ireland, respectively. In these countries, the main sources were broccoli and green peas **(O'Neill et al., 2001)**. Additionally, according to a U.K. study, green lettuce, peas, broccoli, watercress, and spinach are potentially major sources of lutein, along with carrots, cucumber, beans, and Brussels sprouts **(Scott et al., 1996)**. Though watercress and spinach are very good sources of lutein, a relatively low percentage of the respondents consumed them.

According to a study in Japan, the best sources of lutein and zeaxanthin are spinach, cucumber, egg, spring onion, and Chinese onion **(Fukushima et al., 2021)**. **Vargas-Murga et al. (2016)** and **Carnauba et al. (2021)** conducted studies that show that in Brazil, the main sources of lutein are pumpkin, kale, and green leafy vegetables, and the main sources of zeaxanthin are orange, corn, and mango. Meanwhile, a research study in Spain found that lutein and zeaxanthin obtained from fruits (not from green leafy vegetables and egg yolks) play an important role in the prevention of age-related macular degeneration **(Estévez-Santiago, Beltrán-de-Miguel and Olmedilla-Alonso, 2016)**. According to **Durec et al. (2019)**, almost a third of respondents from Slovakia eat fruit one to three times a week, 26% of respondents consume fruit once a day, 23% from time to time and 20% several times a day. At the same time, the majority of respondents prefer to eat fresh fruit.

According to **Langhansova et al. (2021)**, European ferns can contribute to the human diet as a rich source of lutein. Thus, we found differences among the sources of lutein and zeaxanthin. In Russia (particularly in Moscow), the main source of lutein and zeaxanthin was eggs only.

## CONCLUSION

Regardless of the age of the students and the season of the survey, red raw tomatoes and ketchup (for lycopene), and eggs (for lutein and zeaxanthin) were the leading sources of non-vitamin carotenoids. These food sources were present in the daily diet of less than 15% of students living in the megapolis. There were no additional reported food sources of lycopene (red and pink grapefruits, and watermelon), lutein, or zeaxanthin (green leafy vegetables, pumpkin, sprouts, corn, spring onion, and squashes) in the diets of most respondents. Seasonal differences in the consumption of watermelon and persimmon did not significantly contribute to the total level of carotenoids because the inclusion of these products in the diet was noted in only 10.4 – 11.8% of respondents. This is the first study to examine the consumption dietary sources of non-vitamin carotenoids among young adults living in the megapolis. The next step of our research will analyze the content of lycopene, lutein, and zeaxanthin in their diet.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of IM Sechenov First Moscow State Medical University (Sechenov University) (protocol #03-20, dated February 19, 2020).

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# THE TECHNOLOGICAL PROPERTIES OF WINTER WHEAT GRAIN DURING LONG-TERM STORAGE

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## ABSTRACT

In the world, the demand for quality and safe grain products is increasing. The need to preserve wheat in the event of a natural disaster requires the study of optimal storage times of grain without degrading technological indexes. The purpose of the work was to study the dynamics of technological properties of winter wheat grown after peas, clover, corn for silage and the industrial, ecological, biological growing systems during 1, 3, and 5 years of storage in the conditions of the ordinary granary. The absence of significant differences in the technological parameters of the grain of wheat grown at industrial and ecological systems, but significantly lower indicators at a biological growing system was found. The highest hectolitre weight obtained when wheat grain was grown after corn for silage, and vitreousness – after the clover. Significantly higher biochemical parameters of grain and alveographic properties of flour were for the cultivation of wheat after peas, which provided additional accumulation of protein substances. There are no significant changes in the indicator of hectolitre weight during the grain storage. Other indicators increased significantly after 1 year of storage (on average by 10 – 30%). For further storage, vitreousness growth was insignificant. After 5 years of storage, the falling number significantly increased (on 21% compared to the initial values and on 7% – after 3 years of storage). The content of protein, gluten in the grain, and alveographic properties of flour significantly decreased after 5 years of storage. The possibility of obtaining grain of wheat with high technological parameters for a more safe ecological growing system was established. It was also confirmed to need for grain storage up to 1 year to improve quality indicators and it was established that it safely stored for 3 years.

**Keywords:** wheat grain; technological properties; growing systems; predecessors; term of storage

## INTRODUCTION

Wheat is the leading crop in temperate countries **(Shewry, 2009)**. It is a source of nutrition for 35% of the world's population. Wheat currently ranks first amongst cultivated plants in terms of cultivation area and production **(Kurt Polat, Cifci and Yagdi, 2016)**. Its success depends partly on its adaptability and high yield potential. The gluten protein fraction of wheat, which confers the viscoelastic properties that allow the dough to be processed into bread, pasta, noodles, and other food products. Wheat also contributes essential amino acids, minerals, and vitamins, and beneficial phytochemicals components to the human diet **(Smetanska et al., 2021)**.

The quality of wheat grain is influenced by genetic factors, growth location, agricultural measures, conditions of harvest, transport, and postharvest storage of grain **(Dziki et al., 2017)**.

One of the most effective measures to improve the quality of the grain is the correct scientifically based fertilizer system. The main factor constraining the improvement of the quality of the grain is the lack of nitrogen in the soil. It is an integral and indispensable part of amino acids, proteins, chlorophyll, enzymes. An important agrotechnical measure to improve the quality of wheat grain is the proper selection of predecessors. Each field crop, depending on the vegetation and farming uses different amounts of water and nutrients and differently affects the physical properties of the soil, which creates different conditions for the cultivation of the next crop. Also important is the timely fight against weeds, protection against diseases and pests **(Kovalyshyna et al., 2020a)**.

Wheat is also an important crop in organic farming. Organic agriculture is of particular interest concerning healthy and ecologically friendly produced food because inputs of chemicals are not allowed. With the increasing consumer pressure to reduce the use of pesticides and fertilizers in food production systems, the demand for organic foods continues to rise **(Kovalyshyna et al., 2020b)**.

Harvest of wheat received once a year and the need to provide food to the population is every day. It is known that grain quality may change during storage. Therefore, it is necessary to create conditions that would ensure the storage of products with the least loss of its quality.

Wheat stored for disaster relief has the potential of being stored for extremely long periods, which may result

in undesirable changes in milling and baking quality **(Marzec, Cacak-Pietrzak and Gondek, 2011)**.

Increasing the production and storage of wheat grain is a prerequisite for ensuring food safety of the country and normal consumption of food by the population. Therefore, this study aimed to evaluate different growing systems and precursors for obtaining environmentally safe grain of wheat with high technological parameters necessary for the production of bakery products. Furthermore, we also studied the influence of storage term on wheat grain quality and the possibility of using it for bread production after 1, 3, and 5 years of storage.

## Scientific hypothesis

The quality of wheat grain depends on many factors. There are varietal characteristics, soil, climatic conditions, farming systems, post-harvest handling, duration, and regimes of storage. The farming system is one of the most important factors that influence grain quality. An ecological growing system will supply to reduce the cost of production and obtain quality and safe grain products. High technological indicators of wheat grain quality will be kept up for 1 – 3 years of storage.

## MATERIAL AND METHODOLOGY

### Samples

The grain contains 14.3% protein, 29.2% gluten, deformation energy – 365 x $10^{-4}$ J, general baking estimation – 4.2 marks. It is the wheat of valuable. The direction of use is grain for the food industry.

### Chemicals

No chemical reagents were used for the studies.

### Animals and Biological Material:

For the study, a variety of soft winter wheat Poliska 90 was used. The owner of the variety is the Selection-Genetic Institute "Agriculture" of the National Academy of Agrarian Sciences of Ukraine. Variety is erythrosperm.

### Instruments

The moisture content of grain was determined by drying in the air oven SESH-3MU (company LLC "OLIS").

"Mass per hectolitre" was determined on a litter chondrometer with a falling load PH-2 (company LLC "OLIS").

The protein content was determined by Kjeldahl device UDK 129 with manual electronic control (company Velp Scientifica).

The number of falling was determining by device FALLING NUMBER 1900 (company Perten's).

The energy of the dough deformation, dough elasticity, and dough extensibility was determining by Alveo-consistograp NG of the company Chopin Technologies.

Grains were milled on Buhler MLU-202 Laboratory Flour Mill of the Buhler's company.

Samples were weighted by laboratory scales ADA320 (AXIS) from the company "Scales of AXIS Ukraine" with the 2nd class of accuracy.

### Laboratory Methods

The grain samples were analyzed for the moisture content of the grain to **ISO 712:2009** and test weight (called "mass per hectolitre") to **ISO 7971-1:2009**. The percentage of the vitreous kernel was determined according to the method given in **ICC Standard No. 129**. **(1980)** The percentage of vitreous kernels is determined by examining the cross-section of the grains. Vitreous grains appear dark and translucent, while opaque grains appear yellow and starchy. The percentage of vitreousness represents the mean of the 50 kernels × 2.

The grain samples were analyzed for the total content of crude protein in dry matter of grain according to the **ISO 20483:2013**, the content of wet gluten in dry matter of grain to the **ISO 21415-1:2006**, and the falling number method according to Hagberg-Perten to the **ISO 3093:2009**.

The remaining grains were milled on the Bühler laboratory mill (MLU-202 type). Wheat flour was used to determine deformation energy, dough elasticity, and dough extensibility.

### Description of the Experiment

#### Field experiment

Grain is grown on experimental fields of the stationary experiment of the Separated subdivision of NUBIP of Ukraine "Agronomic Research Station", which is located in the Kyiv region. According to the natural-agricultural zoning of Ukraine, this territory is classified as a forest-steppe zone.

The area of research is a zone of sufficient moisture, with a warm temperate and humid climate. Winter is mild, cloudy, with frequent thaws, and the summer is mostly warm, moderately humid. Autumn and spring, in general, have a protracted and unstable character. But prevailing warm spring, with sufficient margin (160 – 80 mm), productive moisture in the meter soil layer. Average monthly air temperature in January – February ranges from 4 to 8 °C. In the summer, the average monthly air temperature ranges from 15 to 20 °C. The absolute maximum reaches 39 – 40 °C. The period with an average monthly temperature above 5 °C concurs with the vegetation period of the main crops and has a period of 200-215 days. Years of the investigation were characterized by slight deviations from perennial norms of temperature and precipitation.

Soils on experimental plots are typical black earth. The content of humus in the arable layer is 3.67 ±0.13%, the pH of the aqueous extract is 6.7, the pH of the salt extract – 6.3, the absorption capacity – 28.9 mg-equivalent per 100 g of soil. Supply of hydrolysis nitrogen compounds for Tyurin and Kononovoy in a layer 0 – 15 cm – high, 15 – 30 cm – raised; mobile phosphorus according to Chirikov in a layer 0 – 15 cm and 15 – 30 cm – average, exchangeable potassium is also average in both layers. The water of soil is deposited at a depth of 6 m and does not affect the process of soil formation. The research included the study of the impact of growing systems (industrial, ecological, biological), predecessors (clover, peas, corn on silage), and storage of experimental samples of grain during five years.

Under the industrial system, 12 tons of organic and 300 kilograms of the active substance of mineral fertilizers were introduced per hectare of arable soil in crop rotation, and the protection of crops was carried out by industrial pesticides. In the ecological model, priority measures were organic fertilizers of 24 t per ha, mineral fertilizers used for 150 kg per ha, and crops were protected by biological means and industrial pesticides on the criterion of the ecological

and economic edge of the number of harmful organisms. The biological model of the agricultural system was provided only by a possible norm of organic fertilizers of 24 tons per hectare of arable soil in crop rotation, and the protection of crops was carried out only by biological means.

The area of the experimental plot in the field experiment was 280 $m^2$, accounting – 240 $m^2$, repetition of the experiment four-time. The placement of options is systematic. The experimental cultures were grown in 10 field crop rotations. The changes of crops in the crop rotation are as follows: clover – winter wheat – sugar beet – corn for silage – winter wheat – corn for grain – peas – winter wheat – sugar beet – barley + clover.

**Sample preparation:** The research was conducted in 2012 – 2017 based on the laboratories of the department technology of storage, processing, and standardization of plant products after named prof. B. V. Lesik NUBIP of Ukraine and State Enterprise «Ukrmetrteststandart».

**Number of samples analyzed:** Experimental samples of grain designed to study the duration of storage were placed in linen bags of 3 kg. Samples for each variant were taken in sufficient quantities to provide a sample for each period of storage for the quality estimation of the grains.

**Number of repeated analyses:** Samples of grain in a dry state (humidity up to 14%) were stored in an ordinary granary (storage conditions of commodity grain).

**Number of experiment replication:** Quality indicators of wheat grain after 1, 3, and 5 years of storage were determined in all variants provided by the research program. Analyzes were performed in three replicates.

### Statistical 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. The obtained data were further subjected to statistical analysis and the consequent evaluations were analyzed for a variance (ANOVA). The statistical differences between the studied variants were estimated through Tukey's test. The Pearson correlation analysis was also carried out on these data. Statistical tests were evaluated at a significance level of $\alpha = 0.05$ using Statistica 6.0 software (StatSoft, Inc., Tulsa, USA).

## RESULTS AND DISCUSSION

### Physical parameters

The main factor determining the orientation and intensity of physiological and biochemical processes during the storage of grain is its moisture content. Safety long-term storage of wheat grain is possible only at a moisture content not exceeding 14% **(Leary et al., 2018; Ramanathan et al., 2018)**.

In our studies, the moisture content of wheat grain was 13.34 ±0.47% on average. Throughout the shelf life, the grain moisture of the studied variants did not change significantly (<0.9%) and was dependent on the relative humidity of air (Table 1). The research determined the effect on the grain moisture of the storage period and the precursors at the ecological and biological systems (Table 2).

Filling grain or grain with a high hectolitre weight has more endosperm and a higher yield of flour. Under unfavorable conditions for the formation of grain or seeds, the mass of peels in comparison with the weight of the grain increases, and the endosperm decreases **(Dorofee, 1972; Bharti et al., 2016)**.

Before the storage, the wheat grain of all the studied variants had a very high hectolitre weight. The highest initial hectolitre weight values were in wheat grown after corn for silage of 81.73 kg and the lowest after the clover of 76.69 kg. The test criterion F (ANOVA) showed the most significant impact on this indicator was precursors at the ecological and biological growing systems.

No significant difference in hectolitre weight was in the grain of wheat grown at the industrial and ecological systems. We partially confirm of our data the research **Dziki et al. (2017)**, about the absence of a clear impact of the growing system on hectolitre weight **(Bober et al., 2020)**. The lowest hectolitre weight values were in wheat grains grown at the biological system of 79.27 kg, which significantly differed from the grain grown at two other systems. These deny data of **Bilsborrow et al. (2013)** about higher hectolitre weight at organic farming compared to conventional. At the same time, our studies point out a significant impact on the hectolitre weight of agricultural systems at all the predecessors (Table 2), thereby confirming data of other scientists **(Zheplinska et al., 2020)**.

During storage of wheat grain, changes in hectolitre weight fluctuations depend on relative humidity and its changes were not significant. After three years of storage of wheat grains, almost all of the studied variants had a slight increase in hectolitre weight and after five – on the contrary, a slight decrease. Although several previously conducted studies by different scientists indicated that after 6 – 12 months of storage there is a significant reduction in hectolitre weight **(Varella et al., 2017; Jafary, Szabo and Niks, 2006)**.

In our study, the test criterion F confirmed the absence of effect on the hectolitre weight of the shelf life.

The consistency of grain is of great importance for flour processing. The higher the vitreousness is then the greater the number of the fraction can be obtained from the grain, and in the future, it provides a larger yield of varietal flour. 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 best ability to give good yield to the cereals and high of flour varieties was at a grain of winter wheat grown after the clover (85% vitreousness), the worst at a grain – after corn for silage (69%). Equally, high vitreousness indexes were in grain grown at the industrial and ecological farming system and significantly lower than the biological system.

In the same way, confirming the data on the effect on the vitreousness index of the amount of fertilizer applied and the content of nitrogen in the soil at different predecessors **(Khaneghah et al., 2018; Kirilenko, 2014)**.

In general, for all agricultural systems, the vitreousness of the grain was quite high, which makes it possible to obtain high yields of cereals and flour.

**Table 1** Quantitative evaluation of physical parameter of the winter wheat (LSD, α = 0.05).

| Parameter | | Moisture (%) | | | Hectolitre weight (kg) | | | Vitreousness (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $\overline{x}$ | $d_{min}$ | signi-ficance | $\overline{x}$ | $d_{min}$ | signi-ficance | $\overline{x}$ | $d_{min}$ | signi-ficance |
| Term of storage | 0 (before storage) | 13.80 | 0.23 | a | 81.09 | 1.73 | a | 56 | 5.13 | a |
| | 1 year of storage | 13.74 | | a | 80.42 | | a | 86 | | b |
| | 3 years of storage | 12.91 | | b | 81.06 | | a | 86 | | b |
| | 5 years of storage | 13.81 | | a | 80.61 | | a | 87 | | b |
| Predecessor | Clover | 13.44 | 0.50 | a | 79.69 | 0.66 | a | 85 | 5.59 | a |
| | Peas | 13.34 | | a | 80.96 | | b | 81 | | a |
| | Corn for silage | 13.28 | | a | 81.73 | | c | 69 | | b |
| Growing systems | Industrial | 13.34 | 0.50 | a | 81.27 | 0.66 | a | 83 | 5.59 | a |
| | Ecological | 13.35 | | a | 81.85 | | a | 80 | | a |
| | Biological | 13.37 | | a | 79.27 | | b | 73 | | b |

Note: $\overline{x}$ = average values of the indicators; $d_{min}$ = least significant difference.

**Table 2** ANOVA, values of the test criterion F for results of selected physical parameters of winter wheat.

| Factor | | Quality parameter | | |
|---|---|---|---|---|
| | | moisture | hectolitre weight | vitreousness |
| Growing systems | | | | |
| Industrial | predecessor | $1.77^{n}$ | $1.24^{n}$ | $79.40^{*}$ |
| | term of storage | $46.40^{*}$ | $1.16^{n}$ | $313.22^{*}$ |
| Ecological | predecessor | $7.00^{*}$ | $31.75^{*}$ | $8.15^{*}$ |
| | term of storage | $96.33^{*}$ | $2.33^{n}$ | $24.74^{*}$ |
| Biological | predecessor | $17.52^{*}$ | $44.76^{*}$ | $24.10^{*}$ |
| | term of storage | $16.00^{*}$ | $3.01^{n}$ | $34.56^{*}$ |
| Predecessor | | | | |
| Clover | growing systems | $4.35^{n}$ | $22.14^{*}$ | $1.95^{n}$ |
| | term of storage | $15.01^{*}$ | $1.17^{n}$ | $27.41^{*}$ |
| Peas | growing systems | $1.45^{n}$ | $16.79^{*}$ | $2.15^{n}$ |
| | term of storage | $10.38^{*}$ | $0.32^{n}$ | $9.23^{*}$ |
| Corn for silage | growing systems | $1.30^{n}$ | $29.20^{*}$ | $3.34^{n}$ |
| | term of storage | $20.24^{*}$ | $3.77^{n}$ | $10.60^{*}$ |

Note: *statistically significant α = 0.05; n – statistically insignificant.

The glassiness is conditioned by the presence of protein. For insufficient quantity of it create a dense consistency (protein-starch complex), which shows virtuousness "transparent" for light penetration for light consistency **(Vlasenko, Koliuchyi and Chebakov, 2005)**.

We found that after one year of storage of wheat grain, all options were to strengthen the consistency, which was expressed by a quantitative increase in the vitreousness index by 30%. This can be explained by the interaction of protein and starchy substances during the post-harvest maturing of grain **(Kozub et al., 2017; Pirko et al., 2012)**.

Previous studies of the effective term of storage on the vitreousness of the grain indicate that vitreousness, in general, is a stable indicator **(Mushtruk et al., 2020a, Palamarchuk et al., 2019)**. In our studies, also, in the three or five years of storage of wheat grain, the vitreousness remained almost unchanged.

The test criterion F indicated a significant effect on the vitreousness of wheat grain storage term and precursors and the absence of impact on growing systems (Table 2).

**Biochemical parameters**

The protein content of wheat grain varied significantly depending on the predecessor and growing systems (Figure 1, Table 3). The highest protein content in wheat grain was provided by the predecessor peas – 14.54%, that was explained by the ability of peas in the process of vegetation to accumulate nitrogen, which is necessary for the

formation of protein in the grain **(Sukhenko et al., 2019)**. The predecessors of the clover and corn for silage provided significantly less protein in the grain.

A high index of protein content was in the grain of wheat grown for both the industrial system of agriculture – 14.55%, for the ecological – 14.23% and significantly lower than the biological – 12.72%. These results are in agreement with the results obtained by several scientists in previous years **(Zheplinska et al., 2021a)**. However, **Kihlberg et al., (2004)** found that wheat grain grown under normal agricultural systems had lower protein content than organic. Also, there is the notion of the absence of influence of agricultural systems on protein content, or their influence was less significant than the influence of other factors **(Mayer et al., 2015)**.

In our study, test criterion F (ANOVA) showed a significant effect on the protein content of all investigated factors (Table 4). The agricultural systems were the most influential on protein content, especially at the predecessor corn for silage. Great influence on the protein content had predecessors at the biological growing system.

It is important to preserve grain protein for a long time. After one year, the protein content increased significantly compared with the initial values (by 0.35%), which is explained by the transition of simple amino acids to complex proteins in the process of post-harvest maturing **(Park et al., 2015)**. Three years of storage ensured the stability of this indicator. However, **Oleinik and Davidyuk (2009)** scientists noted a tendency to decrease the protein content of grain after 24 months of storage by 0.2 – 0.5%. After five years, the protein content has significantly decreased compared to the first year of storage and has not significantly differed from the initial data.

According to **Menkova et al. (1995)** in the grains of winter wheat, stored for eight years under normal conditions, noticeable changes in the biochemical composition did not observe. Insignificant changes in the content of protein during storage other scientists were noted **(Ceseviciene and Masauskiene, 2009)**.

The main part of the proteins that included the grain presented the solutions in alcohol and alkali – glutenin and gliadin, which form the so-called gluten.

As can be seen from Table 3, wheat grain, grown for all agricultural systems, according to the predecessor peas contained the maximum amount of gluten (32.68%), in corn – the minimum (28.27%). The difference in the content of gluten in wheat grain was significant among all the predecessors. Most scientists explain this by the fact that winter wheat is demanding to the conditions of moisture and fertilizer, so its highest yields obtained when placed after perennial herbs, bean-cereal grass mixtures, and peas for grain **(Palamarchuk et al., 2020)**.

The amount of gluten was significantly higher in grain grown at the ecological system (33.23%) compared to industrial (32.58%). The content of gluten in wheat grain grown at the biological system was significantly lower than at the ecological (6.6%) and industrial (5.95%). This confirms by scientists that the traditional system provides a significantly higher index of gluten than organic. In this case, the limiting factor in the system of organic cultivation is the provision of nitrogen **(L- Baeckström, Hanell, and Svensson, 2004)**.

The dynamics of gluten content during storage are controversial. The variation in the content of gliadin and glutenin is probably due to changes in protein aggregation and their solubility. **Kibar (2015)** founded that the amount of gluten increases during the first two months and subsequently decreases. Other researchers have found a significant reduction in the content of gluten in wheat grains after three and eight months of storage. In our studies, during three years, the changes of gluten content in wheat grain were not significant, although the index increased somewhat after a year of storage and slightly decreased after three years. The amount of gluten in the grain decreased significantly after five years of storage compared to the first year (by 3.15%).

The test criterion F (ANOVA) showed the greatest influence on wet gluten content in grain the predecessors at the industrial growing system (Table 4). Also, was influenced strongly by the investigated parameter of growing systems, especially at the grain grown after the clover and corn for silage. The effect on the wet gluten content in grain of the predecessors and the term of storage at the biological system and also the term of storage at the predecessor peas were not essential.

The changes that occur with starch in grain significantly affect the quality of products. Formation and cleavage of starch is carried out by amylolytic enzymes, the degree of activity in grain determined by the index of the falling number. The winter wheat grain of all the variants had a low activity of amylolytic enzymes. The high falling number with insignificant difference between the variants of research, was in the grain grown for all agricultural systems and predecessors.

Significant changes in the falling number were after one year of storage of grain wheat – the index increased on 28 seconds. Further storage was characterized by a gradual increase in the falling number. Again, a significant increase was noted after five years of storage – by 28 seconds compared to the third year of storage and by 72 seconds compared to the initial data.

By our studies, we confirmed and supplemented the results of other scientists regarding the decrease in alpha-amylase activity and the increase in the falling number with an increase in the storage period of wheat grain.

Also, **Linina and Ruza (2016)** noted the dependence of the falling number from weather conditions, storage duration, and the amount of nitrogen fertilizer. **Sulek and Cacak-Pietrzak (2018)** argued that the activity of amylolytic enzymes is more dependent on the variety than cultivation technology. In our research, the test criterion F (ANOVA) shown a statistically significant effect on the falling number predecessors, growing systems, and term of storage (Table 4).

**Quantitative evaluation of flour alveograph properties of the winter wheat**

The evaluation of wheat flour at the mechanized or automated baking production has rheological properties of the dough, in particular deformation energy (W) or the energy used to tear the dough semi-finished. It determines the area of the alveogram and characterizes the baking power of the flour **(Zheplinska et al., 2021b)**.

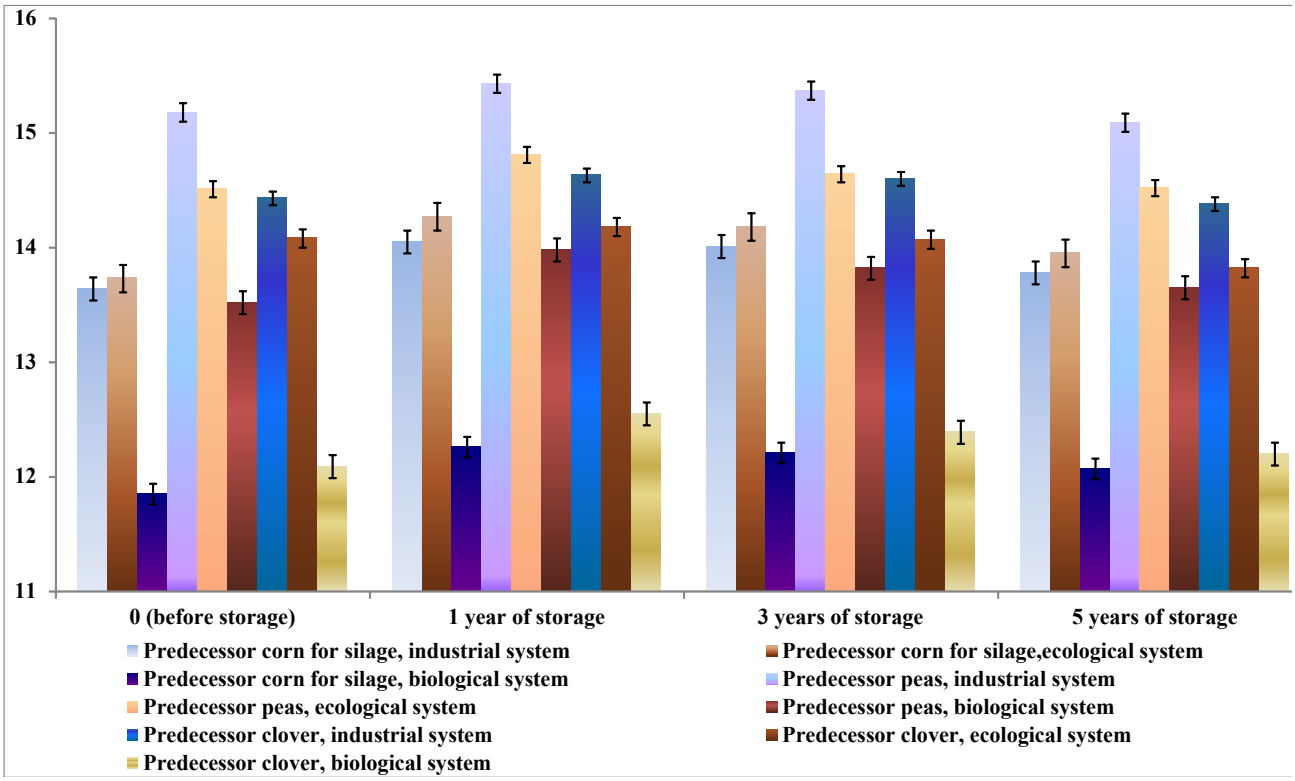


**Figure 1** Protein content in winter wheat grain during stored (%).

**Table 3** Quantitative evaluation of biochemical parameters of the winter wheat (LSD, α = 0.05).

| Parameter | | Protein content in grain (%) | | | Wet gluten content in grain (%) | | | Falling number in grain (seconds) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $\overline{x}$ | $d_{min}$ | signi-ficance | $\overline{x}$ | $d_{min}$ | signi-ficance | $\overline{x}$ | $d_{min}$ | signi-ficance |
| Term of storage | 0 (before storage) | 13.67 | 0.29 | a | 30.62 | 3.03 | ab | 312 | 22.3 | a |
| | 1 year of storage | 14.02 | | b | 32.51 | | a | 340 | | b |
| | 3 years of storage | 13.92 | | ab | 30.77 | | ab | 356 | | b |
| | 5 years of storage | 13.72 | | a | 29.36 | | b | 384 | | c |
| Predecessor | Clover | 13.62 | 0.18 | a | 31.49 | 1.49 | a | 350 | 30.05 | ab |
| | Peas | 14.54 | | b | 32.68 | | b | 363 | | a |
| | Corn for silage | 13.33 | | c | 28.27 | | c | 332 | | b |
| Growing systems | Industrial | 14.55 | 0.18 | a | 32.58 | 1.49 | a | 352 | 30.05 | a |
| | Ecological | 14.23 | | b | 33.23 | | a | 350 | | a |
| | Biological | 12.72 | | c | 26.63 | | b | 342 | | a |

Note: $\overline{x}$ = average values of indicators; $d_{min}$ = least significant difference.

The specific deformation energy of the dough varies widely from 30 to 800 x $10^{-4}$ J. In our studies, the highest rates of deformation energy were at the growing of grain after the peas' predecessor, insignificantly smaller than the clover (33 x $10^{-4}$ J) and significantly lower than corn for silage (45 x $10^{-4}$ J). Deformation energy indices did not differ significantly during the cultivation of grain at industrial and ecological growing systems (Figure 2, Table 5). Significantly lower were the indicators at the biological system: 41 x $10^{-4}$ J compared with the industrial system and 38 x $10^{-4}$ J compared with the ecological system. This agrees with the data of scientists about significantly higher baking properties in the traditional system, compared with organic, but denies the data of other scientists about the insignificant impact of growing systems on these indicators. At the same time, confirming the findings of **L-Baeckström, Hanel and Svensson (2004)**, that nitrogen is the most limiting factor for the organic growing system.

**Rose et al. (2011)** found that grain of wheat retains its nutritional qualities up to 32 years, although they noticed a negative correlation of indicators with the time of storage. **Mushtruk et al. (2020b)** indicated a possible improvement of rheological properties during storage, especially at elevated temperatures. But **González-Torralba et al. (2013)** set only fewer changes of alveographic parameters during storage after 180 – 240 days.

In our studies, after one year of grain storage, there was a significant increase in the deformation energy (on 74 x $10^{-4}$ J), after three years there was a slight decrease (on 14 x $10^{-4}$ J) and after five years a significant decrease (on 42 x $10^{-4}$ J) compared to each previous term.

The test criterion F (ANOVA) showed a significant influence of all investigated factors on the selected flour alveograph properties of winter wheat (Table 6). The greatest impact on deformation energy had storage period at the industrial system and peas predecessor. Also, predecessors at the industrial and biological systems of agriculture had a great influence on this index.

Dough extensibility (L) is the diameter of the test bubble during bursting. This indicator is due to the resistance of the gluten-free frame to spatial reorientation during dough formation and it is a converse characteristic to dough elasticity.

Indicators of dough extensibility differed considerably at the cultivation of wheat grain after clover and peas (on 2 mm), but at industrial and ecological growing systems (on 1 mm). Significantly lower dough extensibility observed in growing wheat grain after corn for silage (9 – 13 mm) and a biological system (6 – 7 mm). The grain storage process characterized a significant increase in the dough extensibility after one year of storage (on 11 mm) and a significant decrease – after five years (9 mm). This confirmed the data of **Kolyanovska et al. (2019)**, which indicated the possible improvement of rheological properties during storage and data by **Rose et al. (2011)** – on the gradual decline of these indicators during long-term storage.

The most important for dough extensibility had the precursors at cultivating grain at the industrial agricultural system. Also, the great impact growing systems and storage periods on the dough extensibility indicator had on growing grain after peas.

Dough elasticity (P) is the maximum pressure created inside the dough at the time of its mechanical destruction during inflation in the form of bubbles. The value of this index quantitatively characterized the resistance of the gluten of the yeast dough to deform during fermentation and dough arise **(Sheiko et al., 2019)**.

Indicators of dough elasticity differed significantly in the cultivation of wheat at industrial and ecological growing systems (on 3 mm) and substantially for the biological system (13 – 16 mm). It confirmed data by **Krejčířová et al. (2007)** about twice the amount of glutenins, which is responsible for the elasticity of the dough, in the grain grown under the traditional system, as compared to the organic. The indicator varies considerably depending on the predecessor. At the same time, the highest values were in the cultivation of wheat grain after peas, which provided a higher content of nitrogen for the formation of proteins in wheat grains.

The tendency of changes in dough elasticity was similar to deformation energy. The most significant changes in dough, elasticity were noted after one year of storage – it increased by 46 mm in comparison with the initial data. After three years of storage, we observed an insignificant decrease in the indicator. Significant reduction in dough elasticity was only after five years of wheat grain storage – on 23 mm compared with the third year of storage.

The greatest impact on the dough elasticity had the terms of storage at predecessors' peas and clover and also at the biological system of agriculture **(Sukhenko et al., 2017)**.

Table 7 presents the correlation coefficients between the physical, biochemical parameter of grain and alveograph properties of flour the winter wheat after 5 years of storage.

Pearson's correlation coefficients confirmed the strong negative effect of moisture on the hectolitre weight of wheat grain ($r = -0.74$), which researchers noted in past years. Simultaneously hectolitre weight had the highest positive correlation with protein content in grain ($r = 0.68$) and a noticeable positive correlation with deformation energy ($r = 0.53$).

It was found that the vitreousness index was very positively correlated with wet gluten content in grain ($r = 0.96$) and strongly with the following indices: deformation energy, dough extensibility, and protein content ($r \geq 0.80$). This is confirmed by the data of the direct dependence of the content of the protein, gluten, and hence the baking properties of the flour from the vitreousness grain **(Mushtruk et al., 2021b)**.

The results showed, that falling number in grain has a strong positive relationship with dough elasticity, dough extensibility, and wet gluten content in grain ($r > 0.70$). This is a confirmation that the quality of starch and the activity of amylolytic enzymes of wheat grain and have a direct effect on the quality of the dough.

The main indicator of flour power is the quantitative-physical properties of gluten and protein content, which confirm the correlation coefficients. Protein content in grain is very positively correlated with the deformation energy ($r = 0.96$) and strongly with dough extensibility, dough elasticity, and wet gluten content in grain ($r > 0.70$). Wet gluten content in grain had the highest positive correlation with dough extensibility ($r = 0.82$).

**Table 4** ANOVA, values of the test criterion F for results of selected biochemical parameters of winter wheat.

| Factor | | Quality parameter | | |
|---|---|---|---|---|
| | | Protein content in grain (%) | Wet gluten content in grain (%) | Falling number in grain (seconds) |
| Growing systems | | | | |
| Industrial | predecessor | 549.29* | 449.51* | 22.07* |
| | term of storage | 19.96* | 14.90* | 25.56* |
| Ecological | predecessor | 32.23* | 28.96* | 5.48* |
| | term of storage | 5.19* | 5.50* | 10.62* |
| Biological | predecessor | 2169.63* | 1.33n | 13.84* |
| | term of storage | 76.05* | 2.69n | 18.23* |
| Predecessor | | | | |
| Clover | growing systems | 632.10* | 116.55* | 7.92* |
| | term of storage | 7.43* | 5.77* | 29.67* |
| Peas | growing systems | 658.53* | 20.31* | 8.93* |
| | term of storage | 21.90* | 2.60n | 19.06* |
| Corn for silage | growing systems | 3089.34* | 110.47* | 18.98* |
| | term of storage | 85.27* | 13.46* | 27.71* |

Note: *statistically significant α = 0.05; n – statistically insignificant.

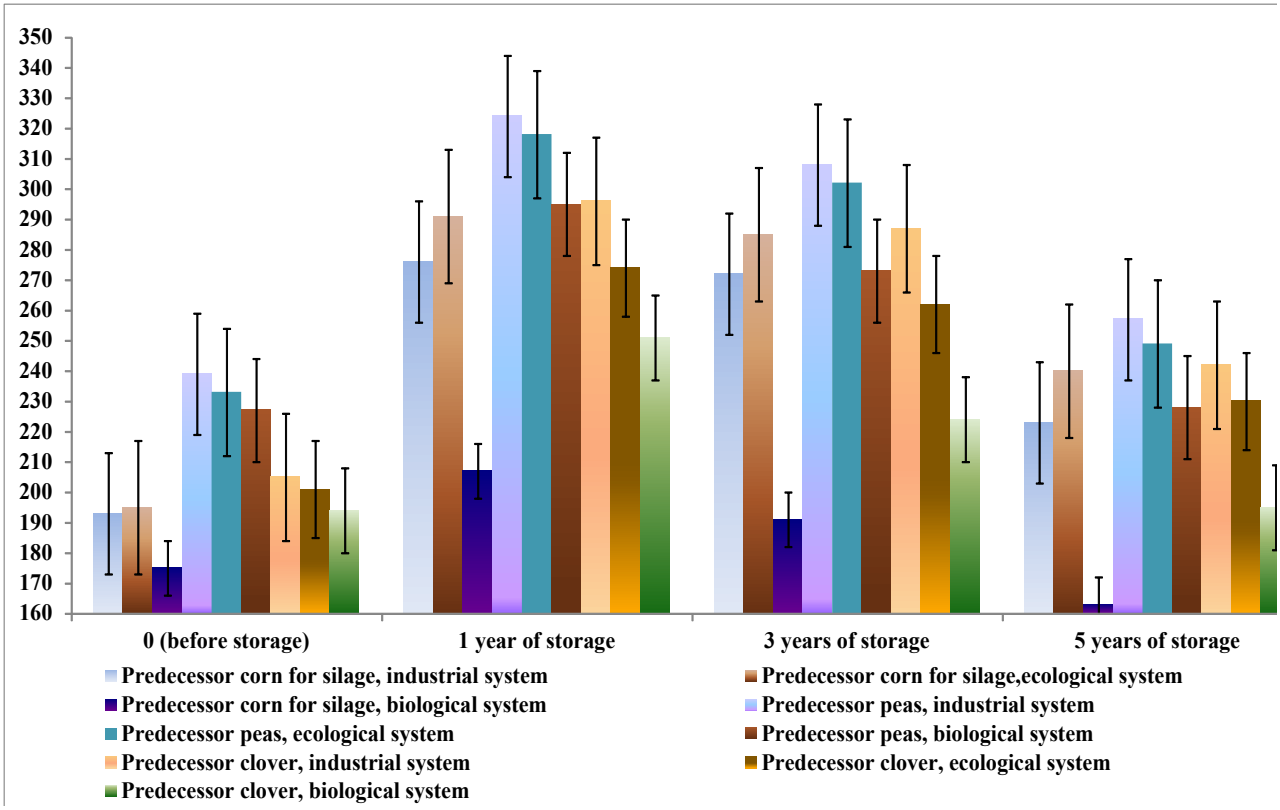


**Figure 2** Deformation energy of flour during stored of winter wheat grain (10 – 4 J).

**Table 5** Quantitative evaluation of flour alveograph propertiesof the winter wheat (LSD, α = 0.05).

| Parameter | | Deformation energy ($10^{-4}$ J) | | | Dough extensibility (mm) | | | Dough elasticity (mm) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $\overline{x}$ | $d_{min}$ | signi-ficance | $\overline{x}$ | $d_{min}$ | signi-ficance | $\overline{x}$ | $d_{min}$ | signi-ficance |
| Term of storage | 0 (before storage) | 207 | 31.03 | a | 73 | 5.42 | a | 118 | 13.78 | a |
| | 1 year of storage | 281 | | b | 84 | | b | 164 | | b |
| | 3 years of storage | 267 | | b | 80 | | bc | 159 | | bc |
| | 5 years of storage | 225 | | a | 75 | | ac | 136 | | d |
| Predecessor | Clover | 238 | 35.71 | ab | 80 | 4.99 | a | 144 | 11.39 | a |
| | Peas | 271 | | a | 82 | | a | 157 | | b |
| | Corn for silage | 226 | | b | 73 | | b | 132 | | c |
| Growing systems | Industrial | 260 | 35.71 | a | 81 | 4.99 | a | 150 | 11.39 | a |
| | Ecological | 257 | | a | 80 | | a | 147 | | a |
| | Biological | 219 | | b | 74 | | b | 134 | | b |

Note: $\overline{x}$ = average values of indicators; $d_{min}$ = least significant difference.

**Table 6** ANOVA, values of the test criterion *F* for results of selected flour alveograph properties of winter wheat.

| Factor | | Quality parameter | | |
|---|---|---|---|---|
| | | Deformation energy ($10^{-4}$ J) | Dough extensibility (mm) | Dough elasticity (mm) |
| Growing systems | | | | |
| Industrial | predecessor | 77.77* | 124.64* | 87.38* |
| | term of storage | 227.60* | 58.27* | 123.00* |
| Ecological | predecessor | 16.31* | 33.81* | 31.13* |
| | term of storage | 63.47* | 42.25* | 111.64* |
| Biological | predecessor | 70.82* | 39.36* | 87.32* |
| | term of storage | 28.83* | 44.05* | 186.75* |
| Predecessor | | | | |
| Clover | growing systems | 13.70* | 19.56* | 81.00* |
| | term of storage | 26.13* | 16.23* | 297.09* |
| Peas | growing systems | 23.02* | 107.48* | 86.64* |
| | term of storage | 135.20* | 84.69* | 311.59* |
| Corn for silage | growing systems | 17.08* | 29.27* | 57.06* |
| | term of storage | 10.54* | 81.87* | 236.58* |

Note: *statistically significant α = 0.05; n – statistically insignificant.

**Table 7** Pearson's correlation coefficients between the physical, biochemical parameter of grain and alveograph properties of flour the winter wheat after 5 years of storage.

| Quality parameter | M | H | V | FN | PC | WG | W | L | P |
|---|---|---|---|---|---|---|---|---|---|
| M | 1.00 | | | | | | | | |
| H | -0.74* | 1.00 | | | | | | | |
| V | -0.38 | 0.45* | 1.00 | | | | | | |
| FN | -0.27 | 0.06 | 0.66* | 1.00 | | | | | |
| PC | -0.60* | 0.68* | 0.80* | 0.61* | 1.00 | | | | |
| WG | -0.40* | 0.44* | 0.96* | 0.75* | 0.74* | 1.00 | | | |
| W | -0.46* | 0.53* | 0.84* | 0.57* | 0.96* | 0.73* | 1.00 | | |
| L | -0.35 | 0.32 | 0.88* | 0.77* | 0.90* | 0.82* | 0.94* | 1.00 | |
| P | -0.28 | 0.27 | 0.68* | 0.82* | 0.84* | 0.66* | 0.83* | 0.91* | 1.00 |

Note: *Statistically significant coefficients (a = 0.05). M – moisture of grain, H – hectolitre weight, V – vitreousness, FN – falling number in grain, PC –protein content in grain, WG – wet gluten content in grain, W – deformation energy, L – dough extensibility, P – dough elasticity.

## CONCLUSION

These studies have shown the possibility of obtaining grain of wheat with high technological parameters necessary for the production of bakery products with less cost and in better safety ecological growing system.

The best predecessor for the formation of high biochemical and alveographic indexes in wheat grain was peas, which provided additional protein accumulation and increased gluten content.

The indicator quality of hectolitre weigh does not significantly change during the grain storage, but other indicators had increased significantly after 1 year of storage (on average by 10 – 30%). The vitreousness of grain during the further storage increased insignificantly.

The number of falling, after 5 years of storage, significantly increased (on 21% compared to the initial values and on 7% – after 3 years of storage). The protein content, grain's gluten, and alveographic properties of flour significantly decreased after 5 years of storage.

It has been established that under ordinary granary conditions the best technological parameters of wheat form after 1 year of storage and hold them during 3 years of storage.

Greater impact on the vitreousness and the falling number was of the term of storage and on the indicators of protein and gluten content – growing systems and predecessors. It was established a very strong positive bond of vitreousness with the amount of gluten and the content of protein with energy deformation.

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This article does not contain any studies that would require an ethical statement.

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# PRODUCT LINE OF SELECTED AGRICULTURAL ENTITY IN SLOVAKIA AND SUGGESTION FOR ITS EXPANSION BASED ON ANALYSIS OF CONSUMERS' INTEREST IN DAIRY AND MEAT PRODUCTS

*Jana Kozáková, Erika Hornáčková*

## ABSTRACT

Studies on primary agricultural production in Slovakia aimed mainly at biological, economic, or market point of view and there is a lack of managerial approach providing a specific recommendation for farmers according to their production lines or planning process. As a reaction, we are providing a practically oriented study based on the selected livestock producer, which is aimed at statistical significances as an establishment for further recommendations for management and marketing of this entity. The paper aims to analyze the views and preferences of customers of the monitored company, which sells its meat and dairy products in its stores and verify the expected success of the planned expansion of the product lines. Regarding this, the online questionnaire study was conducted on a sample of 202 respondents. Realized statistical analysis based on the Kruskal-Wallis nonparametric test and subsequent Bonferroni post hoc correction signify that meat products, fresh meat, and slaughter specialties are the most favorite products among customers of the monitored company. Fresh milk, on the other hand, is not as attractive as a meal for them. However, selling it from vending machines placed out of the store would increase the sales. Dairy products of own production should be even more successful, as we assume they would be as interesting for customers as the currently expanding portfolio of meat products. The benefit of this study lies in the consideration of the practical use of the obtained results in the business practice of the monitored company. Regarding this, we are providing a set of recommendations, according to the planning of their product lines and connected marketing tools for their successful placement on the market.

**Keywords:** consumers; livestock; company store; dairy production; meat; Slovakia

## INTRODUCTION

Agriculture plays an important role in society. In Europe, it is not just an industry with a sole task to produce food. Farmers and agriculture systems play an essential and increasingly important role in protecting the landscape, and rural environment, and contributing to the social and economic development of rural areas. According to **Kotler and Armstrong (2004)**, the farm itself can be considered as a set of factors of production that are subsequently used to produce primary products and services. They are the units of labor, economic, and technological organization and their products are the outcomes of the transformation of the production factors: labor, land, and capital. According to **Jahnátek and Ladvenicová (2011)**, every farm aims to make the production process as successful as possible with the effort to maximize the effectiveness and minimize the difficulty of the performed activities. The complex of interconnected management activities involved in the production of a particular product is considered to be production management **(Chary, 2017)**. It is a managing transformation process that changes labor, capital, materials, information, and other inputs into products and services **(Hill, 2012)** with an aim for the right quality, number, time, and production costs **(Kumar and Suresh, 2006)**. In agribusiness, this process should be designed in a closed cycle, where plant production is closely related to livestock production. Outputs of plant production are at the same time the inputs for animal production (in a form of feed and stems) and outputs of animal, production are used back in plant production (in a form of fertilizers), while both should act environmentally friendly and improve the energy and nutrition cycle **(Bettencourt et al., 2015)**.

Livestock production provides more than one-third of the human protein needs and is the main provider of livelihood in almost all developing countries **(Sakadevan and Nguyen, 2017)**. Farm animals, poultry, and fish are biological transformers of low-quality feed into high-quality proteins and highly bioavailable and essential minerals in the human diet. Livestock production thus plays an important role in improving human nutrition, growth, and health, as well as economic, political, and social stability in society **(Wu, Bazer and Lamb, 2020)**. Livestock plays a crucial role in human development as well

and has hitherto been of great importance in many diverse breeding systems. In the future, livestock production will present considerable opportunities and risks to the food security of the growing human population, as well as to the climatic effects that depend to a large extent on the agricultural system **(Hosey and Melfi, 2018)**. Farmers also breed livestock to transform it into a wide range of outcomes (such as milk, meat, pulling force, manure fertilizer, fuel, hides, wool, fiber, or animals) which can be used for local consumption or sales **(Rushton, 2008)**. Therefore, the personality and attitudes of farmers must be linked to animals, their health, wellbeing, productivity, and management. To reach beneficial results, farm managers should have a high degree of technical knowledge, skills for solving problems, be perceptive and responsible, have the situation under control, and have a good relationship with animals **(Adler, Christley and Campe, 2019)**.

Milk is a biological fluid that contains all significant nutrients and a certain number of substances for given species and conditions **(Keresteš and Selecký, 2003)**. Increasing the consumption of dairy products to the recommended level is one practical change that could significantly improve the content of certain vitamins and minerals in the body and have a positive impact on health **(Scholz-Ahrens, Ahrens and Barth, 2020)**. This applies to calcium, magnesium as well as vitamin A. All dairy producers strive to improve the quality of milk, which can be achieved through various management procedures **(Nolan, 2017)**. Optimal management of dairy farming should focus on milk production which is sustainable and responsible to animal welfare, and social, economic, and environmental perspectives. Each of these aspects is interrelated with one other and has an impact on sustainability, health, and workforce productivity **(Hagevoort, Douphrate and Reynolds, 2013)**. Since the 1990s, the Slovak dairy industry has undone many significant changes related to the reduction of herds of cattle and milk cows, decrease in the quantity of the milked milk, low purchase prices of milk, and decline in a great number of milk producers and processors **(Nagyová et al., 2021)**. Profitable dairy producers must have a great ability to identify problems or opportunities and then, based on their experience and judgment, look for solutions. However, adequate and timely information is of crucial importance for today's managerial decision-making **(Onozaka, Nurse and McFadden, 2010)**.

Over the last few years, supply chains have become increasingly complex, multilevel, and geographically widespread. However, there is still increasing downward pressure on costs without further competing priorities for providing excellent customer service and offering high-quality products and services **(Wilhelm et al., 2016)**. As a result, companies are looking for new technologies and advanced tools to help them navigate complexity **(Gunasekaran et al., 2017)**. In agribusiness, external pressures on better environmental conditions, animal welfare, and food safety practices, together with the internal pressures faced by the conventional food system have led to the development of new supply chains, too **(Ilbery and Maye, 2005)**. The agri-food supply chains are characterized by unique characteristics, compared to the conventional supply ones. Therefore, adapted managerial systems based on high product differentiation, seasonality, the short life cycle of perishable goods, specific requirements of transport, storage conditions, and quality and safety in compliance with national and international regulations are needed **(van der Vorst, Beulens and van Beek, 2000)**. One of the most discussed types of supply chains in Slovak agribusiness is a yard sale. A yard sale can be understood as the selling of products from primary production directly to consumers or indirectly through traders, other farmers, or retailers. **Wagner (2000)** describes two forms of direct selling of agricultural products. The first is a direct selling to final consumers or households in a form of selling from the yard, selling in a field or an orchard, local marketplace, and self-collection. The second form of direct sales is selling through various channels, for example, sales to smaller processing companies such as bakeries and retailers, and wholesalers. **Supeková (2014)** describes yard sales as an economic activity aimed at producing and selling certain foods, specialty products, or typical traditional products. In this type, products can be delivered by local supply chains which support the local producers, while promoting a certain kind of culture in the country. **Pretty (1998)** describes selling from the farm shop as an important way of selling directly to customers. However, he is highlighting that setting up and running such shops can be very costly and often may not bear fruit, since it is aimed at customers who come to the farm on their own. In connection with this, **Šimčák, Piszczalka and Bialeková (2003)** add, that an important feature of the sale from the yard is that the revenues remain in the area and are further used for the development of the region. As a result, agriculture receives more funding, which helps to expand activities and create a positive impact on rural areas and employment. In addition, **Supeková (2014)** mentioned, that direct sale from the yard contributes to the development of agrotourism and countryside and diversification of agricultural production and expansion of the offer of the regional specialties and traditional products.

The operation of milk vending machines is a specific form of selling from the yard. This represents a short supply chain because there are no intermediaries between the farmer and the final consumer and a close supply chain. After all, all the participants are geographically close to each other **(Gallaud and Laperche, 2016)**. In line with customers' wishes to buy local foods and consume natural unprocessed foods, consumers appreciate this distribution channel and at the same time can buy products cheaper. To meet this demand for 'freedom of choice', farmers have increased their sales using self-service vending machines for sale on farms, outside supermarkets, in public squares, car parks, or along crowded streets and cheese factories **(Giacometti et al., 2012)**. In recent years, there has been a significant increase in the development of the vending machine industry. For many farmers, revenues from this become necessary since the price of milk barely covers the production costs of farms. Fortunately, in developed countries, consumers' interest in raw milk is attributed to current trends in organic food and traditional local products **(Tremonte et al., 2014)**. Therefore, vending machines generally appear to be a dynamically developing sector of the economy with fierce competition on the market **(Hampel, Jůzová and Matulíková, 2012)**. Still, this type of selling faces objections in many countries, even though

the legislation has been pushed slowly **(Giacometti et al., 2012)**. Moreover, vending machines alone cannot solve the crisis in the dairy industry. Farmers must help with teaching consumers to buy and consume high-quality and health-promoting milk **(Šmehýlová, 2010)**. The quality of milk in vending machines is guaranteed by strict rules. Machines allow the sale of raw milk only for twenty-four hours, the temperature of the storage unit and the condition of the milk machine can be checked any time via application (farmer) or key card (inspectors) **(TMR, 2016)**. Moreover, under the HACCP (Hazard Analysis and Critical Control Points) system, food business operators shall ensure that all the stages of food production, processing, and distribution under their control comply with the hygiene requirements of **Regulation (EC) No 852/2004**. Successful implementation of the HACCP-based procedures requires full cooperation and deployment of employees, who, concerning this, should undergo training **(Egan et al., 2007)**. In addition, since the vending machines dispense milk in a raw state, the farmer is legally obliged to order consumers to cook it before consumption **(Böhnlein et al., 2021)**.

The main objective of this paper is to analyze consumers' interest in the products of the monitored company sold in the company's store and make suggestions for the future by examining eight factors of consumption (gender of respondents, their age, social status, income, number of people in their household, distance from the company's store, frequency of purchase, and awareness of the product range) and twelve preferences of respondents (the type of products they prefer, whether they buy meal, milk, meat products, specialties, grease, pâté and ready meal for consumption, frequency of buying milk in the company's store, their potential interest in buying new dairy products or milk from a vending machine, whether the monitored company plans to expand the product line and lastly, their suggestions on new products offered). For this purpose, we are analyzing answers of 202 respondents of an online questionnaire study who were customers of the examined company.

Some relevant prior studies are strongly aimed at biological, economic, or marketing conditions of the primary agricultural production, without specific recommendations for selected farmers according to their product lines or provision of details for their planning process. We consider this lack of managerial approach as a big disadvantage for Slovak farmers and as a reaction, we are providing a practically oriented study based on the situation of the selected livestock producer and aimed at the statistical significances as a base for further recommendations for management and marketing of this entity.

Our findings indicate that meat products, fresh meat, and slaughter specialties are the most favorite products among customers of the monitored company. Fresh milk, on the other hand, is not as attractive as a meal for customers, but selling fresh milk from vending machines placed out of the store would increase the sales. The dairy products of their production should be even more successful, as we assume they would be as interesting for customers as the currently expanding portfolio of meat products.

The paper is structured as follows: the introductory part provides an overview of literature sources dealing with entrepreneurship in the primary agricultural production, specifically in animal production focused on the production of fresh meat and milk with the addition of sale of the agricultural products directly from the farm. Next, we are setting a scientific hypothesis and a goal for this study. According to them, we are describing the used material and methodological procedure including its limitations. After, we are introducing the monitored company and describing the outcomes of the implemented analysis and their implications for the business practice of the monitored company. In the end, we are summarizing our outcomes, regarding the description of customers and their interest in the selected products, including recommendations for the production and marketing of the monitored company.

## Scientific Hypothesis

The study is conducted to analyze the demand of the monitored company and make some suggestions for the future. For this purpose, the article analyzes both frequencies of the examined variables and differences between them, as well as preferences of respondents based on the general hypothesis H0 and the set of alternative hypotheses HA connected with the general one (H1 – H8), each specified, according to one factor of consumption (Q1 – Q8):

H0: There is no difference between the observed variables (factors of consumption Q1 – Q8 and the preferences of respondents Q9 – Q13).

HA: There is a difference between the observed variables (factors of consumption Q1 – Q8 and the preferences of respondents Q9 – Q13).

For our research, this alternative hypothesis was extended to the set of specific hypotheses derivated from HA (Table 1). Specific alternative hypotheses (H1 – H8) pointed to the differences between factors Q1 – Q8 and preferences of respondents Q9 – Q13. A computed *p*-value lower than the significance level alpha = 0.05 indicates rejection of the null hypothesis H0, and acceptance of the alternative hypothesis HA and vice versa.

## MATERIAL AND METHODOLOGY

The goal of this study is to analyze the demand of the monitored company and make suggestions for the future. The article examines eight factors of consumption (Q1 Gender, Q2 Age, Q3 Distance, Q4 Social Status, Q5 Household, Q6 Income, Q7 Frequency, Q8 Awareness) which can be at the same time considered as (sorting) characteristics of customers and twelve preferences of respondents (Q9 Consumption Preferences, Q9A Consumption of Meal, Q9B Consumption of Milk, Q9C Consumption of Meat Products, Q9D Consumption of Specialties, Q9E Consumption of Grease, Q9F Consumption of Pâté, Q9G Consumption of Ready Meal, Q10 Milk products, Q11 Milk Frequency, Q12 Milk Machine, Q13 Missing Product) which express respondents' interest in consumption (and buying) of the selected products of the monitored company.

**Table 1** Specific hypotheses derivated from HA.

| HA | *There is a difference between…* | Factor of consumption | *…and the* | Preferences of respondents |
|---|---|---|---|---|
| $H_1$ | | Q1 Gender | | Q9 Consumption- Preferences |
| $H_2$ | | Q2 Age | | Q9A Consumption Meal |
| $H_3$ | | Q3 Distance | | Q9B Consumption Milk |
| $H_4$ | | Q4 Social Status | | Q9C Consumption Meat Products |
| $H_5$ | | Q5 Household | | Q9D Consumption Specialities |
| $H_6$ | | Q6 Income | | Q9E Consumption Grease |
| $H_7$ | | Q7 Frequency | | Q9F Consumption Pate |
| $H_8$ | | Q8 Awareness | | Q9G Consumption Ready Meal |
| | | | | Q10 Milk products |
| | | | | Q11 Milk Frequency |
| | | | | Q12 Milk Machine |
| | | | | Q13 Missing Product |

**Table 2** Design of questionnaire.

| Title | Code | Description | Options |
|---|---|---|---|
| Gender | Q1* | Gender | 1- Man, 2 - Woman |
| Age | Q2* | Age | 1 - 18-24, 2 - 25-34, 3- 35-44, 4- 45-54, 5 - 55 and more |
| Distance | Q3* | Distance from home to the monitored store | 1 -Less than 10 km, 2 – less than 20 km, 3 - less than 30 km, 4 - 50 and more |
| Social Status | Q4* | Social inclusion of respondent | 1 - Student, 2 - Working student, 3 - Employee, 4 - Pension / Maternity leave, 5 - Entrepreneur / Self-employed |
| Household | Q5* | Number of members in the household | 1 - 1 person, 2 - 2-3 people, 3 - 4-5 2 people, 4 -6 and more people |
| Income | Q6* | Common monthly income | 1- less than 900 EUR, 2- 900 – 1500 EUR, 3 -1500 EUR and more |
| Frequency | Q7* | Frequency of shopping in monitored store | 1 - Every day, 2 - Several times a week, 3 - A couple of times a month, 4 - A couple of times a year |
| Awareness | Q8* | awareness of the respondent about the current offer of the monitored store | 1- Yes, 2 - No |
| Consumption-Preferences | Q9 | The most preferred product of consumption from the portfolio of monitored store | 1 - Fresh meat and milk, 2 -Meat and meat products, 3 - Meat and meat specialties |
| Consumption Meal | Q9A | Consumption of fresh meat (beef, veal, pork, smoked) from monitored store | 1- Yes, 2 - No |
| Consumption Milk | Q9B | Consumption of fresh milk from monitored store | 1- Yes, 2 - No |
| Consumption Meat Products | Q9C | Consumption of meat products (Hams, dry sausages, sausages, sausages) from monitored store | 1- Yes, 2 - No |
| Consumption Specialities | Q9D | Consumption of slaughterhouse specialties (press, slaughterhouse porridge, liver) from monitored store | 1- Yes, 2 - No |
| Consumption Grease | Q9E | Consumption of ointment and greaves from monitored store | 1- Yes, 2 - No |
| Consumption Pate | Q9F | Consumption of pâtés and sterilized meat from monitored store | 1- Yes, 2 - No |
| Consumption Ready Meal | Q9G | Consumption of ready meals (cabbage, soup soup) from monitored store | 1- Yes, 2 - No |
| Milk products | Q10 | The potential interest of the respondents in new dairy products produced by the monitored company (cottage cheese, butter, yogurt, cheese) | 1- Yes, 2 - No |
| Milk Frequency | Q11 | Frequency of purchase of fresh milk in the monitored store | 1 - Every day, 2 - Several times a week, 3 - A couple of times a month, 4 - A couple of times a year |
| Milk Machine | Q12 | Potential increase in the consumption of raw cow's milk in case the subject place a milk vending machine outside the store | 1- Yes, 2 - No |
| Missing Product | Q13 | Missing product in the portfolio of monitored store | 1- Yes, 2 - No |

Note: *factors of consumption.

**Description of the Experiment**

**Questionnaire preparation:**

The study was conducted in a form of an online questionnaire **(Raclaw, Barchas-Lichtenstein and Bajuniemi, 2020)** created by Google Forms.

**Number of questions analyzed:** 13.

The design of the questionnaire is summarized in Table 2. The questionnaire includes closed questions only **(Rowley, 2014)**. The first part of them Q1 – Q8 includes sorting questions and the following Q9 – Q13 are the questions directed at the preferences of respondents.

**Conducting a questionnaire survey:** Due to the current pandemic situation **(Cato et al., 2020; Wang, 2021)**, the questionnaire was distributed online by social media **(Kayam and Hirsch, 2012)** based on stratified randomization **(Kim and Shin, 2014)**. We addressed members of the local Facebook communities with the support of the examined company to kindly ask their customers to participate and share the questionnaire via their profile. In the process, roughly 4000 local users of Facebook were addressed, which can be approximated to 2000 potential responding households (with an assumption of two adults/users per household). Therefore, we can say that we addressed 2000 respondents out of which 207 returned a filled form. The return rate was calculated as 10.35%. After adjusting the data **(Wapstra, Audi and Thibault, 2003)**, the sample was narrowed to 202 respondents.

For this article, we addressed exclusively customers of the examined producer, since the designed questionnaire aimed at their preferences in buying products in the company store. The population size **(Kaliyadan and Kulkarni, 2019)** was estimated from the number of inhabitants of the 50 km radius around the company's stores (considered in the questionnaire). This was derived from the number of inhabitants of two regions (region of Nové Mesto nad Váhom – 62,531 inhabitants + region of Myjava 26,895 inhabitants). Together, it is 89,426 inhabitants from 17,885 households (the estimated number of people per household is 4), which indicates the same number of potential customers, since we assume one respondent per household. Therefore, with a 95% of probability that our sample accurately reflects the attitudes of the population and a 7% margin of error, a sample of 202 respondents can be considered representative **(Krejcie and Morgan, 1970)**.

## Statistical Analysis

For this study, the use of statistical analysis was indispensable. The methodological procedure was based on the initial finding that the data have a normal distribution and no autocorrelation. Subsequently, we indicated statistically significant differences between the factors of consumption and the preferences of respondents and verified the established hypotheses. The found differences were subsequently subjected to a pairwise comparison.

Initially, the Shapiro Wilk test of normality **(Shapiro and Francia, 1972)** was used to verify, if the sample comes from a normal distribution. The Shapiro Wilk procedure shows the Sig. value for all dependent variables 0.000, which indicates that the data significantly deviate from a normal distribution. This outcome was proved also by partial Sapiro Wilk tests for factors Q1 – Q8, which establish that the variables are not normally distributed for each level of independent variables.

To indicate, whether there is an autocorrelation between variables, the Durbin–Watson test on autocorrelation **(Watson and Durbin, 1951)** was used. This was based on the least-squares residual vector, which causes that distribution depends on the regression matrix **(Abrahamse and Louter, 1971)**. The Durbin-Watson procedure for the variables Q1 – Q8 (Table 3) shows outcomes between 1.5 and 2.5 and therefore, we can conclude that the data do not autocorrelation with one another.

Subsequently, under the non-normally distributed data we had, the Kruskal-Wallis nonparametric statistical test, which assesses the differences among three or more samples on a single non-normally distributed variable **(Kruskal and Wallis, 1952)**, was applied. We use the test with an assumption of statistically significant differences between groups of the independent variables Q1 – Q8 and continuous or ordinal dependent variables Q9 – Q13 (Table 4 – Table 11). After finding significance, according to the group of variables Q1 – Q8, we used the Bonferroni post hoc test to study the relationship between the variables **(Lee and Lee, 2018)** closely. The test was applied to pairwise comparisons of the significant relationships found by Kruskal-Wallis, with an assumption that the data are a random sample from a normal population. The post hoc analysis was conducted as a pairwise comparison of the dependent variables and factors, based on the estimated marginal means with the mean difference significant at the 0.05 level, using the adjustment for multiple comparisons (Table 12 – Table 26). The *p*-value calculated in these analyses was adjusted for Bonferroni correction and therefore, it was not necessary to correct it additionally. The pairwise comparisons were applied to significance between factors of consumption (Q1 – Q8) and preferences of respondents (Q9 – Q13) identified by the Kruskal-Wallis test, excluding combination of dependent variable Q11 Milk Frequency and factor Q7 Frequency because of an apparent link between them, although no autocorrelation between respondents' responses has been found. Nevertheless, in our opinion, this combination does not yield valuable results for interpretation.

For the statistical analysis and calculating of the data frequencies, the Software IBM SPSS Statistics Subscription 1.0.0.1447 was used.

## RESULTS AND DISCUSSION

The monitored entity is an agricultural cooperative established in 1991 from the original one which started in 1949. The company's main activities include the production, processing, and sale of agricultural products. It is a long-term member of the agricultural sphere in Slovakia and has a tradition in the region. In its production, it focuses on crop and livestock and thereby achieves optimal diversification **(De Roest, Ferrari and Knickel, 2018)** of primary agricultural production. Currently, it manages 1500 hectares of land of which 1000 hectares are of arable land, where traditional crops grow, while 70% of plant production goes to feed the livestock **(Church, 1991)**. From the livestock sector, the subject is engaged in pig breeding **(Whittemore, 1980)** and cattle farming oriented towards combined meat and milk production **(Foley et al., 1972)**.

**Table 3** Durbin-Watson test of autocorrelation for variables Q1 – Q8.

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate | Durbin-Watson |
|---|---|---|---|---|---|
| Q1 | .379[a] | 0.144 | 0.054 | 0.457 | 2.273 |
| Q2 | .556[a] | 0.309 | 0.237 | 1.079 | 2.080 |
| Q3 | .370[a] | 0.137 | 0.047 | 0.746 | 1.905 |
| Q4 | .513[a] | 0.263 | 0.187 | 0.672 | 2.108 |
| Q5 | .434[a] | 0.188 | 0.104 | 0.614 | 1.908 |
| Q6 | .505[a] | 0.255 | 0.177 | 0.552 | 1.909 |
| Q7 | .700[a] | 0.490 | 0.437 | 0.529 | 1.986 |
| Q8 | .406[a] | 0.165 | 0.078 | 0.360 | 1.888 |

**Table 4** Kruskal-Wallis Test – Grouping Variable: Q1 Gender.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 0.214 | 0.9 | 0.494 | 0 | 0.178 | 0.572 | 0.011 | 0.9 | 0.964 | 0.408 | 1.42 | 0.058 |
| df | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Asymp. Sig. | 0.643 | 0.343 | 0.482 | 0.986 | 0.674 | 0.450 | 0.915 | 0.343 | 0.326 | 0.523 | 0.233 | 0.810 |

**Table 5** Kruskal-Wallis Test – Grouping Variable: Q2 Age.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 0.675 | 2.082 | 3.678 | 4.105 | 5.095 | 0.565 | 2.153 | 2.331 | 1.271 | 0.936 | 11.616 | 10.295 |
| df | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Asymp. Sig. | 0.954 | 0.721 | 0.451 | 0.392 | 0.278 | 0.967 | 0.708 | 0.675 | 0.866 | 0.919 | 0.020 | 0.056 |

**Table 6** Kruskal-Wallis Test – Grouping Variable: Q3 Distance.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 2.505 | 3.193 | 5.74 | 2.228 | 0.577 | 2.582 | 1.394 | 1.039 | 2.224 | 6.52 | 3.305 | 2.336 |
| df | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Asymp. Sig. | 0.474 | 0.363 | 0.125 | 0.527 | 0.902 | 0.461 | 0.707 | 0.792 | 0.527 | 0.089 | 0.347 | 0.506 |

**Table 7** Kruskal-Wallis Test – Grouping Variable: Q4 Social Status.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 8.9 | 3.13 | 5.938 | 4.495 | 4.367 | 4.47 | 5.428 | 0.898 | 2.273 | 6.898 | 4.737 | 2.949 |
| df | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Asymp. Sig. | 0.088 | 0.536 | 0.204 | 0.343 | 0.359 | 0.346 | 0.246 | 0.925 | 0.686 | 0.141 | 0.315 | 0.566 |

**Table 8** Kruskal-Wallis Test – Grouping Variable: Q5 Household.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 1.512 | 4.274 | 5.997 | 2.029 | 6.942 | 24.937 | 4.838 | 3.98 | 2.298 | 6.49 | 2.443 | 5.298 |
| df | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Asymp. Sig. | 0.680 | 0.233 | 0.112 | 0.567 | 0.074 | 0.000 | 0.184 | 0.264 | 0.513 | 0.030 | 0.486 | 0.151 |

**Table 9** Kruskal-Wallis Test – Grouping Variable: Q6 Income.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 0.081 | 2.574 | 1.694 | 0.736 | 1.496 | 7.111 | 8.746 | 0.836 | 1.777 | 0.723 | 2.44 | 0.278 |
| df | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Asymp. Sig. | 0.961 | 0.276 | 0.429 | 0.692 | 0.473 | 0.029 | 0.013 | 0.658 | 0.411 | 0.697 | 0.295 | 0.870 |

**Table 10** Kruskal-Wallis Test – Grouping Variable: Q7 Frequency.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 4.287 | 14.533 | 9.223 | 7 | 13.727 | 11.382 | 2.759 | 10.271 | 6.259 | 64.606 | 6.847 | 13.773 |
| df | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Asymp. Sig. | 0.232 | 0.002 | 0.026 | 0.072 | 0.003 | 0.010 | 0.43 | 0.016 | 0.100 | 0.000 | 0.077 | 0.003 |

The company processes approximately 3,000 pigs per year. In dairy farming, it focuses on the longevity of cows **(VanRaden and Klaaskate, 1993; Pritchard et al., 2013)** to reach the intermediate period below 390 days and to show favorable production parameters. The average performance of the herd **(Coleman, Thayne and Dailey, 1985)** varies around 8,000 kg of milk per cow. Also, the subject owns a slaughterhouse and since 2015, it sells slaughter meat and products in its store. The offer based on fresh products was designed primarily for residents of the surrounding area. But the shop is regularly visited by inhabitants of nearby villages and towns, too. Therefore, the company decided to facilitate access to their products for people from the wider surroundings, and in 2018, the second store was opened. In these stores, they sell own-produced (1) pork (head, lobe, neck, shoulder, loin, tenderloin, belly, thigh, stock, tail, knee, leg and meat smoked by traditional technology on beech wood, (2) beef (neck, chest, shoulder, high and low filler, true sirloin, ribs, groin, and tail), (3) veal and (4) chicken meat. In addition, they offer meat products, traditional hams various types of sausages, slaughter specialties (porridge, stems), fat, grease, pâté, and sterilized meat. Newly, there are also ready-made dishes (ready meal) such as cabbage soup or soup stick. Fresh cow's milk, which can be sucked into a plastic bottle or own container, is also a stable part of the product line. The milk dispensing rack is made for self-service dosing into bottles. For the future, the monitored entity is considering placing the milk vending machine **(Doležalová et al., 2014)** outside the store, which presupposes an increase in milk sales, as it will be closer to consumers and due to this, it will not be necessary to visit the store for its purchase. In addition, due to the extraordinary success of meat products and specialties, the entity is considering the production of its own dairy products. Therefore, this paper analyzes the views and preferences of customers of the monitored entity on the currently offered products and verifies the expected success of the planned changes and expansion of the product line.

The benefit of this study lies in the consideration of the practical use of the obtained results in the planning of the production base of the monitored company. In addition, its contribution is theoretical in the expansion of the knowledge of management of the primary agricultural production in Slovakia and points out the need to strengthen the position of livestock production in the economy of the individual agricultural entities and country. As the world population continues to grow, the main challenge facing society is to maintain a strong and viable food system that is closely linked to the well-being and comfort of food-producing animals **(Wilson and Burton, 2015)** and to create strong connections between cultural and biological values **(Dobrovodská et al., 2019)** in rural society. This study's object is the primary agricultural producer who contributes to the fulfillment of the mentioned functions in its wider region. It focuses on the production of fresh and high-quality products, affordable and geographically available to people in the region. This effort is therefore directly dependent on the requirements of customers. It is the scientific processing that is an appropriate tool for analyzing the customers' requirements and preferences and we believe that this study, conducted in an academic approach, will bring practical benefits to the next steps in the business of monitored subject. Regarding this, we have analyzed the answers of 202 respondents – customers of the monitored company. An online questionnaire study aimed at their preferences in buying products offered in the company's store was implemented. The questionnaire includes a set of sorting questions (Q1 – Q8), which helps us to understand the structure of the company's customers in a more detailed way, together with the questions (Q9 – Q13), which are aimed at consumers' preferences for products of the monitored store.

According to the product-oriented set of questions, we can conclude that the most preferred products of consumption (Q9 Consumption – Preferences) from the portfolio of the monitored store are meat and meat specialties, which is the reason for visiting the store for 62.4% of customers (126 out of 202). An additional 24.8% of them (50 out of 202) are interested mainly in meat and meat products (specialties excluding). And just the rest of 12.9% (26 out of 202) are interested in fresh milk and meat. In line with this, **Cupák, Pokrivčák and Rizov (2015)** described demand for dairy products as own-price elastic, which indicates that such goods are perceived as luxuries. Described structure of the customer's interest confirms that the recent expansion of the product portfolio of meat products and slaughter specialties was a good step, as these are the reasons for visiting the store for more than 80% of customers.

The specific composition of the customer's preferences in buying products of the monitored store is shown in Figure 1 and Figure 2. This proves that meat products, fresh meat, and slaughter specialties are the most favorite products among customers of the monitored company. The study of **Thornton (2010)** expected similar customer preferences and predicted a continuous increase in the demand for livestock products, driven largely by human population growth, income growth, and urbanization for the next three decades, at least.

Fresh milk, on the other hand, is not as attractive as a meal for customers. Just 1.5% of them (3 out of 202) buy fresh milk in the monitored store every day, 16.3% (33 out of 202) buy it several times a week and 34.7% (70 out of 202) buy it a couple of times a month. Almost half of the examined customers, 47.5% (96 out of 202), buy fresh milk just a couple of times a year. Such insufficient consumption of milk and dairy products was highlighted also by **Kubicová, Predanocyová and Kádeková (2019)**, who assumed that in Slovakia, it is just at the level of 70% of the recommended intakes. Therefore, we see a solution for the monitored company in a start-up of production of their dairy products. We are recommending this even though since 1990, we have significantly reduced the production and consumption of dairy products in Slovakia, which resulted in two to three times fewer consumption of acidic products and cheese than in the developed countries of Europe **(Herian, 2019)**.

**Table 11** Kruskal-Wallis Test – Grouping Variable: Q8 Awareness.

| | Q9A Meal | Q9B Milk | Q9C Meat product | Q9D Specialty | Q9E Grease | Q9F Pate | Q9G Ready meal | Q9 Preferences | Q10 Milk products | Q11 Milk Frequency | Q12 Milk Machine | Q13 Missing Product |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kruskal-Wallis H | 2.986 | 1.283 | 1.2 | 0.326 | 4.204 | 6.433 | 0.593 | 1.004 | 8.95 | 6.594 | 0.036 | 1.953 |
| df | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Asymp. Sig. | 0.084 | 0.257 | 0.313 | 0.568 | 0.040 | 0.011 | 0.441 | 0.316 | 0.003 | 0.010 | 0.850 | 0.162 |

**Table 12** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9B Milk vs Factor: Q7 Frequency.

| (I) Q7 Frequency | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | 0.252 | 0.225 | 1 | -0.348 | 0.853 |
| | 3 | -0.052 | 0.219 | 1 | -0.635 | 0.531 |
| | 4 | -0.094 | 0.228 | 1 | -0.703 | 0.514 |
| 2 | 1 | -0.252 | 0.225 | 1 | -0.853 | 0.348 |
| | 3 | -.304* | 0.084 | 0.002 | -0.527 | -0.082 |
| | 4 | -.347* | 0.107 | 0.008 | -0.63 | -0.063 |
| 3 | 1 | 0.052 | 0.219 | 1 | -0.531 | 0.635 |
| | 2 | .304* | 0.084 | 0.002 | 0.082 | 0.527 |
| | 4 | -0.042 | 0.091 | 1 | -0.286 | 0.201 |
| 4 | 1 | 0.094 | 0.228 | 1 | -0.514 | 0.703 |
| | 2 | .347* | 0.107 | 0.008 | 0.063 | 0.63 |
| | 3 | 0.042 | 0.091 | 1 | -0.201 | 0.286 |

**Table 13** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9C Meat product vs Factor: Q7 Frequency.

| (I) Q7 Frequency | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -0.13 | 0.195 | 1 | -0.65 | 0.389 |
| | 3 | -0.226 | 0.189 | 1 | -0.73 | 0.278 |
| | 4 | -0.389 | 0.197 | 0.302 | -0.915 | 0.137 |
| 2 | 1 | 0.13 | 0.195 | 1 | -0.389 | 0.65 |
| | 3 | -0.096 | 0.072 | 1 | -0.288 | 0.097 |
| | 4 | -.258* | 0.092 | 0.033 | -0.504 | -0.013 |
| 3 | 1 | 0.226 | 0.189 | 1 | -0.278 | 0.73 |
| | 2 | 0.096 | 0.072 | 1 | -0.097 | 0.288 |
| | 4 | -0.163 | 0.079 | 0.244 | -0.373 | 0.048 |
| 4 | 1 | 0.389 | 0.197 | 0.302 | -0.137 | 0.915 |
| | 2 | .258* | 0.092 | 0.033 | 0.013 | 0.504 |
| | 3 | 0.163 | 0.079 | 0.244 | -0.048 | 0.373 |

**Table 14** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9E Grease vs Factor: Q7 Frequency.

| (I) Q7 Frequency | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -0.052 | 0.178 | 1 | -0.525 | 0.421 |
| | 3 | -0.261 | 0.172 | 0.789 | -0.72 | 0.198 |
| | 4 | -0.317 | 0.18 | 0.48 | -0.796 | 0.163 |
| 2 | 1 | 0.052 | 0.178 | 1 | -0.421 | 0.525 |
| | 3 | -.209* | 0.066 | 0.011 | -0.384 | -0.033 |
| | 4 | -.264* | 0.084 | 0.011 | -0.488 | -0.041 |
| 3 | 1 | 0.261 | 0.172 | 0.789 | -0.198 | 0.72 |
| | 2 | .209* | 0.066 | 0.011 | 0.033 | 0.384 |
| | 4 | -0.056 | 0.072 | 1 | -0.248 | 0.136 |
| 4 | 1 | 0.317 | 0.18 | 0.48 | -0.163 | 0.796 |
| | 2 | .264* | 0.084 | 0.011 | 0.041 | 0.488 |
| | 3 | 0.056 | 0.072 | 1 | -0.136 | 0.248 |

**Table 15** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9E Grease vs Factor: Q8 Awareness.

| (I) Q8 Awareness | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -.150* | 0.072 | 0.04 | -0.292 | -0.007 |
| 2 | 1 | .150* | 0.072 | 0.04 | 0.007 | 0.292 |

**Table 16** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9F Pate vs Factor: Q5 Household.

| (I) Q5 Household | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | 0.155 | 0.158 | 1.00 | -0.268 | 0.577 |
| | 3 | 0.159 | 0.159 | 1.00 | -0.264 | 0.583 |
| | 4 | .818* | 0.191 | 0.00 | 0.309 | 1.328 |
| 2 | 1 | -0.155 | 0.158 | 1.00 | -0.577 | 0.268 |
| | 3 | 0.004 | 0.055 | 1.00 | -0.143 | 0.152 |
| | 4 | .664* | 0.12 | 0.00 | 0.344 | 0.983 |
| 3 | 1 | -0.159 | 0.159 | 1.00 | -0.583 | 0.264 |
| | 2 | -0.004 | 0.055 | 1.00 | -0.152 | 0.143 |
| | 4 | .659* | 0.12 | 0.00 | 0.338 | 0.98 |
| 4 | 1 | -.818* | 0.191 | 0.00 | -1.328 | -0.309 |
| | 2 | -.664* | 0.12 | 0.00 | -0.983 | -0.344 |
| | 3 | -.659* | 0.12 | 0.00 | -0.98 | -0.338 |

**Table 17** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9F Pate vs Factor: Q6 Income.

| (I) Q6 Income | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -0.159 | 0.124 | 0.601 | -0.458 | 0.14 |
| | 3 | -0.002 | 0.121 | 1 | -0.295 | 0.29 |
| 2 | 1 | 0.159 | 0.124 | 0.601 | -0.14 | 0.458 |
| | 3 | .157* | 0.059 | 0.025 | 0.015 | 0.299 |
| 3 | 1 | 0.002 | 0.121 | 1 | -0.29 | 0.295 |
| | 2 | -.157* | 0.059 | 0.025 | -0.299 | -0.015 |

Our proposal assumes that customers could be more interested in dairy products than in fresh cow's milk itself, just as it is with meat products, in which customers are more interested than in fresh meat. Moreover, potential interest in new dairy products of the monitored company (cottage cheese, butter, yogurt, cheese), Q10, was expressed by 186 out of 202 asked customers, which indicates the niche on this local market and creates potential space for expansion. On the other hand, the potential increase in the consumption of raw cow's milk, in case the subject places a milk vending machine outside the store was shown just by 98 out of 202 customers, which still provides an opportunity but not as clear one as in the case of dairy products (Table 2).

This was indicated also by the outcomes of **Nagyová et al. (2021)**, who described a highly positive response of consumers (more than 94%) to the consumption of the Slovak traditional cheeses and dairy products. However, it must be mentioned that the recent worldwide consumption of animal-based products has decreased in favor of plant-based food products, under the current trend of the global expansion of alternative food **(Prytulska, 2021)**. Also, when expanding the product range, the monitored company should be aware of the main factors determining the consumption of milk and dairy products considered by **Kubicová, Predanocyová and Kádeková (2019)** as are quality, composition, price, durability, and nutrition data. These variables form consumer's attitudes and were thus found to be an important predictor of local food purchase behavior **(Feldmann and Hamm, 2015)**.

According to gender, we can say that customers of the examined company are mostly women, 136 out of 202 (67.3%), which corresponds with the outcomes of **Ubrežiová et al. (2019)**, who states that women tend to buy dairy products more often than men. This fact should be taken into consideration when planning marketing and choosing retail strategies, customized for female clients **(Bakewell and Mitchell, 2003; Silverstein and Sayre, 2009; Hawkins and Mothersbaugh, 2010**). On the other hand, we do not recommend modifying the offered assortment and adapting it to the generally accepted preferences of female customers, given that we are analyzing products purchased for the whole family and therefore, the preferences of other household members should be considered, too. This is supported also by the results of the statistical examination out of which the Kruskal-Wallis test (Table 4) showed no statistically significant differences in the preferences of men and women involved in our study since *p*-values of all the examined variables were higher than significant 0.05. Therefore, in the case of grouping the variable Q1, we are accepting the null hypothesis H0 and rejecting the alternative hypothesis H1. According to this, we consider the personal preferences of the one who is buying as not decisive, since products of the monitored company are foods for the whole family. Thus, the composition of the purchase reflects the customs of the family as a community and is based on the observances and traditions grown within the family **(MacGregor, 1995)** without a link to the preferences of a particular gender. On the other hand, this outcome could be enhanced by the fact that the study was carried out at the time of the applied lockdown restricting the mobility of persons, which theoretically excluded more than one household person from buying and thus promoting their preferences.

From the age point of view, customers of the examined company are more diverse. We stratified five age groups (Table 5) which reflect the life stages of respondents. In the first group, there were young adults from 18 to 24 years of age, who typically still stay at their parent's house **(White, 1994)**. Even though they often have at least a partial source of their own income **(Szendrey and Fiala, 2018)**, their basic maintenance (food including) is often financed by their parents. This age group formed 11.9 % of respondents (24 out of 202). The second relatively low represented group (12.9%) was of age 55 and more (26 out of 202), which can be influenced by the fact, that the rural population (the study was carried out in an area with almost exclusive representation of rural settlements) in this age category maintains the tradition of domestic slaughters **(Edwards, 2010)** and self-made meat products **(Talon et al., 2007),** which results in a lower need for buying them. Age groups from 35 to 44 (21.8%) and 45 to 54 years (22.3%) are significantly higher represented. Respondents from the group of 35 to 44 year old (44 out of 202) are typically from families who have children in elementary schools or of teenage age and thus, their preferences affect the shopping habits within the family. Subsequently, respondents from the group of 45 to 54 years of age typically have children of adult age, yet dietary needs remain common. Adults at the age of 25 – 34 (32.2%) have the highest representation among the customers. In our opinion, the reason for this is that they are parents of younger children and thus try to be responsible, well informed, and rational, which results in their efforts to ensure high quality, fresh and local products for their family, since the home food environment is particularly important during childhood by making healthful foods available and accessible, modeling healthful eating behaviors, and encouraging children to choose healthful foods. Due to this, parents help them develop dietary patterns that will serve them well throughout their lives **(Ray and Klesges, 1993)**. It must be mentioned that we found no significant differences in the frequency of shopping ($p = 0.705$) between mentioned age groups and therefore, there are no differences in the frequency of purchases between customers of different ages, either. Different age groups are represented in different numbers. However, significant differences were found (Table 5) in the case of Q12 for which we can reject the null hypothesis and adopt the alternative hypothesis H2. The Kruskal-Wallis test showed a statistically significant difference in attitudes towards the placement of a new milk vending machine among different age groups $\chi2\ (2) = 11.616$ with $p = 0.020$. The subsequent post hoc test showed that this significant difference was between the age group 1 and 4 (Table 25), which means that the customers of age groups of 18 to 24 years old and of 45 to 54 years old have different attitudes ($p = 0.017$) towards the milk machine placed out of the existing company's shop.

**Table 18** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9F Pate vs Factor: Q7 Frequency.

| (I) Q7 Frequency | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -0.052 | 0.186 | 1 | -0.548 | 0.443 |
| | 3 | -0.252 | 0.18 | 0.982 | -0.733 | 0.229 |
| | 4 | -0.317 | 0.188 | 0.567 | -0.819 | 0.186 |
| 2 | 1 | 0.052 | 0.186 | 1 | -0.443 | 0.548 |
| | 3 | -.200* | 0.069 | 0.025 | -0.384 | -0.016 |
| | 4 | -.264* | 0.088 | 0.018 | -0.499 | -0.03 |
| 3 | 1 | 0.252 | 0.18 | 0.982 | -0.229 | 0.733 |
| | 2 | .200* | 0.069 | 0.025 | 0.016 | 0.384 |
| | 4 | -0.064 | 0.075 | 1 | -0.265 | 0.136 |
| 4 | 1 | 0.317 | 0.188 | 0.567 | -0.186 | 0.819 |
| | 2 | .264* | 0.088 | 0.018 | 0.03 | 0.499 |
| | 3 | 0.064 | 0.075 | 1 | -0.136 | 0.265 |

**Table 19** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9F Pate vs Factor: Q8 Awareness.

| (I) Q8 Awareness | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -.191* | 0.075 | 0.012 | -0.339 | -0.043 |
| 2 | 1 | .191* | 0.075 | 0.012 | 0.043 | 0.339 |

**Table 20** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9G Ready meal vs Factor: Q6 Income.

| (I) Q6 Income | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -.242* | 0.089 | 0.022 | -0.458 | -0.027 |
| | 3 | -.263* | 0.087 | 0.009 | -0.473 | -0.052 |
| 2 | 1 | .242* | 0.089 | 0.022 | 0.027 | 0.458 |
| | 3 | -0.02 | 0.043 | 1 | -0.123 | 0.083 |
| 3 | 1 | .263* | 0.087 | 0.009 | 0.052 | 0.473 |
| | 2 | 0.02 | 0.043 | 1 | -0.083 | 0.123 |

**Table 21** Pairwise Comparisons – Bonferroni: Dependent Variable: Q9 Preferences vs Factor: Q7 Frequency.

| (I) Q7 Frequency | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | 0.126 | 0.329 | 1 | -0.751 | 1.003 |
| | 3 | 0.287 | 0.319 | 1 | -0.564 | 1.138 |
| | 4 | 0.633 | 0.333 | 0.354 | -0.255 | 1.522 |
| 2 | 1 | -0.126 | 0.329 | 1 | -1.003 | 0.751 |
| | 3 | 0.161 | 0.122 | 1 | -0.164 | 0.486 |
| | 4 | .507* | 0.155 | 0.008 | 0.093 | 0.922 |
| 3 | 1 | -0.287 | 0.319 | 1 | -1.138 | 0.564 |
| | 2 | -0.161 | 0.122 | 1 | -0.486 | 0.164 |
| | 4 | 0.346 | 0.133 | 0.061 | -0.009 | 0.702 |
| 4 | 1 | -0.633 | 0.333 | 0.354 | -1.522 | 0.255 |
| | 2 | -.507* | 0.155 | 0.008 | -0.922 | -0.093 |
| | 3 | -0.346 | 0.133 | 0.061 | -0.702 | 0.009 |

**Table 22** Pairwise Comparisons – Bonferroni: Dependent Variable: Q10 Milk products vs Factor: Q8 Awareness.

| (I) Q8 Awareness | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -.152* | 0.05 | 0.003 | -0.251 | -0.054 |
| 2 | 1 | .152* | 0.05 | 0.003 | 0.054 | 0.251 |

**Table 23** Pairwise Comparisons – Bonferroni: Dependent Variable: Q11 Milk Freqency vs Factor: Q5 Household.

| (I) Q5 Household | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | 0.149 | 0.327 | 1 | -0.722 | 1.021 |
| | 3 | 0.227 | 0.328 | 1 | -0.647 | 1.101 |
| | 4 | 0.864 | 0.394 | 0.178 | -0.187 | 1.915 |
| 2 | 1 | -0.149 | 0.327 | 1 | -1.021 | 0.722 |
| | 3 | 0.078 | 0.114 | 1 | -0.227 | 0.383 |
| | 4 | .714* | 0.247 | 0.026 | 0.055 | 1.373 |
| 3 | 1 | -0.227 | 0.328 | 1 | -1.101 | 0.647 |
| | 2 | -0.078 | 0.114 | 1 | -0.383 | 0.227 |
| | 4 | 0.636 | 0.249 | 0.067 | -0.026 | 1.299 |
| 4 | 1 | -0.864 | 0.394 | 0.178 | -1.915 | 0.187 |
| | 2 | -.714* | 0.247 | 0.026 | -1.373 | -0.055 |
| | 3 | -0.636 | 0.249 | 0.067 | -1.299 | 0.026 |

**Table 24** Pairwise Comparisons – Bonferroni: Dependent Variable: Q11 Milk Freqency vs Factor: Q8 Awareness.

| (I) Q8 Awareness | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -.368* | 0.146 | 0.013 | -0.657 | -0.079 |
| 2 | 1 | .368* | 0.146 | 0.013 | 0.079 | 0.657 |

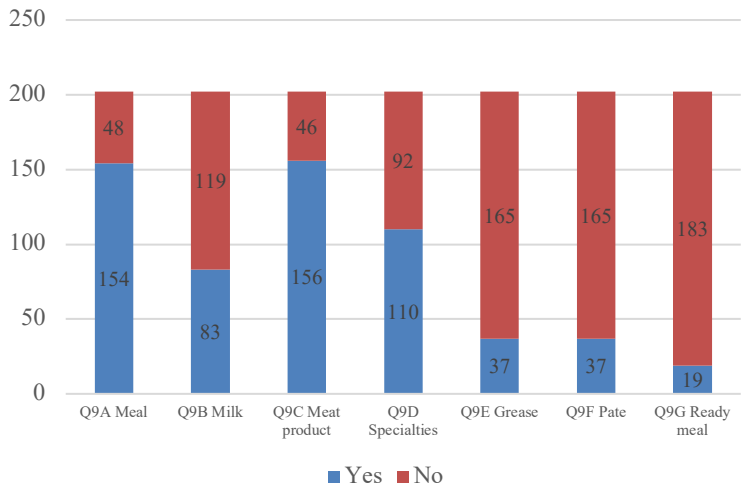


**Figure 1** Frequencies of Q9A – Q9G.

These outcomes highlighted no necessity to adjust the product portfolio to different age groups of customers, but it must be perceived with caution in the plans, regarding selling raw milk in vending machines and taking the age of customers into consideration. The third examined variable was distance Q3. The choice of this variable was based on the outcomes of **Javanmard and Hoseini (2013)**, who proved a significantly positive relationship between short distance and customers' satisfaction. Our findings show that the majority of customers (74.8%) are from the neighborhood of ten kilometers (151 out of 202), which shows similarities with the outcomes of **Onozaka, Nurse and McFadden (2010)**, who identified that 82% of people buy local fresh products, which is vital information, regarding the aim of a marketing strategy. In connection with this, we strongly recommend pointing to the benefits of the local fresh production intended primarily for the direct domestic consumers. The rest of the customers live far from the company's store. Concerning distance of more than 10 kilometers but less than 20, 17.8% of them arrive in the store (36 out of 202) and concerning the distance of 30 kilometers, 2.5% of them arrive at the store (5 out of 202). Surprisingly, due to the products of the monitored company, 5% of the examined customers (10 out of 202) do not hesitate to overcome the longest distance of 30 to 50 kilometers. However, in the case of this variable, no significance was found. The Kruskal-Wallis test (Table 6) showed no statistically significant differences in the preferences of customers from different distances since *p*-values of all the examined variables were higher than 0.05 and therefore, for the variable Q3, we are accepting the null hypothesis H0 and rejecting the alternative hypothesis H3. It must be mentioned that from the distance point of view, **Bond, Thilmany and Bond (2009)** recommended increasing security and loyalty towards the current local customers by emphasizing the availability of fresh, superior, vitamin-rich, and locally grown products. To attract new customers who do not currently have a preference for purchasing directly, producers may find greater success by locating shop into the convenient-to-reach venues, showcasing a variety of colorful offerings, and working to enhance the overall aesthetic appeal of the market locations.

A similar statistical outcome as in the case of distance Q3 was found also in the case of the variable Q4 social status. Here we are accepting the null hypothesis H0 and rejecting the alternative hypothesis H4 out of the same reasoning (Table 7). Nevertheless, it can be concluded that just 4% of customers (8 out of 202) are students (with no own income) and an additional 4.5% of them (9 out of 202) are working students with their income. Surprisingly, just 6.4% are entrepreneurs or self-employers (13 out of 202) and 10.4 % of them are on a pension or maternity leave (21 out of 202). Therefore, we can say that most of the customers are employees (151 out of 202) and we can assume that this 74.8% represent adults with regular income, which allows them to buy products of the monitored company.

As the next examined variable, the Q5 household was chosen to identify, whether there is a difference in customer's preferences connected with the number of people, their household consists of. One-person households are represented among customers of the monitored store only by 3% (6 out of 202), similarly to households of more than six people, by 5.4% (11 out of 202). The majority of customers, 48% (97 out of 202), are from families of two to three people, and surprisingly, a little lower number of them (88 out of 202) are from four to five-member families (44%). This distribution supports the general fact that most people of productive age (our most represented group of respondents) live in three to five-member households **(SOSR, 2020)**. However, we believe that in the case of our respondents, it is also due to the increased interest in fresh meat and dairy products by families with children who are trying to apply a responsible approach to their eating habits **(Pedersen, Grønhøj and Bech-Larsen, 2012)**. Moreover, in the case of this variable (Q5 Household), the Kruskal-Wallis test (Table 8) showed a significant difference in buying Pâté Q9F, $\chi 2$ (2) = 24.937 and $p$ = 0.000. The post hoc Bonferroni test showed a specific significance between families of more than six people and every other examined family type with the $p$ = 0.000 (Table 16). This outcome indicates that the examined company should apply a different approach in selling their pâté to bigger households (more than six people). But, because this type of family is represented just by 5.4%, we do not recommend taking any specific actions. According to the Kruskal-Wallis test (Table 8), there was also a significant difference in the frequency of buying Q11 fresh raw milk ($\chi 2$ (2) = 6.49 and $p$ = 0.030), which under a deeper analysis (the Bonferroni post hoc test) showed a pairwise difference (Table 23) between households of two to three people and households with more than six people ($p$ = 0.026). For the factors Q9F Pâté and Q11 Milk Frequency, we can therefore accept the alternative hypothesis H5. This indicates that the examined company should apply a different approach when selling fresh milk to these types of families. But again, due to a low share of more than six people families among customers of this company, we consider special activities too costly with a high risk that they will not be effective.

Generally, consumers' income and food prices are understood as the main influential factors affecting food consumption in Slovakia **(Benda-Prokeinová and Hanová, 2016)**. Regarding this, the variable Q6 Income was included in the analysis to detect the income level of customers, which can help to understand their demand and price elasticity. According to this, we set three levels of customers' income. Monthly income under 900 EUR occurred less often, in just 5.9% of them (12 out of 202). Monthly income of 900 to 1500 EUR occurred in 38.1% of customers (77 out of 202) and surprisingly, the highest level of income which was set over 1500 EUR per month occurred in more than a half (55.9%) of the examined customers (113 out of 202). These outcomes indicate that the products of the monitored company are popular with people from a higher income group, given that the average nominal monthly wages of an employee in the Slovak economy (in 2020) was 1133 EUR **(SOSR, 2021)**. Additional statistical analysis (Table 9) shows significant differences in buying pâté and ready meal by different income groups of customers. In case of Q9F Pâté, it was with $\chi 2$ (2) = 7.111 and $p$ = 0.029.

**Table 25** Pairwise Comparisons – Bonferroni: Dependent Variable: Q12 Milk Machine vs Factor: Q2 Age.

| (I) Q2 Age | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -0.258 | 0.118 | 0.298 | -0.592 | 0.077 |
| | 3 | -0.341 | 0.125 | 0.068 | -0.695 | 0.013 |
| | 4 | -.394* | 0.124 | 0.017 | -0.747 | -0.042 |
| | 5 | -0.173 | 0.139 | 1 | -0.568 | 0.222 |
| 2 | 1 | 0.258 | 0.118 | 0.298 | -0.077 | 0.592 |
| | 3 | -0.083 | 0.097 | 1 | -0.357 | 0.191 |
| | 4 | -0.137 | 0.096 | 1 | -0.409 | 0.136 |
| | 5 | 0.085 | 0.115 | 1 | -0.24 | 0.41 |
| 3 | 1 | 0.341 | 0.125 | 0.068 | -0.013 | 0.695 |
| | 2 | 0.083 | 0.097 | 1 | -0.191 | 0.357 |
| | 4 | -0.054 | 0.104 | 1 | -0.349 | 0.242 |
| | 5 | 0.168 | 0.122 | 1 | -0.177 | 0.513 |
| 4 | 1 | .394* | 0.124 | 0.017 | 0.042 | 0.747 |
| | 2 | 0.137 | 0.096 | 1 | -0.136 | 0.409 |
| | 3 | 0.054 | 0.104 | 1 | -0.242 | 0.349 |
| | 5 | 0.221 | 0.121 | 0.689 | -0.122 | 0.565 |
| 5 | 1 | 0.173 | 0.139 | 1 | -0.222 | 0.568 |
| | 2 | -0.085 | 0.115 | 1 | -0.41 | 0.24 |
| | 3 | -0.168 | 0.122 | 1 | -0.513 | 0.177 |
| | 4 | -0.221 | 0.121 | 0.689 | -0.565 | 0.122 |

**Table 26** Pairwise Comparisons – Bonferroni: Dependent Variable: Q13 Missing Product vs Factor: Q7 Frequency.

| (I) Q7 Frequency | | Mean Difference (I-J) | Std. Error | Sig.[b] | 95% Confidence Interval for Difference[b] | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| 1 | 2 | -0.1 | 0.212 | 1 | -0.664 | 0.464 |
| | 3 | -0.348 | 0.205 | 0.552 | -0.895 | 0.2 |
| | 4 | -0.406 | 0.215 | 0.361 | -0.977 | 0.166 |
| 2 | 1 | 0.1 | 0.212 | 1 | -0.464 | 0.664 |
| | 3 | -.248* | 0.078 | 0.011 | -0.457 | -0.039 |
| | 4 | -.306* | 0.1 | 0.015 | -0.572 | -0.039 |
| 3 | 1 | 0.348 | 0.205 | 0.552 | -0.2 | 0.895 |
| | 2 | .248* | 0.078 | 0.011 | 0.039 | 0.457 |
| | 4 | -0.058 | 0.086 | 1 | -0.287 | 0.171 |
| 4 | 1 | 0.406 | 0.215 | 0.361 | -0.166 | 0.977 |
| | 2 | .306* | 0.1 | 0.015 | 0.039 | 0.572 |
| | 3 | 0.058 | 0.086 | 1 | -0.171 | 0.287 |

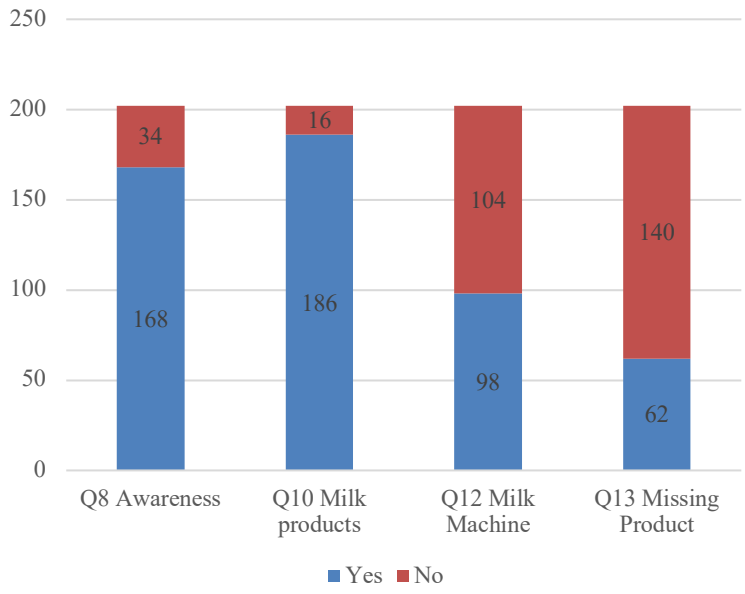


**Figure 2** Frequencies of Q8, Q10, Q12, and Q13.

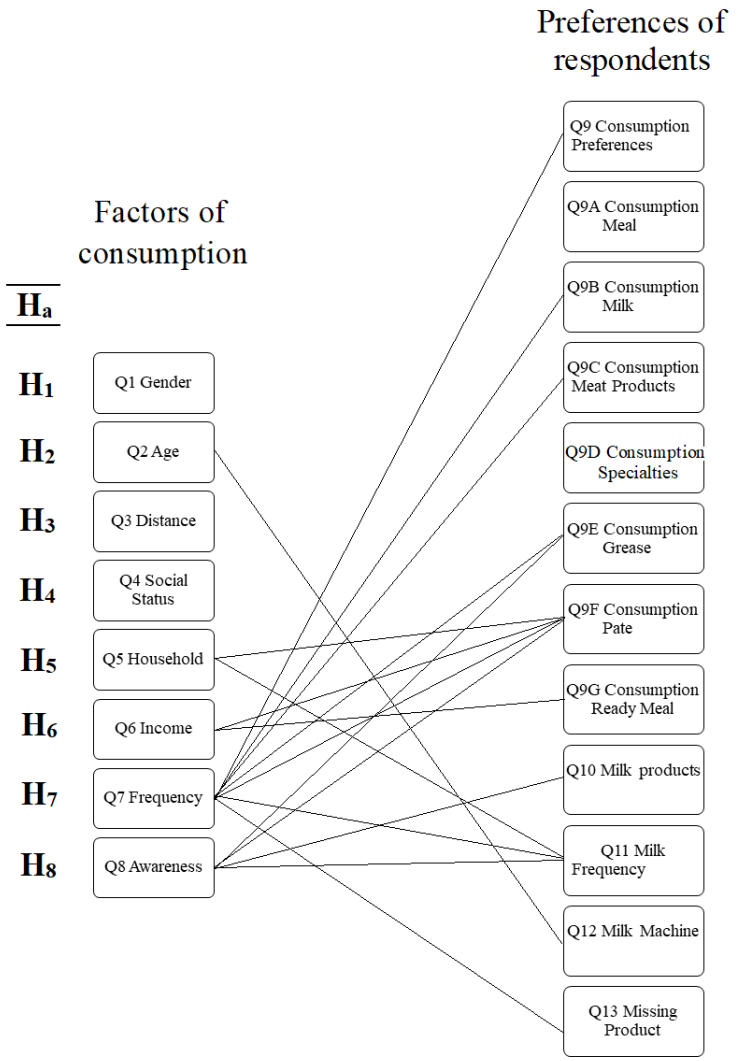


**Figure 3** Graphical Conclusion.

The subsequent post hoc testing shows a difference (Table 17) between income groups of 900 to 1500 EUR and over 1500 EUR ($p$ = 0.025). This pairwise comparison indicates the need for a different approach when selling pâté to customers with income under or over 1500 EUR. A similar difference was found by the Kruskal-Wallis test (Table 9) in buying Q9G ready-made dishes ($\chi2$ (2) = 8.746 and $p$ = 0.013) with a pairwise difference (Table 20) between the lowest income group (under 900 EUR) and the other two income groups (with income of 900 to 1500 EUR, $p$ = 0.022 and with income over 1500 EUR, $p$ = 0.009). According to this, we can accept the alternative hypothesis H6 for two factors Q9F Pâté and Q9G Ready Meal.

These outcomes show the need for a different approach to customers with different incomes in case of a supply of specific products such as pâté and ready meal. Even though we identified this specificity only in the case of two products from the current portfolio, we recommend being aware of this variable in the future, too, when the monitored company will consider expanding the product portfolio and including other special products in it, too. This statement is supported by the outcomes of **Ubrežiová et al. (2019)**, who found the significance of the impact of the customer's average income per month on their willingness to pay for high-quality and relatively expensive dairy products. On the order hand, **Kádeková et al. (2017)** confirmed that 18% of respondents buy most meat and meat products of organic quality and 13% of them fully prefer dairy products of organic quality, which indicates a possibility for the monitored company to succeed when certifying production, even though the certified organic products will be of higher price.

Despite some statistical significance in the case of previous factors of consumption, the highest number of differences was found in connection with the factor Q7 Frequency. This showed the frequency of shopping in the monitored store and it was divided into four alternatives with the following outcomes. Just 2.5% of respondents (5 out of 202) visit the monitored shop every day, even though they sell fresh products of everyday consumption. This outcome is understandable, regarding lockdown applied at the time of the survey. Still, 22.8% of respondents visit the shop several times a week (46 out of 202) and 56.9% a couple of times a month (115 out of 202). Just 17.8% (36 out of 202) represent rare visitors, who visit the store just a couple of times a year. The Kruskal-Wallis test (Table 10) proved significant differences between these frequencies of shopping in connection with six factors (Q9, Q9B, Q9C, Q9E, Q9F, and Q13). In such cases, we can reject the null hypothesis and accept the alternative hypothesis H7. Differences in frequencies when buying (Q9B) milk ($\chi2$ (2) = 14.533 and $p$ = 0.002) were found (Table 12) between buying several times a week and a couple of times a month ($p$ = 0.002) and buying several times a week and a couple of times a year ($p$ = 0.008). Similarly, differences (Table 10) in frequencies when buying (Q9C) meat products ($\chi2$ (2) = 9.223 and $p$ = 0.026) were found (Table 13) between buying several times a week and a couple of times a year ($p$ = 0.033). Also, in frequencies (Table 10) when buying (Q9E) grease ($\chi2$ (2) = 13.727 and $p$ = 0.003), differences were found (Table 14) between buying several times a week and a couple of times a month ($p$ = 0.011) and several times a week and a couple of times a year ($p$ = 0.011). In frequencies (Table 10) when buying (Q9F) pâté ($\chi2$ (2) =11.382 and $p$ = 0.010), differences were found (Table 18) again between buying several times a week and a couple of times a month ($p$ = 0.025) and several times a week and a couple of times a year ($p$ = 0.018). In frequencies considering (Q9 Preferences) the most preferred product of consumption (Table 10) from the portfolio of the monitored store ($\chi2$ (2) = 10.271 and $p$ = 0.016), differences were found (Table 21) between buying several times a week and a couple of times a year ($p$ = 0.008). Lastly, we found differences between the frequency of visiting the monitored store and opinions of respondents on the missing product (Table 10) in the portfolio ($\chi2$ (2) = 13.773 and $p$ = 0.003). In pairwise comparison, it was (Table 26) again between buying several times a week and a couple of times a month ($p$ = 0.011) and several times a week and a couple of times a year ($p$ = 0.015). According to this, we can conclude that significant differences were found between the examined frequencies of visiting the monitored store, mostly in case of buying several times a week and a couple of times a month (Q9B, Q9E, Q9F, and Q13) and several times a week and a couple of times a year (Q9, Q9B, Q9C, Q9E, Q9F, and Q13). The high number of these differences indicates a strong need for adjusting the selling strategy, regarding the frequency of visiting the store and using specific marketing tools for customer groups, according to the frequency of their purchases, at least for products as fresh milk and fresh meat, grease, and pâté. Also, this approach would be essential in connection with expanding the portfolio by selling raw milk in vending machines outside the store or by other new products which are currently missing in the portfolio.

Finally, some significance was found in the case of the factor Q8 Awareness, which expresses the awareness of the respondents of the current offer of the monitored store. This question is simply dichotomic and shows that 83.2% of respondents (168 out of 202) are aware and feel to be well informed about the current offer of the monitored store and the rest of 16.8% (34 out of 202) feel not to be. Specifically, differences in customer's awareness were found in the case of the variables Q9E, Q9F, Q10, and Q11, for which we can reject the null hypothesis and accept the alternative hypothesis H8. Significance was found by the Kruskal-Wallis test (Table 11) and proved by the Bonferroni pairwise comparisons with the same $p$-value since Q8 was dichotomic. Mentioned significant differences in customer's awareness were in case of the products Q9E Grease ($\chi2$ (2) = 4.204 and $p$ = 0.040); Q9F Pâté ($\chi2$ (2) = 6.433 and $p$ = 0.011); Q10 Milk products ($\chi2$ (2) = 8.95 and $p$ = 0.003) and Q11 Milk Frequency ($\chi2$ (2) = 6.594 and $p$ = 0.010). This outcome shows a gap in customer's information about the current offer. Since all the examined customers are proficient in the use of social networks and information and communication technologies, we suggest the monitored company consider, for example, distribution of an information leaflet with the current offer through a local group on Facebook (as this method has also proven itself in the distribution of the questionnaire research). This suggestion is supported by **Jahn and Kunz (2012)** as well as **Huang and Chen**

**(2018)** who highlighted that consumer engagement and loyalty to the brand increase if a seller can satisfy their motivations or needs via social networking services since according to **Shawky et al. (2020)**, customers interact with brands through social media for several reasons such as access to the customer service and content, product information, brand engagement, entertainment, and promotion.

**Limitations**

The article is based on the online questionnaire study since we believe that in the current epidemiological situation, it is the only safe way to conduct it. However, the form of the study can affect the characteristics of the analyzed sample, since, in this way, we addressed only customers who were at the same time users of social media. Nevertheless, despite the widespread opinion that older people are not familiar with information and communication technologies **(Dickinson, Arnott and Prior, 2007)** and that social media users are mostly younger people **(Mellon and Prosser, 2017)**, we believe that our study faithfully describes the reality of the researched subject (customers' age structure included). This presumption is also supported by the outcomes of **Velšic (2020)** who proved that in Slovakia, the proportion of Internet users is above average, especially among respondents under 54 years of age. In addition, **Huang and Chang (2020)** stated that in recent years, there has been a great surge in the number of Social networking services users and **Greenwood, Perrin and Duggan (2016)** added that more than 50% of Internet users use two or more Social networking services (social media platforms). Moreover, provided research sample is relatively age-balanced and its composition corresponds roughly to the age composition of the customers of the store declared by its employees.

The study is methodically based on the assumption that only one person from a household was involved in the questionnaire (which the research participants were concerned about). We are aware that possible failure to comply with this condition could significantly reduce the relevance of the results achieved, but we believe that this has not been attained. Moreover, the study was carried out at the time of the applied lockdown, restricting mobility, which theoretically excluded more than one person from a household from visiting the store and thus, becoming a customer of the monitored store.

The analysis shows the possibility of expanding the portfolio of dairy products. Interest in buying raw cow's milk from milk vending machines placed outside the store was claimed by 48.51% of customers. This supported the outcome of **Nagyová et al. (2021)**, which states that the most remarkable factor, which has a positive impact on the purchase of cow`s milk, is its origin. In the case of dairy products such as cottage cheese, butter, yogurt, and cheese, it was 92.07%, which indicates that customers could be more interested in dairy products than fresh cow's milk itself, just as it is in the case of the new selling meat products.

When planning the marketing tools and choosing the retail strategies, we recommend for the monitored company to customize these for female clients (because they shop more often) but with the awareness that they do the shopping for the whole family, not just themselves. At the same time, we want to point to the benefits of local fresh production intended primarily for direct domestic consumers. We identify no necessity to adjust the product portfolio to different age groups of customers, but caution must be the subject in the future, regarding selling raw milk in the vending machine and considering the age of customers. We also find the application of different approaches suitable, when selling fresh milk to households of two to three people and six-plus people (for this type of family, it is also applicable, when selling pâté), but due to the low share of these families among customers, we consider the special activities useless.

On the other hand, it would be essential to apply a different approach to customers with various incomes in case of a supply of specific products such as pâté and ready meal from the actual portfolio and we recommend being aware of this variable in the future, when including other special products, too. A specific selling strategy should be used also regarding the frequency of purchases, at least for products as fresh milk and fresh meat, grease and pâté, and in the future for milk from the vending machine and other new products. We believe that the distribution of an information leaflet with the current offer in an electronic form would be an effective and cheap way to do this, since COVID-19 has accelerated changes in the way we share information, with a shift towards social media use **(Jimenez-Sotomayor, Gomez-Moreno and Soto-Perez-de-Celis, 2020)**. Specifically, we recommend using social media (e.g., a local Facebook group) since this social networking service offers various sorts of information to their audience **(Huang and Chang, 2020)** and can aim locally.

This study expands the limited published research on the managerial practice of the primary agricultural producers in Slovakia. Previous studies were based on an economic analysis **(Šimčák, Piszczalka and Biaelková, 2003; Jahnátek and Ladvenicová, 2011; Cupák, Pokrivčák and Rizov, 2015)** or a biological approach **(Keresteš and Selecký, 2003; Doležalová et al., 2014; Herian, 2019)**. However, there is a lack of managerial point of view in a form of studies examining the production process and its management in business entities of the primary agricultural production or livestock production. On the other hand, we found a solid base of actual and purely marketing oriented studies **(Kádeková et al., 2017; Kubicová, Predanocyová and Kádeková, 2019; Ubrežiová et al., 2019; Nagyová et al., 2021)**. Our study follows these and connects the managerial approach aimed at the selected aspects of livestock production with product finalization and its placement on the market, with marketing as a last step of the production management. In this way, we can help local suppliers of fresh and high-quality agricultural products to succeed on the market, which can lead to the changes of whole Slovak food supply and result in substantial changes in diet quality and overall health of people **(Byrd-Bredbenner, Abbot and Cussler, 2009)**, since the current reduced consumption and thus reduced milk production has an adverse impact on agriculture, livestock production and, in particular, on human health **(Herian, 2019)**.

For further research, we recommend enlarging the number of the examined entities on a geographical or production basis and provide a comparison between them. Also, we can see a possibility for comparison with other subjects

of a similar product range, using the database of RIAFE (Research Institute of Agricultural and Food Economics). This would allow study at the level of production area and/or country but considering the incomplete data it provides, we consider it impossible in the current conditions.

The benefit of this study is seen in the practical use of the obtained results in the planning of the production base of the monitored company. In addition, its contribution is theoretical in expanding the knowledge base of management of the primary agricultural production in Slovakia and points out the need to strengthen the position of livestock production in the economy of individual agricultural entities. We believe that this study, conducted through an academic approach, will bring practical benefits for the business of the monitored company.

Our findings will be used both in the academic and commercial spheres. Results will be provided to the representatives of the monitored company as a base for the further planning process. We believe that our outcomes encourage the expansion of the product range and help with choosing suitable marketing tools. The results will be used also in the teaching process of the universities involved in the project KEGA 005SPU-4/2019.

## CONCLUSION

The paper aimed to analyze the views and preferences of customers of the livestock agribusiness entity which sells its meat and dairy products in its stores. Concerning this, we conducted an online questionnaire study on the sample of 202 respondents – customers of the monitored company to analyze their preferences and verify the expected success of the planned expansion of the product lines.

Our outcomes show that the most preferred products from their portfolio are meat and meat specialties, which is the reason for visiting the store for 62.4% of customers. Therefore, the recent expansion of the product portfolio of meat products and slaughter, specialties was a good step, as these are interesting for 80% of customers. Fresh milk, on the other hand, is not as popular, since almost half of the customers (47.5%) buy it just a couple of times a year.

A detailed analysis of the examined customers of the monitored store shows that most women (67.3%), adults between 25 and 34 years of age (32.2%) with regular income since they are employees (74.8%), members of families of two to three people (48%) or four to five people (44%), from the neighborhood of ten kilometers (74.8%), with monthly income over 1500 EUR (55.9%), are the consumers, visiting the shop of the monitored company a couple of times a month (56.9%) and the majority of them (83.2%) are aware and feel to be well informed about its current offer.

Statistical analysis pointed to the differences between the factors of consumption (gender, age, distance, social status, household, income, frequency, and awareness) and the preferences of respondents when buying the company's products (meal, milk, meat products, meat specialties, grease, pâté, and ready meal). Concerning this, the set of eight alternative hypotheses was examined with the result of accepting just five of them as variables.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE ORGANOLEPTIC QUALITY OF LIQUID FOOD FORMULA MADE FROM SNAIL (*PILA AMPULLACEA*), TEMPEH, AND MORINGA LEAVES

*Fery Lusviana Widiany, Mochammad Sja'bani, Susetyowati, Emy Huriyati*

## ABSTRACT

This study aims to determine the organoleptic quality of liquid food formula made from snail (*Pila ampullacea*), tempeh, and moringa (*Moringa oleifera*) leaves. The study was conducted in Yogyakarta, Indonesia. It involved 25 moderately trained panelists and also 5 trained panelists, who met the criteria. The measured variable was the organoleptic quality, which included aspects of color, texture, taste, and aroma. The formula tested was a powder formula made from the snail, tempeh, and moringa leaves. The proportion of snail flour, tempeh flour, and moringa leaves flour was 50:30:20. It was found that more than 50% of panelists liked the liquid food formula based on color, texture, taste, and aroma. Independent t-test to determine the difference between the organoleptic quality of the two groups showed $p = 0.710$ for color, $p = 0.335$ for texture, $p = 0.603$ for taste, and $p = 0.880$ for aroma. In conclusion, most of the panelists liked the liquid food formula products made from snail, tempeh, and moringa leaves based on the organoleptic quality result. There was no difference between the organoleptic quality studies of the two groups in the aspect of color, texture, taste, and aroma.

**Keywords:** liquid food formula; moringa leaves; organoleptic quality; snail (*Pila ampullacea*); tempeh

## INTRODUCTION

Protein-energy wasting is highly prevalent in hemodialysis patients **(Ikizler, 2013)**. Management of nutritional aspects of hemodialysis patients includes a comprehensive combination of preventive maneuvers to diminish protein and energy depletion. The recommended dietary protein and calorie intake for sedentary hemodialysis patients are 1.2 – 1.4 $g.kg^{-1}$ and 30 – 35 $kcal.kg^{-1}$, respectively **(Kopple, 2001)**.

An important consideration regarding strategies for increasing dietary protein intake in hemodialysis patients is the potential for increased intake of several potentially harmful elements, especially phosphorus **(Kovesdy, Shinaberger and Kalantar-Zadeh, 2010)**. Nutritional support for hemodialysis patients with high protein and low phosphorus content is required.

This nutritional support can be fulfilled from functional foods that utilize Indonesian local food. The making of formula from local food enables the community to optimize local food into more nutritious food so that it can be produced at the household scale **(Pratiwi et al., 2021)**. Functional food can be in the form of natural food or food that contains one or more certain components that have a functional effect on the health and welfare of consumers **(Tur and Bibiloni, 2016)**.

Functional foods can be made from modification of some local food ingredients in food to provide health benefits. Several local food ingredients in Indonesia have a high protein content but meet phosphorus to protein ratio below 16, that are snail (*Pila ampullacea*), tempeh, and moringa (*Moringa oleifera*) leaves.

*Pila ampullacea* is a freshwater snail that is widespread in freshwater, some of which are known to be in brackish water. Snail contains higher protein and calcium but low in phosphorus than other sources of animal protein. Snail contains vitamin D3 (cholecalciferol) 21 $\mu g.100g^{-1}$ edible weight. Snails have high protein content, but low phosphorus content, so they are good for giving to hemodialysis patients **(Subekti, 2004)**.

Modification of vegetable protein can be done by utilizing tempeh which is made from Indonesian local soybean. The digestibility value of tempeh is 83% better than soybean which is only 75%. Tempeh also contains isoflavones, arginine, branched-chain amino acids, linoleic acid, and linolenic acid which are higher than other vegetable protein sources **(Kholidah and Pardjanto, 2011)**.

For the fulfillment of antioxidants, the preparation of liquid food formula is modified with the addition of *Moringa oleifera* leaves. Moringa is a plant that can grow quickly, live long, flower all year round, and withstand extreme heat conditions. Moringa leaves have great health benefits and are often modified into a variety of foods. Moringa leaves contain antioxidant flavonoids, ascorbic

acid, carotenoids, and phenolics **(Abdull Razis, Ibrahim and Kntayya, 2014)**.

Previous studies related to providing oral nutritional support to hemodialysis patients have been carried out, but there has been no research that utilizes a combination of local food based on a mixture of animal protein sources, vegetable protein sources, and vegetables as a formula for oral nutritional support for hemodialysis patients. Previous research has never been conducted on providing nutritional support to patients using a combination of three local foods at once. This oral nutritional support formula derived from a mixture of snail, tempeh, and moringa leaves has a novelty in clinical nutrition science in the dietary management of hemodialysis patients, which is suitable with the recommendations for the protein requirements from high biological value protein source can only be met by 50%, while the rest is met from the vegetable protein source **(Fouque, 2003)**.

The snail, tempeh, and moringa leaves each has weaknesses in terms of organoleptic. The snail has a fishy taste, while the tempeh and moringa leaves have an unpleasant taste and aroma. Therefore, it is necessary to analyze the organoleptic quality when these three local food ingredients are mixed into modified formulas with a certain proportion to meet the nutritional needs of hemodialysis patients. The manufacture of functional food made from local food requires an organoleptic analysis first so that it can be well received by consumers.

This study was conducted to determine the results of the organoleptic quality of liquid food formulas made with the basic ingredients of a combination of snail, tempeh, and moringa leaves. This formula was made in the form of a powder formula that can be made into liquid food. This liquid food formula will be tested on hemodialysis patients so it requires an organoleptic analysis phase first in healthy people.

## Scientific Hypothesis

This project was carried out to determine the organoleptic quality of liquid food formula made from snail (*Pila ampullacea*), tempeh, and moringa leaves. We estimate that this liquid food formula product has a good organoleptic quality and will be liked by most of the panelists based on the aspect of color, texture, taste, and aroma. We expect that there is no difference in the organoleptic quality of liquid food formula between the moderately trained panelists and the trained panelists.

## MATERIAL AND METHODOLOGY

### Samples

The sample in this study was the liquid food formula. The formula tested was a powder formulation made from the snail, tempeh, and moringa leaves. Each ingredient was floured first, then mixed into a formula. The proportion of snail flour, tempeh flour, and moringa leaf flour in this formula was 50:30:20, and it was called the basic formula. Several food additives were added, such as corn sugar, canola oil, powder cinnamon, and rice flour. As many as 32 g of basic formula was mixed with 7.5 g of corn sugar, 8 mL of canola oil, 0.6 g of powder cinnamon, and 26 g of rice flour.

The study involved 25 students of the Undergraduate Nutrition Study Program of Universitas Respati Yogyakarta as moderately trained panelists who have met the inclusion criteria aged >18 years, liked full liquid food, did not experience taste problems, did not experience an allergy to the snail, tempeh, and moringa, and passing the food technology course. This study also involved 5 lecturers from three different universities in the Special Region of Yogyakarta, Indonesia, as trained panelists, who have an interest in nutrition and food technology.

### Chemicals

Analysis of macronutrient content was carried out at the Chem-Mix Pratama Laboratory, Yogyakarta, Indonesia. Analysis of micronutrient content was carried out at the Analytical Chemistry Laboratory of Gadjah Mada University, Yogyakarta, Indonesia. Analysis of amino acid content was carried out at the PT. Saraswanti Indo Genetech Laboratory, Bogor, Indonesia. The analytical method and the nutritional content of the formula are shown in Table 1.

### Animals and Biological Material

This study did not use animals or biological materials. However, the oral nutritional support formula used as a sample in this study was analyzed for germ numbers. Analysis of germ numbers was carried out at the Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta, Indonesia.

The results of the analysis of the number of germs in the oral nutritional support formula were reported to have met the quality requirements of the drug according to the Regulation of the Head of the Food and Drug Supervisory Agency, Republic of Indonesia, namely the total plate number of bacteria of $3.05 \times 10^3$ colonies per g (normal $\leq 10^6$ colonies per g), yeast mold of $2.5 \times 10^1$ colonies per g (normal $\leq 10^4$ colonies per g), and did not contain pathogenic bacteria such as *Staphylococcus aureus*, *Salmonella*, *Escherichia coli*, *Plasmodium aeruginosa*.

### Instruments

The variable of this study was the organoleptic quality, which included aspects of color, texture, taste, and aroma. Organoleptic quality was defined as the result of panelist preference analysis of the taste, aroma, texture, and color of the liquid food formula product from the snail, tempeh, and moringa leaves.

The organoleptic quality was measured using a 6-point scale organoleptic test form instrument. The organoleptic quality parameters include devilish (score 1), bad (score 2), a little bad (score 3), a little good (score 4), good (score 5), and excellent (score 6).

### Laboratory Methods

PT. Saraswanti Indo Genetech Bogor is an accredited testing laboratory based on **ISO/IEC 17025: 2017**, while the Chem-Mix Pratama Laboratory and the Analytical Chemistry Laboratory of Gadjah Mada University have not been accredited to ISO standards. The analysis method used for the nutrient content of the formula has been mentioned in Table 1.

### Description of the Experiment

**Sample preparation:** As many as 36 g powder formula on each serving can be diluted with 125 mL hot water (90 – 96 °C) to form a liquid food formula. The liquid food formula will be given in two servings a day for hemodialysis patients.

**Number of samples analyzed:** 1
**Number of repeated analyses:** 2
**Number of experiment replication:** 1

### Statistical Analysis

The organoleptic quality data for each aspect were analyzed using the IBM SPSS Statistics 22 program. Data were presented as mean ± standard deviation of the mean (SDM).

The statistical analysis was conducted to determine whether there was a difference between the organoleptic quality result of the two groups of panelists. The Shapiro-Wilk test was used to determine the normal distribution of the data. The result showed that the data in the two groups for all aspects were normally distributed ($p \geq 0.05$) so that statistical analysis was carried out using an independent t-test. The hypothesis could be accepted if the result of the independent t-test showed $p \geq 0.05$.

## RESULTS AND DISCUSSION

The oral nutritional support formula product made from a mixture of snail, tempeh, and moringa leaves has been analyzed for nutritional content and meets the dietary requirements for hemodialysis patients. The dietary requirements for hemodialysis patients include high protein, rich in amino acids, high in calcium, but low in phosphorus. The recommended ratio of phosphorus to protein is <16. If it is higher than 16, it is suspected that it can increase mortality in end-stage kidney disease **(Sja'bani, 2015; Barril-Cuadrado, Puchulu and Tomero, 2013)**. This oral nutritional support formula has a phosphorus: protein ratio of 6.9, so it has an excellent quality based on that aspect. Although it has good nutritional content for hemodialysis patients, it is necessary to consider the organoleptic quality of the formula product so that it can be consumed by hemodialysis patients optimally.

The analysis of preference for the organoleptic aspects of the liquid food formula made from the snail, tempeh, and moringa leaves was carried out on two groups of the panelist, as well as moderately trained panelist group and trained panelist group.

The organoleptic quality result in Table 2 shows that the mean score given by the two groups of panelists on all organoleptic aspects was above 3.0, which means that the panelists liked the liquid formula product based on all organoleptic aspects. Acceptance or rejection of food depends entirely on whether it is by the expectations and needs of consumers **(Mosca et al., 2015)**.

The frequency distribution of moderately trained panelists based on each aspect of the organoleptic quality is shown in Figure 1. It was found that most of the moderately trained panelists like liquid food formula products made from the snail, tempeh, and moringa leave based on aspects of color, texture, taste, and aroma. However, a small proportion of panelists did not like the formula product. Panelists who did not like the formula product considered that there were still weaknesses in several aspects of the organoleptic test. These weaknesses include the color of the product which was considered too dark, the texture that was still slightly thick after dissolving it with hot water, there was still a little fishy taste and bitter after-taste, and a slightly unpleasant aroma in the formula product.

Meanwhile, the organoleptic quality on the trained panelists showed slightly different results from the moderately trained panelists. The frequency distribution of trained panelists based on each aspect of the organoleptic quality is shown in Figure 2.

Figure 2 shows that most of the trained panelists liked the liquid food formula product made from the snail, tempeh and moringa leaves based on the aspects of color, taste, and aroma, but did not like the texture of the formula product. However, in the assessment of the texture aspect, 20% of the panelists chose the excellent category. A small proportion of panelists who did not like formula products based on several organoleptic aspects regarding the weaknesses of the formula product, that were the color of the product was considered too dark, the texture was still slightly thick after dissolving with hot water, there was still a little fishy taste and after-taste bitter, and a little unpleasant aroma in formula products.

The researchers continued to carry out the analysis to determine whether there was a difference between the organoleptic quality result of the two groups of the panelist. Figure 3 shows that statistically, there is no difference in the organoleptic quality based on color, texture, taste, and aroma of the liquid food formula product in the two groups of panelists ($p \geq 0.05$), which is shown with $p = 0.710$ for color, $p = 0.335$ for texture, $p = 0.603$ for taste, and also $p = 0.880$ for aroma.

The most important component of food for the consumer is its sensory characteristics, including taste, aroma, color, texture, and overall acceptance **(Aberoumand, 2015)**. Sensory characteristics, including taste, aroma, texture, and appearance of food specifically influence consumer decisions about food material preferences **(Kostyra et al., 2016)**.

### Color

Color is a very important component in determining the quality and degree of acceptance of food. Food that is considered delicious and has a good texture will not be eaten if it has an unsightly color or has deviated from the color it should have. Determination of food quality depends on several factors, but before other factors are considered visually, the color factor appears first to determine its quality **(Winarno, 2002)**. The function of the color of food, the product is very important because it can affect consumer tastes and be able to arouse appetites **(Rahmayanti, Ningtyias and Baroya, 2020)**.

Most of the panelists like the color of liquid food formula products in this study. The product's color is blackish brown like coffee. Most of the panelists prefer the food they like. Since ancient times, certain types of food have been consumed specifically because of the pleasures they offer consumers, such as ice cream, coffee, alcohol, and chocolate. These foods are usually consumed to provide a positive state of mind or to reduce negative thought states **(Kim, Lee and Kim, 2016)**.

A small proportion of panelists disliked the color of the liquid food formula products in this study because they preferred lighter colors. In the manufacture of liquid food formula products, the formation of brown color is caused by chemical changes that occur during the process of processing the formula, namely the browning reaction.

**Table 1** The daily nutritional content of the formula.

| Nutrients | Unit | Content per 100 g of edible weight | Analysis method |
|---|---|---|---|
| Energy | kcal | 259.78 | Total energy from protein, fat and carbohydrate |
| Protein | g | 16.37 | Micro kjeldahl |
| Fat | g | 6.23 | Soxhlet extraction |
| Carbohydrate | g | 34.68 | By different |
| Calsium | mg | 1538.32 | Atomic absorption spectrophotometry |
| Iron | mg | 10.43 | Atomic absorption spectrophotometry |
| Sodium | mg | 53.57 | Atomic absorption spectrophotometry |
| Potassium | mg | 141.28 | Atomic absorption spectrophotometry |
| Phosphorus | mg | 112.18 | UV-visual spectrophotometry |
| Vitamin D3 | μg | <0.67 | 18-5-1/MU/SMM-SIG (HPLC) |
| Glutamic acid | g | 2.1 | 18-5-17/MU/SMM-SIG (UPLC) |
| Aspartic acid | g | 1.1 | 18-5-17/MU/SMM-SIG (UPLC) |
| Arginine | g | 2.2 | 18-5-17/MU/SMM-SIG (UPLC) |
| Leucine | g | 1.7 | 18-5-17/MU/SMM-SIG (UPLC) |
| Glycine | g | 1.4 | 18-5-17/MU/SMM-SIG (UPLC) |
| Alanine | g | 0.9 | 18-5-17/MU/SMM-SIG (UPLC) |
| Lysin | g | 0.6 | 18-5-17/MU/SMM-SIG (UPLC) |
| Valine | g | 1.0 | 18-5-17/MU/SMM-SIG (UPLC) |
| Proline | g | 0.9 | 18-5-17/MU/SMM-SIG (UPLC) |
| Serine | g | 1.3 | 18-5-17/MU/SMM-SIG (UPLC) |
| Tryptophan | g | 0.1 | 18-5-63/MU/SMM-SIG (HPLC) |
| Isoleucine | g | 0.9 | 18-5-17/MU/SMM-SIG (UPLC) |
| Phenylalanine | g | 1.7 | 18-5-17/MU/SMM-SIG (UPLC) |
| Tyrosine | g | 1.1 | 18-5-17/MU/SMM-SIG (UPLC) |
| Histidine | g | 0.7 | 18-5-17/MU/SMM-SIG (UPLC) |
| Cystine | g | 0.2 | 18-12-38/MU/SMM-SIG (LC MS/MS) |
| Methionine | g | 0.2 | 18-12-38/MU/SMM-SIG (LC MS/MS) |
| Threonine | g | 1.2 | 18-5-17/MU/SMM-SIG (UPLC) |

**Table 2** The result of organoleptic quality between two groups of panelist.

| The aspect of the organoleptic quality | Moderately trained panelist group | | Trained panelist group | |
|---|---|---|---|---|
| | n | Mean (SDM) | n | Mean (SDM) |
| Color | 25 | 4.1 (0.8) | 5 | 4.2 (0.8) |
| Texture | 25 | 3.9 (0.7) | 5 | 3.6 (0.9) |
| Taste | 25 | 3.8 (0.8) | 5 | 4.0 (0.7) |
| Aroma | 25 | 3.8 (0.9) | 5 | 3.8 (0.4) |

Browning in liquid food formula products is due to the Maillard reaction, lipid oxidation, and interactions between amino acids and lipid oxidation products **(Agustini et al., 2014)**.

The browning reaction is an important reaction that occurs during the food processing process. The accumulation of brown pigment is an indication that shows the Maillard reaction to foods containing protein and carbohydrates. Besides causing browning, the Maillard reaction also affects the flavor, texture, and taste of the food through protein cross-linking and degradation **(Agustini et al., 2015)**.

Foodstuffs that have undergone processing or heating can be expected to experience color changes. The process of heating or drying, food changes its physical and chemical quality **(Utami, Fitrianingrum and Haryani, 2012)**. The protein content in food can cause a brown color when drying or heating **(Noviyanti, Wahyuni and Syukri, 2016)**. This liquid food formula product has a high protein content so that the color of the product becomes dark brown.

**Texture**

Food texture is largely determined by the content of water, fat, protein, and carbohydrates. The texture is a pressure sensor that can be observed with the mouth (when bitten, chewed, and swallowed) or touched with fingers **(Noviyanti, Wahyuni and Syukri, 2016)**. In this study, texture observation was carried out by determining the sensory texture based on the pressure on the liquid food formula when touched using a spoon. The texture of this liquid food formula product is slightly thick.

Most of the panelists like liquid food formula products with a slightly thick texture. The slightly thick texture is made because the formula product will be given to hemodialysis patients, which must limit the consumption of fluids. A small proportion of respondents considered the texture less watery, so they did not like liquid food formula products based on texture.

The texture or thickness of the liquid food formula is one of the parameters that determine the quality of the product. This formula product is consumed by drinking so that the respondents assume that the texture of the product must be thin and easy to swallow. However, with the consideration that the liquid food formula must be made with liquid restrictions, then 1 cc of liquid food must contain more than 1 kcal of energy. So that the texture of the formula product is made slightly thick.

The presence of protein content in the raw materials of snail, tempeh, and moringa leaves also affects the texture of the liquid food formula product in this study. Protein has hydration properties that can increase the absorption of water in products **(Rauf, 2015)**.

**Taste**

Taste is the most important factor in determining the decision for consumers to accept or reject a food or food product. Even though the other params are good if the taste is bad or disliked the product will be rejected. There are four basic types of taste recognized by humans, namely salty, sour, sweet, and bitter. Whilst the other flavors are a combination of other tastes **(Soekarto, 2012)**.

Taste refers to the proximal sense that requires direct contact of food with stimuli on the tongue to determine the quality of the food being digested. Basic flavors such as umami, sour, sweet, bitter, and salty are important for signaling nutrient-rich foods. The sweet taste concludes a high concentration of carbohydrates, particularly monosaccharides, whilst salty and savory tastes are associated with protein and electrolytes **(Romagny, Ginon and Salles, 2017)**.

Most of the panelists like the taste of liquid food formula products. The organoleptic analysis showed that a small proportion of panelists did not like the taste of the product. Some comments that if judged in terms of taste, liquid food formula products have an after taste (a little bit bitter). This is because this liquid food formula product uses raw materials for tempeh and moringa leaves which are floured.

Powdered tempeh has a greater unpleasant aroma and causes a bitter after-taste in liquid food formula products. The bitter after-taste can be caused by the hydrolysis of amino acids that occurred in the Maillard reaction during the process of making tempeh flour. There are amino acids that cause a bitter taste such as lysine, arginine, proline, phenylalanine, and valine. The amino acid lysine is an amino acid that has the most bitter taste compared to other amino acids that cause a bitter taste. The compound causing the bitter taste is found in the crude fat fraction. This crude oil has a high acid, peroxide, and thiobarbituric acid (TBA) number, so it is suspected that the cause of the bitter taste is compounded resulting from degradation/oxidation of triglycerides **(Kurniawati and Ayustaningwarno, 2012)**.

The bitter taste of tempeh flour is caused by the presence of glycoside compounds in soybean seeds. Among these glycosides, soyasaponins and sapogenins are the main causes of bitter taste in soybeans and their processed products. Other glycosides that cause off-flavor in soybeans are isoflavones and their aglycone groups. Isoflavone compounds in soy consist of genistin and daidzin, whilst the aglycone groups are called genistein and daidzein, respectively **(Santoso, 2009)**.

Researchers have made several efforts to minimize the bitter after-taste of liquid food formula products. Among them, steaming raw tempeh before processing it into tempeh flour to reduce the bitter taste of the resulting tempeh flour, blanching raw moringa leaves for 5 minutes using hot water with temperature (±80 – 90 °C), and adding corn sugar to the formula. The concentration of sugar affects the taste caused by food products, the more the concentration of sugar, the more it can mask the bitter taste of moringa flour **(Dewi, Suliasih and Garnida, 2016)**. However, based on the results of the organoleptic analysis, there were comments that there was still a little after-taste. bitter in liquid food formula products.

The effect of taste on food acceptance is highly correlated with the personal preferences that people have **(Cho et al., 2015)**. People tend to accept and consume foods that contain a concentration of the taste they like **(Kim et al., 2018)**. The difference in the sensation that occurs between panelists can be caused by differences in sensation received, due to differences in the level of sensitivity of the sensing organs, or due to a lack of knowledge of certain tastes **(Setyaningsih and Apriyantono, 2010)**.

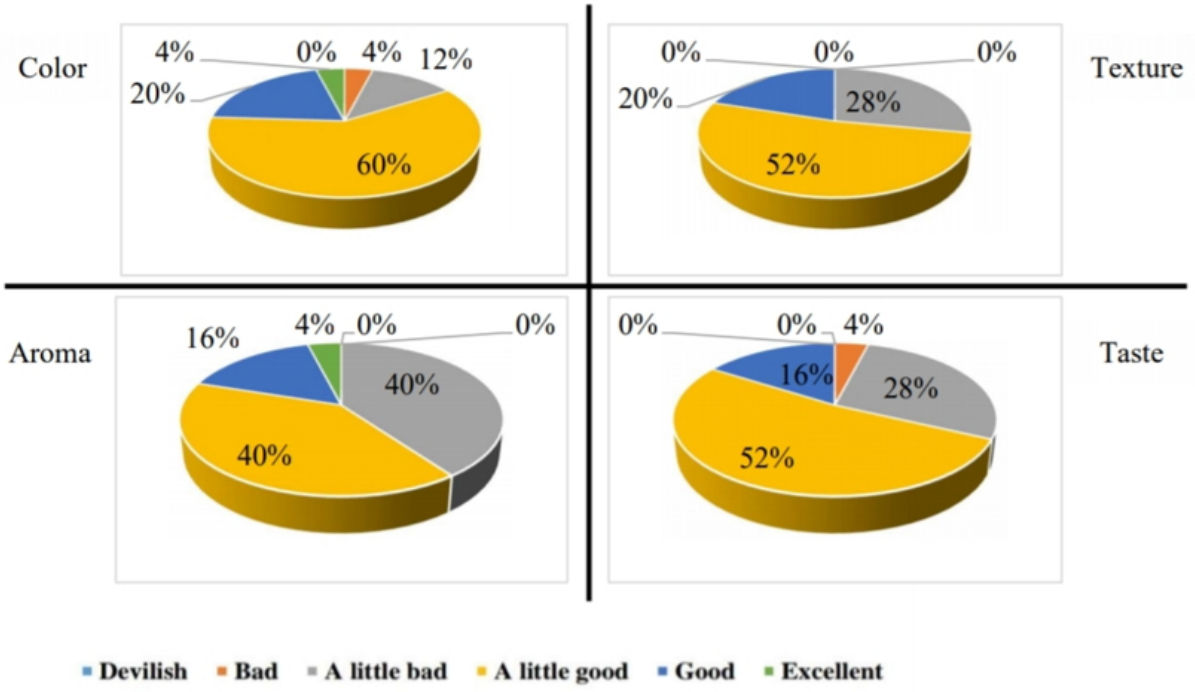


**Figure 1** The organoleptic quality result of liquid food formula in the moderately trained panelist group.

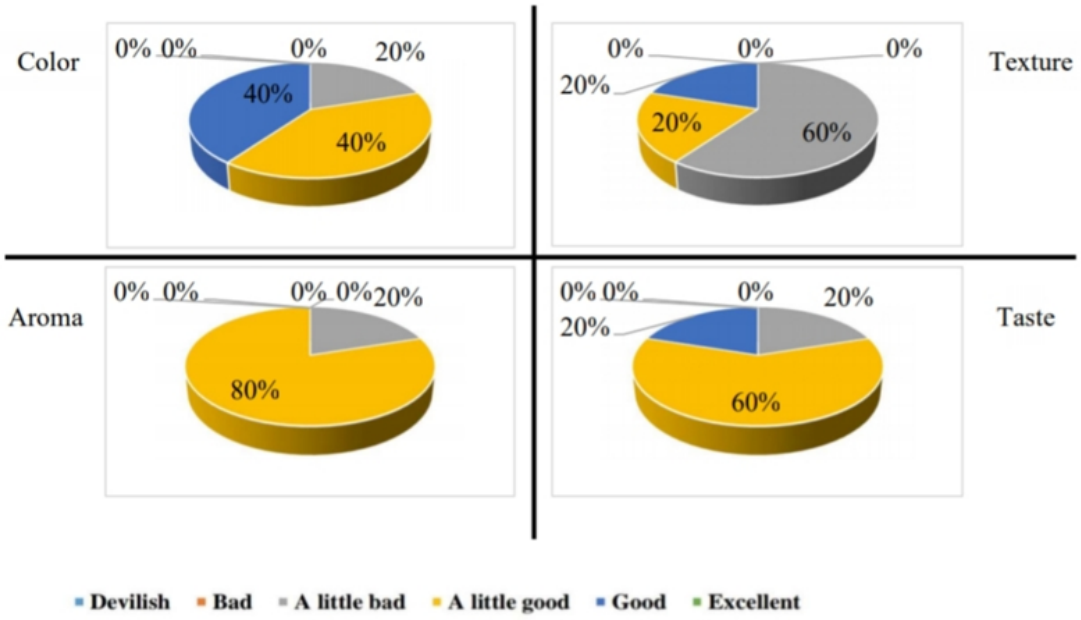


**Figure 2** The organoleptic quality result of liquid food formula in the trained panelist group.

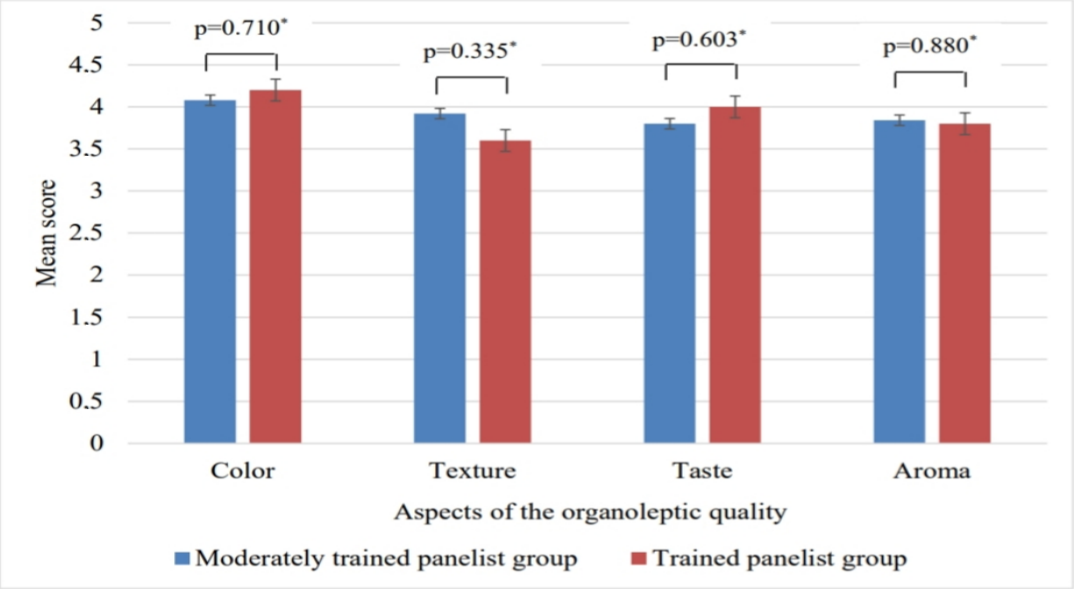


**Figure 3** The difference in mean score of the organoleptic quality result in the two panelist groups. Note: *$p$ – analyzed using independent t-test.

**Aroma**

Aroma determines the delicacy and taste of food. The smell produced from food determines the delicacy of these foods and has to do with the five senses of smell. Aroma has a very important role in determining the degree of assessment and quality of food **(Noviyanti, Wahyuni and Syukri, 2016)**.

The appearance of an aroma or odor is due to the presence of volatile and odorous substances. The protein contained in the material will be degraded into amino acids by the presence of heat. The reaction between amino acids and sugar will produce an aroma, whilst the fat in the material will be oxidized and broken down by heat so that some of the active ingredients caused by the breakdown will react with amino acids and peptides to produce aroma **(Mutiara, Adikahriani and Wahidah, 2012)**.

The smell of food forms important sensory signals and a fundamental component of taste perception and shapes the way people perceive the taste and texture of these foods. Aroma serves as a signal of edible or inedible food, even before consumers see the food **(Sinesio et al., 2018)**. The smell of food directs acceptance towards food which is specifically marked by smell **(Maina, 2018)**.

The results of this study indicated that 60% of panelists liked the aroma of liquid food formula products. A small proportion of panelists did not like the aroma because it still had a slightly unpleasant and fishy aroma. Exposure to retro-nasal aromas has a consistent effect on food acceptability **(Tauferova et al., 2015)**.

The aroma formation of liquid food formula products is thought to be influenced by the raw materials used to make liquid food formula products, which are snail, tempeh, and moringa leaves. The use of snail with the largest proportion compared to tempeh and moringa leaves causes the distinctive aroma of liquid food formula products which is still slightly fishy. However, the use of tempeh and moringa leaves causes an unpleasant aroma to the products produced.

Researchers have made efforts to add corn sugar and a little powder cinnamon to cover up the unpleasant smell, but based on the comments on the organoleptic quality results, it turns out that there was still a slightly unpleasant smell in liquid food formula products. The unpleasant odor of tempeh flour is produced by the presence of lipoxidase enzymes in soybeans. This occurs because the lipoxidase enzyme hydrolyzes or breaks down soybean fat into compounds that cause unpleasant odors, which are classified in the hexanal and hexanol groups. These compounds in low concentrations can cause unpleasant odors **(Santoso, 2009)**.

## CONCLUSION

The liquid food formula made from a mixture of the snail, tempeh, and moringa leaves meets the requirements of the hemodialysis diet and has good organoleptic quality based on aspects of color, texture, taste, and aroma.

It is necessary to conduct clinical trial research on providing an oral nutritional support formula made from a mixture of snail, tempeh, and moringa leaves to hemodialysis patients in hospitals. It is necessary to investigate the effectiveness of this oral nutritional support formula on clinical outcomes, nutritional status, immune status, and inflammatory status of hemodialysis patients.

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The authors declare no conflict of interest.

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# INNOVATIVE APPROACHES TO THE DEVELOPMENT OF A NEW SOUR MILK PRODUCT

*Irina Vlasenko, Valentyna Bandura, Tetyana Semko, Larisa Fialkovska, Olga Ivanishcheva, Vladyslav Palamarchuk*

## ABSTRACT

The topic provides an analysis of the current approach to healthy nutrition and represents a new functional fermented milk drink based on buttermilk containing natural prebiotics - a biologically valuable complex Spirulina platensis. The main tasks of the industry as a holistic system for the management and production of food ingredients and products are outlined. The work highlights the requirements for the quality and context of the functional product manufacture, the main criteria for the consumer choice, and positioning of products in the healthy lifestyle system. The topic covers the most common pro- and prebiotics, including strains. The unique food green microalgae Spirulina platensis as a source of biologically valuable components is proposed for industrial application. A technique for the production of the fermented milk drink based on buttermilk and spirulina as prebiotic was developed and scientifically substantiated. The protein contained in buttermilk is characterized by high nutritional value, exhibits functional properties and can significantly affect the quality of the drink. To confirm this, the possibility of using buttermilk with different protein content from 2.9 to 3.2 to improve the structure of the clot in the composition of the drink and the content of spirulina from 10 to 20% was studied. As single criteria for optimizing the prescription composition of the drink at the different protein content of buttermilk used indicators of product quality - acidity, degree of syneresis, organoleptic parameters. The optimal values of the individual criteria are obtained in different ranges of protein content 3 times 1-2-2; sample 2-3.0; sample 3-3.2%, which allows you to get recommendations for the formulation of a new type of drink.

**Keywords:** buttermilk; prebiotics; probiotics; spirulina; technology; model food composition.

## INTRODUCTION

Nowadays, the regulatory requirements for raw milk are described in the EU Food Code and the regulations of the European Parliament, which provide a detailed explanation of the traceability of raw milk. Regulation (EU) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 describes the general principles and requirements of food law concerning food safety, as well as a package of so-called "hygiene regulations" governing the requirements for milk producers. raw materials, food producers, entities. It should be noted that according to the data, the protein content in milk harvested by processing enterprises is below 3.0% not only from March to May but even in the summer months. The requirements for the control of the qualitative and quantitative composition of the microflora contained in milk are becoming very important. Milk is a good nutrient medium for the development of most microorganisms **(Vlasenko, et al., 2020)**. Specialists divide all microorganisms found in milk into three groups: technically important, pathogenic, and sanitary-indicative microorganisms. Sanitary-indicative microorganisms indicate the degree of contamination of milk and non-compliance with sanitary and hygienic production regimes. Thus, the quality of raw milk supplied to enterprises is one of the main problems that need to be addressed to improve quality and safety, increase yield and reduce the cost of finished products **(Semko, Kolianovska, and Ivanishcheva, 2018)**.

The concept of functional foods as a source of protein, carbohydrates, and microelements consists of consuming products that offer various benefits that go beyond basic nutritional needs **(Martirosyan, Singh, 2015)**.

Functional foods are designed to improve metabolic functions and biochemical parameters of the human body and help prevent non-communicable diseases such as cardiovascular disease (CVD), cancer, type 2 diabetes mellitus, and osteoporosis. Functional dairy products are the main suppliers of probiotic microorganisms, help to restore the gastrointestinal microflora. Strains of bifidobacteria and some lactobacillus species, such as

*Lactobacillus acidophilus, Lactobacillus casei* subsp. *casei, Bifidobacterium SPP. (B. adolescentis, B. animalis, B. bifidum, B. longum, B. breve*), belong to probiotic cultures. They exert a positive effect on the human body and normalize the composition and functions of the bacterial flora in the gastrointestinal tract. **(Breus et al., 2019)**.

The world's leading manufacturers and researchers of functional foods are France, Japan, Mexico, while the United Kingdom, the Netherlands, Germany, and Spain are among the European leaders. **(Vicentini, Liberatore and Mastrocola, 2016)**. About 50% of healthy functional products are dairy products and about 30% are cereals.

Tackling the healthy nutrition issue is the most challenging and urgent task for the society and contemporary public health system. In the XXI century, a strategic line of the food industry consists in the attribution of special-purpose foods to the "healthy" diet.

One of the important components of any fermented milk drink is spirulina, which is widely used to improve the taste and protein content. A new technique for the manufacture of products using spirulina extract will become a new trend in the enrichment of traditional fermented milk drinks that heralds the innovative development of the dairy industry aimed at expanding the range of functional products.

In recent years, Ukrainian scientists have developed several products containing spirulina, although the assortment is still limited and does not meet the demand for such products. Raising consumer awareness of nutrient health benefits and nutritional value of components for potential disease prevention and health improvement will play the most significant role in the global food and nutraceutical market.

Functional products are considered medicinal products that are not only a source of energy and important nutrients but also good for health. Many studies, including some funded by the European Commission (EC), have led to an understanding of the potential mechanisms of biologically active food ingredients able to improve health and possibly reduce the risk of disease while improving overall well-being. Functional foods and nutraceuticals help improve health, reduce healthcare costs and promote economic development in rural areas. The demand for functional foods, especially in developed economies, is soaring now due to better awareness of the health benefits of functional foods and the growing gross income of households. The United States is the world's largest market for functional foods expecting to grow. This growth is mainly driven by the ever-increasing demand for energy drinks and enriched dairy products. Meanwhile, national rules and justifications for claims of no harm to health are among the most challenging problems that the functional foods and nutraceuticals market continues to face **(Daliri and Lee, 2015)**.

Probiotics are considered single or mixed cultures of living microorganisms, which can change the intestinal microbiota and benefit the health of a host once ingested in sufficient quantities **(Tiihonen, Ouwehand and Rautonen, 2010)**. Probiotic products contain live microorganisms over a dose recommended as good for health (5 × 109 CFU/day for 5 days) **(Gupta and Garg, R., 2009)**.

Prebiotics are considered food components intended to selectively stimulate the growth and/or activity of intestinal bacterial strains capable of fermenting, for example, undigested starch, oligosaccharides, and non-starch polysaccharides **(Upadhyay and Moudgal, 2012)**. Synbiotics contain both probiotics and prebiotics and exert beneficial health activity as a result of the synergistic action of the two components **(Scholz-Ahrens, et al., 2016)**.

Products that contain probiotics, including *Lactobacillus* strains and/or *Bifidobacterium* and less often *Escherichia*, *Enterococcus,* or *Bacillus*, include yogurt, kefir, buttermilk, acidophilus milk, sauerkraut, pickled cucumbers, tempeh, miso, and tea fungus **(Zawistowska-Rojek, et al., 2016)**. Prebiotics are considered oligosaccharides such as fructooligosaccharides (inulin), galactooligosaccharides, xylooligosaccharides, cyclodextrins, and lactulose. Significant sources of oligosaccharides are chicory root, topinambur, raw garlic, onions, and whole grains **(Al-Sherajiae et al., 2013)**.

The use of probiotics has been associated with improving immune function. Probiotics have been used for the treatment of gastrointestinal disorders such as constipation, diarrhea, Crohn's disease, irritable bowel syndrome (IBS), and diverticular disease. The health benefits of probiotics are closely associated with a proper balance of microflora in the digestive system **(Clements and Carding, 2018)**.

Probiotics are considered useful once supported by prebiotics in a diet since they can stimulate the growth of bifidobacteria, which numbers are significantly reduced in humans within 55 to 60 years of age **(Stavropoulou, and Bezirtzoglou, 2019)**. The health benefits of prebiotics consist in their ability to stimulate useful digestive activity, including bifidobacterial growth. Some prebiotics, not hydrolyzing in the human gastrointestinal tract, yet having other beneficial properties, such as inulin, which replace fat. Other prebiotics, such as fructans, contribute to the absorption of calcium, magnesium, iron, zinc, copper, and phosphorus. The dosage of fructans that exert the prebiotic effect ranges from 4 to 8 g per day **(Gibson, et al., 2010)**.

The combination of pro- and prebiotics in the diet of elderly people is considered advantageous in terms of the ability to intensify the intestinal growth of bifidobacteria. The increased number of bifidobacteria contributes to the higher concentration of bacterial folic acid, vitamins B 1, B 2, B 6, and nicotinic acid. Therefore, bifidobacteria can be a source of essential nutrients and support the preventive measures and management of gastrointestinal diseases **(Shoaib et al., 2016)**.

The most important group of products containing probiotics are fermented milk products (yogurt, kefir, buttermilk, etc.), cheeses, and tea fungus. Probiotic bacteria are present in some other foods, such as ice-cream, frozen desserts, fermented fruits, vegetable juices, and fermented meat **(Zaręba and Ziarno, 2011)**. Probiotic bacteria are added to sweets, such as jelly candies, in combination with yogurt or chocolate, as well as to chocolate products in combination with lyophilized microorganisms, to grain juices, frozen yogurt, chocolate bars, cookies, and cereals **(Semko, Vlasenko and Palamarchuk, 2020)**. Some probiotics are sold in the form of tablets or capsules. The new products are beverages containing probiotic bacteria in bottle caps, which run in

the bottle contents once the bottle is opened **(Kregiel, 2015)**. Traditional unrefined sources of prebiotics in the diet are soybeans, shelled wheat and barley, and raw oats. The food market also offers a variety of products with the addition of prebiotics and synbiotics, such as fermented dairy drinks, yogurts, fruit drinks, fatty spreads, meat products, mayonnaise, margarine, and pasta **(Liutkevičius et al., 2016)**.

The use of probiotics and their positive effect on the digestive system have been substantiated by scientific data and approved by EU health regulators **(Van Loveren, Sanz, and Salminen, 2012)**.

The purpose of the work is the development of a new functional fermented milk drink based on buttermilk using a complex of biologically valuable natural pro- and prebiotics originating from *Spirulina platensis*.

## Scientific Hypothesis

Development of innovative technology for combining secondary raw materials of the dairy industry with a unique food green algae prebiotic Spirulina platensis. Fermentation with selected strains of probiotic cultures was carried out in the work. The proposed product can complement the range of functional fermented milk drinks in the system of healthy nutrition.

The peculiarity of our proposed application of bacterial leavens is that after high-temperature treatment of buttermilk in the mode (100 ±5ºC) with a holding time of 25 s was cooled to 30 ±2ºC.

One of the important components of our sour milk drink is spirulina, which is widely used to improve the taste and enrich it with protein.

The resulting fermented milk drink is a polydisperse colloidal system in which the buttermilk is a dispersion medium, and the proteins of buttermilk and spirulina - the dispersed phase. The research results show that under the same conditions of the coagulation process with the increasing amount of spirulina added from 10 to 25% changes the acidity of the finished product.

## MATERIAL AND METHODOLOGY

### Samples

The object of the study is the quality indicators of the fermented milk product obtained by acid coagulation of buttermilk proteins using spirulina.

Subjects of research: buttermilk, spirulina, organoleptic, physicochemical parameters (mass fraction of protein, acidity, degree of syneresis).

For the study were prepared 4 samples of the drink with a mass fraction of protein of 2.9-3.2% with different doses of buttermilk and combinations of spirulina in different mass fractions. Spirulina (TM "Food Factor") was used to enrich the drink and form a characteristic thick consistency. TM is registered with the FDA (Food and Drug Administration - US Federal Food and Drug Administration). Doses of leaven (1.8% of the main (manufacturer Center for the Development of Microbiology and Biochemistry "Biochem") and 0.2% additional (manufacturer State Research Enterprise of Bacterial Ferments TIMM, Ukraine). Bacterial cultures are produced by the Center for Development of Microbiology and Biochemistry "Bioc1hem" and ANV produced by the State Research Enterprise of Bacterial Ferments TIMM Kyiv (manufacturer of pro- and prebiotics Ukraine). The bacterial leaven of mesophilic lactic acid bacteria and the additional leaven of thermophilic lactic acid bacteria were used as the main raw materials. The main bacterial leaven includes Lactococcus lactis subsp. lactis, Lactococcus lactis subsp cremoris, Lactococcus lactis subsp diacetilactis, Lenconostoc lactis.

Samples of the drink were prepared in the laboratory of food production VITE KNUTE certified quality management system (certificate No. UA.80050.063 QMS-21 recertified from 21.06.2021) following the classical technological scheme of production of fermented milk product.

### Chemicals

Buttermilk was used as raw material for the production of the drink. Buttermilk is a cream plasma obtained during the separation of butter from cream. The nutritional and dietary value of buttermilk preconditions its complete collection and use for food production whenever possible.

It is formed at the stages of whipping or separation of cream.

The chemical composition of buttermilk depends on the cream fat content, its acidity, temperature, and mechanical treatment. Protein substances of buttermilk are rich in cystine, lysine, methionine, and other essential amino acids. Buttermilk is characterized by a high content of fat-soluble vitamins. The chemical composition of buttermilk is presented in Table 1.

**Table 1** Chemical composition of buttermilk

| Description | Buttermilk (sweet butter) |
|---|---|
| Dry matter content, % | 9.1 (8.3-9.5) |
| Lactose content, % | 4.8 (4.7–4.8 |
| Protein content, % | 3.1 (2.9 – 3.2) |
| Milk fat content, % | 0.4 (0.4 – 0.7) |
| Acidity, °T, after CHFC | 20 (20 – 21) |
| Density, kg/m$^3$ (temperature (20±2) °C) | 1030 (1027 – 1029) |

Pro- and prebiotic starters for production of the fermented milk drink based on buttermilk were selected from concentrates provided by manufacturers of bacterial cultures: Biochem Center for the Development of Microbiology and Biochemistry – a manufacturer of probiotics since 1978 located in an ecologically clean region of Italy, and ANV producer TIMM State Research Enterprise of Bacterial Starters, the Ukrainian (Kyiv) manufacturer of pro- and prebiotics.

**Table 2** Characteristics of lactobacilli used in the work

| Starter name | Manufacturing company | Starter content | Starter type |
|---|---|---|---|
| Basic complex | Biochem Center for the Development of Microbiology and Biochemistry. Department of Biochemistry of Microorganisms Institute of Microbiology and Virology. D.K. Zabolotny NAS of Ukraine, Kyiv, Ukraine. | *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp Lactococcus lactis subsp diacetilactis, Lenconostoc lactis* | Gout-Foud - diplococci, chains of cocci of different length |
| Additional | TIMM State Research Enterprise of Bacterial Ferments TIMM, Kyiv, Ukraine. www.ddpdz.com.ua | *Lactobacillus acidophilus* (inviscid medium) | ANV – cocci, sticks of different length |

Bacterial starter of mesophilic lactic acid bacteria was used as the main raw material, while thermophilic lactic acid bacteria were used as the additional one.

**Animals and Biological Material:**

The main bacterial starter included *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp cremoris, Lactococcus lactis subsp diacetilactis,* and *Lenconostoc lactis.*

The description of lactobacilli starters is presented in Table 2.

One of the unique natural food sources is spirulina, a blue-green microalgae *Spirulina platensis*, which can be successfully used in everyday diet as a source of biologically valuable components. Spirulina is a product with a high concentration of protein (65 - 72%) by weight of algae. Algae contain several essential amino acids. Carbohydrates containing in the amount of 10-15% of the total spirulina weight are well digested and do not exert a negative effect on the pancreas. Spirulina contains almost all vitamins but C and D. It is the richest known source of vitamins A, B12, E, and F. Spirulina has five times more iron than brewer's yeast or dill. The algae contain trace elements featuring antioxidant activity, such as selenium, manganese, zinc, copper, iron, and chromium.

Spirulina is one of the richest sources of carotenoids. It contains about ten different types of carotenoids, including alpha-, beta- and gamma-carotenoids, half of the yellow xanthophylls.

Spirulina contains an average of 40-150 times more B vitamins (B1, B2, B3, B5, B6, B9, B12) than milk, cheese, meat, eggs, and butter.

The chemical composition of spirulina suggests the possibility of producing a wide range of prophylactic dairy products of various forms. This trend in the production of combined dairy products is promising and socially significant. According to marketing research, people choose dairy products containing plant components being guided by three criteria - taste, health, and culture. Once these criteria are met, the foods are considered fit for the main diet. And of course, they must be safe, meet the demand of essential nutrients, as well as contain biologically active components exerting a preventive effect on the human body **(Lange et al., 2020)**.

Producer:

United States Pharmacopeia (USP) Reference Standard (Sigma Aldrich).

This product is provided as delivered and specified by the issuing Pharmacopoeia. All information provided in support of this product, including MSDS and any product information leaflets, have been developed and issued under the Authority of the issuing Pharmacopoeia.

For further information and support please go to the website of the issuing Pharmacopoeia.

**Instruments**

The peculiarity of the introduction of bacterial leavens is that after high-temperature treatment of buttermilk in the mode (100 ±5) oC with a holding time of 25 s and cooled to 30 ±2 oC. The sourdough was prepared on chilled milk, stirred for 5 min to evenly distribute the components of the sourdough cultures **(Semko, Vlasenko and Palamarchuk, 2020)**. Made a different ratio of basic and additional leaven: 1.8% of the main and 0.2% of the additional. Fermentation was performed to a titratable acidity of at least 75-80 ºT (pH = 4.7). Then added fresh-frozen spirulina (1up = 20 grams) (1 sample-10g, 2 sample-15g, 3sample -20g, 4-25g). Samples of sour milk drink were cooled and stored in a refrigerator at a temperature of (4 ± 2) ° C for at least 8 days. After these technological operations, tests were performed. Mixing was performed with a mixer.

Pasteurization of buttermilk in a cheese boiler brand FJ 15 (Figure 1). Samples of the mixture No. 1, 2, 3 and 4.

The acidity of the fermented milk drink expressed in degrees Turner (ºT), and determined by the titrimetric method.

To modify the method of acid coagulation and rationalize the dose of spirulina, a range from 5% to 25% was determined with a step of variation of 5.

Functional and technological characteristics - acidity, the degree of syneresis was determined in the laboratory of VITE. Acidity was determined on a Bunsen apparatus (Figure 1). The titrated acidity of the fermented milk mixture was determined according to GOST 3624-92 and measured in degrees Turner (ºT). Turner degrees mean the number of millimeters of alkali solution 0.1 mol·dm$^3$ spent on neutralizing the concentrate **(Grek et al., 2015)**. The mass fraction of buttermilk protein was determined on an Ecomilk Bond milk analyzer (determination time – 120 sec with printer).

**Laboratory Methods**

Biochemical and physicochemical parameters of buttermilk as raw material and semi-finished products in the process of manufacture of fermented milk drink were studied by conventional methods of analysis set out in the relevant standards and guidelines for microbiological and technical-and-chemical control of fermented milk, as well as methods described in special literature.

The research methods are presented in Table 3.




**Figure 1** Mini pasteurizer cheese factory FJ 15.




**Figure 3** "Ecomilk Bond" to determine in buttermilk and drinks.

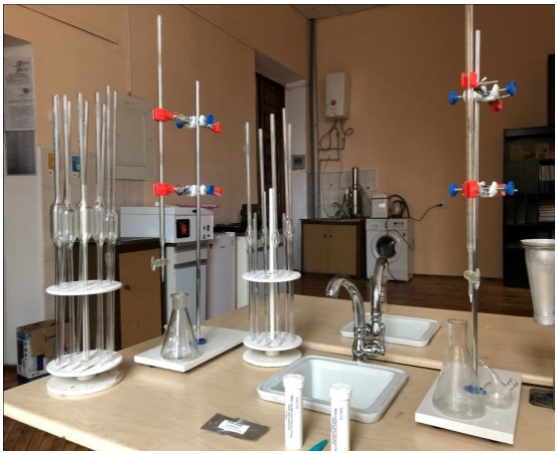

**Figure 2** Apparatus for Bunsen titration.



**Figure 4** Laboratory centrifuge "Orbit".

**Table 3** Research methods.

| Description | The principle of the research method |
|---|---|
| Raw buttermilk sampling and preparation for tests | According to GOST 26809-86 |
| Mass fraction of fat, % | Gerber acid centrifugation method and "Ekomilk Bond" milk analyzer according to GOST 5867-90 |
| Mass fraction of protein, % | Using a milk analyzer "Ekomilk Bond" according to GOST 2579-90 i GOST 23327-78 |
| Active acidity (pH), (units) | Potentiometric method according to GOST 26781-85 |
| Titrated acidity, ◦T | Titrometric method according to GOST 3624-92 |
| Density, g/cm$^3$ | Areometric method according to GOST 3625-84 |
| Milk purity grade, group | Filtering |
| Sampling for microbiological tests | According to GOST 26668-85 |
| The amount of acidophilic bacillus, % | By the method of limiting dilution of sterile skim milk at a temperature of 43°C |
| NMAFAnM, CFU/sm$^3$ | According to GOST 9225-84 and GOST 10444.12-88 |
| Mass fraction of nitrogenous compounds in the fermented milk product, % | By Kjeldahl method modified by ASRIBCM |
| The composition of free amino acids, % | Using amino analyzer Bio-Tronik LC 2000 following treatment with a solution of sulfosalicylic acid |
| Fractional composition of proteins in the fermented milk product, % | Polyacrylamide gel electrophoresis method (Lemley method modified by introduction of urea gel) |

**Table 4** The model food composition of the drink, kg.

| Description | Receipts | | | |
|---|---|---|---|---|
| | No. 1 | No. 2 | No. 3 | No. 4 |
| Buttermilk | 920.0 | 910.0 | 900.0 | 890 |
| Sugar | 50.0 | 55.0 | 60.0 | 65 |
| Spirulina | 10.0 | 15.0 | 20.0 | 25 |
| Starter (1.8 % - basic and 0.2 % - additional) | 20.0 | 20.0 | 20.0 | 20,0 |
| Finisher product output | 1000 | 1000 | 1000 | 1000 |

**Table 5** Organoleptic properties of the fermented milk drink enriched with pro- and prebiotic cultures.

| Description | Model product | | | |
|---|---|---|---|---|
| | No. 1 | No. 2 | No. 3 | No. 4 |
| 1. Appearance and consistency | Uniform thickening | Uniform thickening, minor buttermilk separation | Uniform thickening, granular consistency | Heterogeneous clot with separation of serum drops |
| 2. Taste and odor | Pleasant well-expressed sour milk, without foreign taste and smell | Sour milk, without foreign taste and smell | Specific sour milk, some foreign taste and smell | The specific sour-milk has a foreign herbal taste |
| 3. Color | | White, greenish tinge | | Greenish-gray with spirulina |

**Description of the Experiment**

**Sample preparation:**

While developing the recipe, we set the fo following tasks: to study the technological properties of spirulina intended for the manufacture of fermented milk drinks based on buttermilk; to develop the recipe and technological scheme for the manufacture of finished products; to choose the ratio of microorganisms that would enhance the taste of the product; to study the organoleptic, physical-and-chemical, microbiological properties of the fermented milk drink [8].

The model food composition of the drink is presented in Table 4.

The next stage of the study was to create a technological scheme presented in Figure 5.

The degree of syneresis was not more than 15 cm³ per 100 g of product, and not less than 10 points in the organoleptic evaluation were achieved at different doses.

Therefore, as criteria for optimizing the prescription composition of sour milk drink used characteristics:

- degree of syneresis (C, cm³ / 100 g);
- -acidity of the product (Degrees T);
- organoleptic parameters (OP, points).

Limit indicators of criteria are characteristic of the control sample:

- degree of syneresis - no more than 15 cm ³ / 100 g;
- acidity - not more than 100

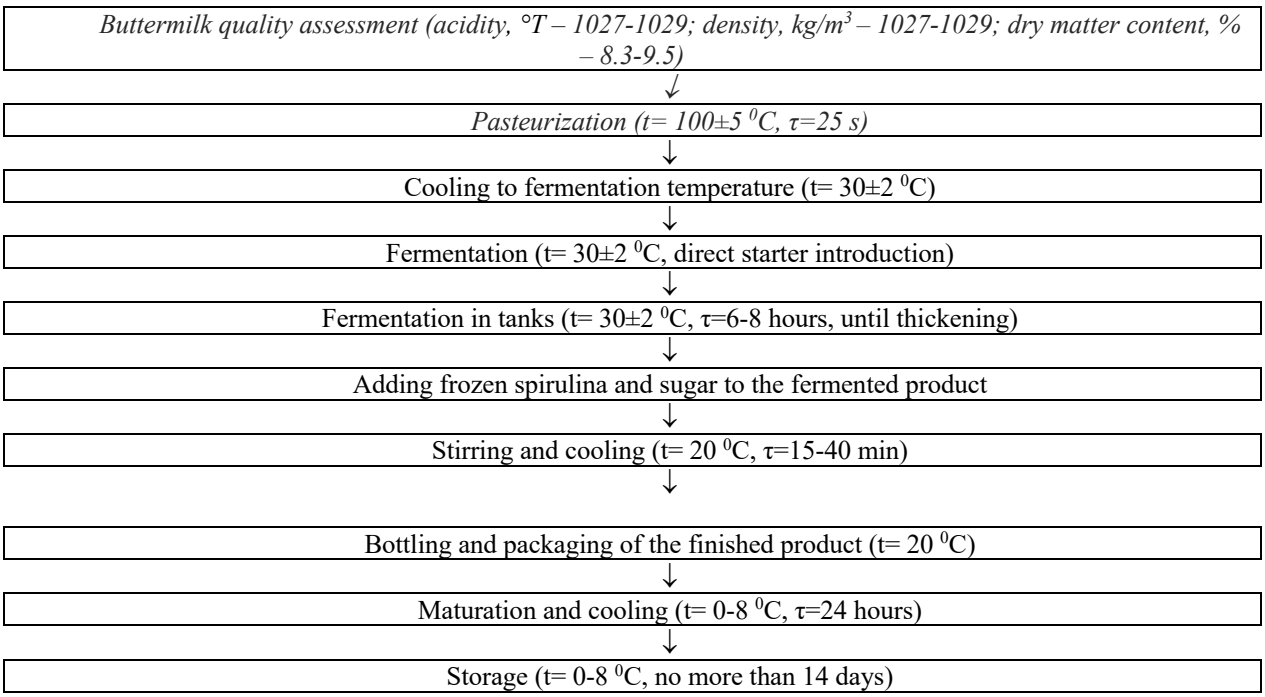


**Figure 5** Process flow diagram for production of the fermented milk product from buttermilk

- organoleptic indicators - from 0 to 10 points.

Independent variables determine the mass fraction of buttermilk fat (F, %) in the range from 0 to 3% and the mass fraction of spirulina application (C, %) in the range from 0 to 40 g.

**Number of samples analyzed:**

As a result of the study, three model products were obtained.

Table 5 contains the results of the organoleptic study of finished product enriched with prebiotic *Spirulina platensis* and probiotic (basic – *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp cremoris, Lactococcus lactis subsp diacetilactis, Lenconostoc lactis;* additional – *Lactobacillus acidophilus).*

Organoleptic characteristics of the drink were determined on a 10-point scale in accordance with the requirements of table. 1.

### Statistical analysis

Mathematical modeling is used in the work. The experiment was repeated three times and mathematically processed using Microsoft Exel 2007 indicators: acidity, degree of syneresis, organoleptic characteristics **(Kolyanovska et al., 2019)**.

The research was performed according to the matrix of experimental planning in accordance with the plan of small factorial experiment SFE-$2^{4-1}$.

The experiments were performed in triplicate. When processing the experimental data, a statistically significant indicator was considered to be the value ($n = 3$, $p \geq 0.05$).

## RESULTS AND DISCUSSION

The authors developed a functional sour milk drink **(Bultosa, 2016)**. The expediency and relevance of the use of secondary raw materials for the production of oil using specialized strains of the microorganism in the production of food products with a functional focus are described. **(Bal'-Prilipko et al., 2016)**.

We present the development **(Guneser, Hosoglu, Guneser and Yuceer, 2020)** of a new functional sour milk drink from buttermilk with the use of natural pro- and prebiotics - with a biologically valuable complex *Spirulina platensis* **(Kavimandan, 2015)**. The chemical composition of sour milk drink enriched with pro- and prebiotic cultures was studied **(Fakhry et al., 2021)**. The main most common pro- and prebiotics, including strains, are specified Lactobacillus acidophilus, Lactobacillus casei subsp. casei, bifidobacterium SPP. (B. Adolescentis, B. animalis, B. bifidum, B. longum, B. breve) bacteria of starting cultures **(Katla, et al., 2001)**. Lactobacilli are used from several different sources **(Klein et al., 2000)** have broad antimicrobial activity. Lactobacilli were used as probiotics in experiments to make the drink. Experiments on the development of fermented milk product according to the approved quality management system ISO 9001 and the developed procedure for tasting the drink contain organoleptic characteristics and a score (from 1 to 10) **(Dal Bello, et al., 2017)**. To assess the basic chemical composition in the literature, different types of products were analyzed **(Hayaloglu et al., 2008)**. Our studies have shown that with increasing administration of spirulina, the acidity of the drink increases disproportionately. With a spirulina content of 10 to 20%, the acidity of the finished product is acceptable. At introduction - 25% acidity for 5 hours makes 87 – 90 °T that testifies to the overestimated acidity of a product. Using the MIR technique, the average fat content of the beverage, which is 0.4%, the content of dry nonfat milk residue - 8.0, the acidity of the beverage 75 – 87 ˚T **(Manuelian et al., 2017)**. Comparisons of proteolysis products obtained with different proteolytic drugs were studied **(Yukalo, Datsyshyn and Storozh, 2019)**. The results of the studies corresponded to the chemical parameters for determining the degree of syneresis (C, $cm^3$/100 g); product acidity (degrees T) and organoleptic parameters (OP, points) **(Madalozzo, Sauer and Nagata, 2015)**. Samples No. 1, 2 and 3 in the work **(Breus et al., 2019)** similar in characteristics and meet the requirements for fermented milk drinks. The composition of buttermilk shows that its use for the production of fermented milk drinks is relevant. In this context, the problem of full and rational use of buttermilk is considered relevant **(Manuelian et al., 2017)**. For the manufacture of sour milk drink **(Polischuk et al., 2020)** the degree of syneresis (serum isolation from the beverage clot) was investigated by centrifugation using a laboratory centrifuge Orbita (Ukraine). Rotation speed 1000 rpm, centrifuge tubes with a volume of 25 $cm^3$ for 5 – 25 min and in terms of serum volume in $cm^3$ per 100 g of product **(Lange et al., 2020)**.

The synergistic properties of beverage clots were evaluated by the amount of whey released during protein coagulation **(Atallaha, Morsyb and Gemiela, 2020)**. The dependence of the syneretic properties of buttermilk on the density of the clot and the duration of centrifugation of sour milk drink was determined. Analyzing the obtained data, it should be noted that the glossy clot with spirulina impairs the syneretic properties of the clot. After centrifugation for 5 – 25 min rennet clots obtained from buttermilk, which underwent two-stage pasteurization at different doses of spirulina, the amount of serum for centrifugation was: variant 1 sample - 77%, 2 samples - 74%, 3 samples - 71%, 4- sample - 68%. The lowest amount of serum was obtained in the sample with the highest dose of spirulina-25 **(Patel et al., 2019)**. In the fourth sample, it was noted that the appearance and consistency have a heterogeneous structure, a clot with the release of serum drops, the taste and smell of grassy, greenish-gray color with spirulina. The overall score of the fourth sample is overestimated. Due to the deterioration of organoleptic parameters, the study of sample №4 was stopped.

Food manufacturers around the world are tasked with improving the quality, biological value, safety, taste, and range of certain foods, including functional foods **(Türkmen, Akal and Ozer, 2019)**. Therefore, the development of the latest technologies for the production of fermented milk drinks based on buttermilk, suitable for all functional groups using the natural prebiotic spirulina, is considered a very promising task **(Bultosa, 2016)**. In particular, on the basis of dairy products you can produce a wide range of health, medical and dietary products.

**Table 6** Chemical composition of the fermented milk drink enriched with pro- and prebiotic cultures.

| Description | Model product | | | |
|---|---|---|---|---|
| | No. 1 | No. 2 | No. 3 | No. 4 |
| Fat content, % | 0.4 | 0.4 | 0.4 | 0.4 |
| The content of dry skim milk residue, % | 8.0 | 8.0 | 8.0 | 8.0 |
| Acidity, ˚T | 75 | 78 | 80 | 87 |
| Temperature, ˚C | 8 | 8 | 8 | 8 |

**Table 7** Microbiological properties of fermented milk drink enriched with pro- and prebiotic cultures.

| Description | Model product | | |
|---|---|---|---|
| | No. 1 | No. 2 | No. 3 |
| *Escherichia coli* bacteria (coliforms), per 0.1 $cm^3$ of the fermented milk drink | Not found | | |
| Molds, CFU per 1 $cm^3$ | Not found | | |
| Pathogenic microorganisms, including Salmonella bacteria, per 25 $cm^3$ | Not found | | |
| The number of viable lactic acid bacteria, CFU per 1 $cm^3$ | $1.0x10^7$ | $1.0x10^7$ | $1.0x10^7$ |

Note: Having analyzed the resulted organoleptic, physical-chemical and microbiological parameters, we found model product No.1 the best one.

**Table 8** Recipes for sour milk drink with different doses of spirulina.

| Name of the component | Recipes | | | |
|---|---|---|---|---|
| | No. 1 | No. 2 | No. 3 | No. 4 |
| buttermilk | 920,0 | 910,0 | 900,0 | 895 |
| Sugar | 50,0 | 55,0 | 60,0 | 60,0 |
| Spirulina | 10,0 | 15,0 | 20,0 | 25 |
| Sourdought (1.8% of the main and 0.2% of the additional) | 20,0 | 20,0 | 20,0 | 20,0 |
| The yield of the finished product | 1000 | 1000 | 1000 | 1000 |

**Table 9** Estimation of quality of samples of drink on organoleptic indicators (the maximum possible points are specified).

| Indicator | Characteristics |
|---|---|
| Consistency, appearance (3 points) | Homogeneous, tender, with a broken clot, moderately dense, without gas formation, with the replacement of the included threads of spirulina. |
| Taste and smell (6 points) | Pure, sour milk, with a taste of green algae-spirulina, odorless. |
| Color (1 point) | Green, for fermented milk drink caused by spirulina |

Therefore, research related to the processing of buttermilk and the search for alternative ways to use it as a raw material for the production of fermented milk drinks, supplemented with prebiotics, deserves to be introduced into production.

In our work, we present a recipe for a sour milk drink based on buttermilk and spirulina **(Atallaha, Morsyb and Gemiela, 2020)**. We have developed a recipe for a functional milk drink based on buttermilk enriched with prebiotic spirulina **(Leiss et al., 2008)**, can be used by dairy companies **(Guneser et al., 2001)**.

Table 6 contains the chemical composition of the fermented milk drink enriched with pro- and prebiotic cultures.

Table 7 contains microbiological properties of fermented milk drink enriched with pro- and prebiotic cultures.

The degree of syneresis (serum isolation from the beverage clot) was studied by centrifugation using a laboratory centrifuge Orbita (Ukraine). Rotation speed 1000 rpm, centrifuge tubes with a volume of 25 $cm^3$ for 5 – 25 min and in terms of serum volume in $cm^3$ per 100 g of product.

The synergistic properties of the beverage clots were evaluated by the amount of serum released during protein coagulation. The dependence of the syneretic properties of the fermented milk clot on the density of the clot and the duration of centrifugation of the fermented milk drink is shown in Figure 6. Analyzing the data obtained, it should

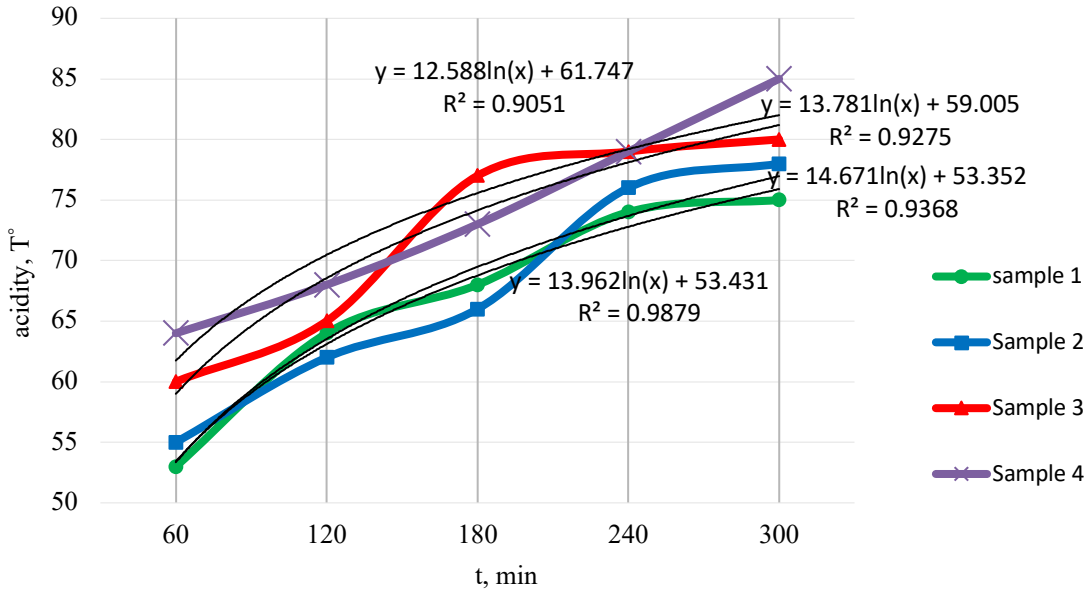


**Figure 7** Dynamics of change in acidity of buttermilk with spirulina.

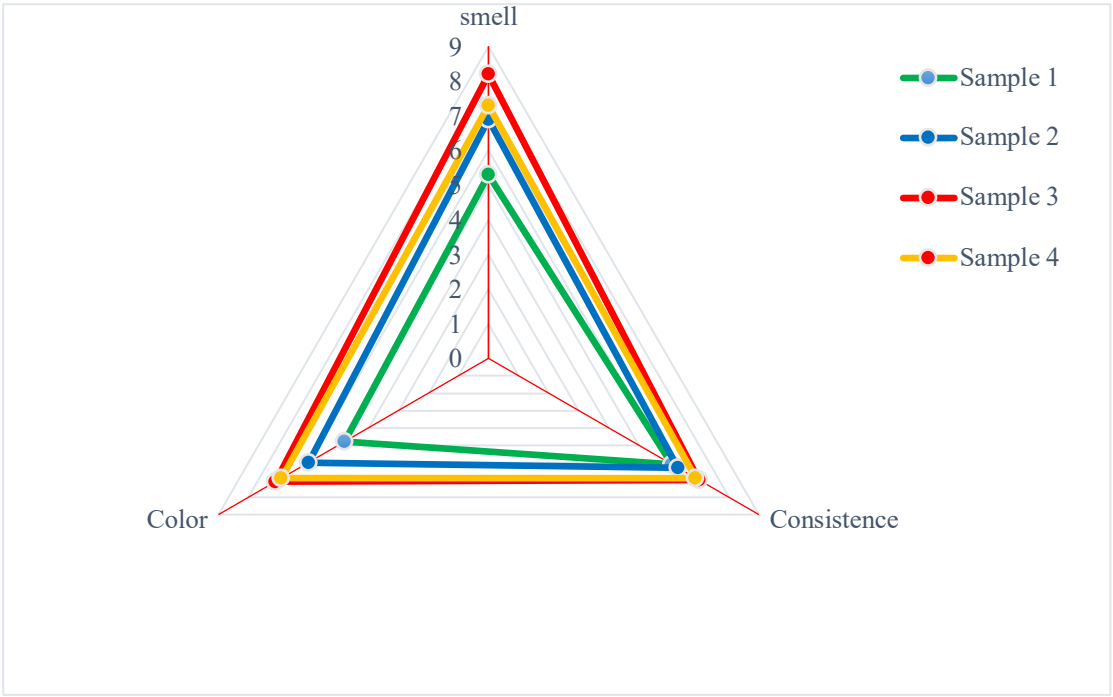


**Figure 8** Comparison of organoleptic characteristics of samples.

be noted that a loose clot with spirulina impairs the syneretic properties of the clot. After centrifugation for 5-25 min rennet clots obtained from buttermilk, which underwent 2-stage pasteurization at different doses of spirulina, the amount of centrifugation serum was: option 1 sample - 77%, 2 sample - 74%, 3 sample - 71%, 4-sample-68%. The lowest amount of serum was obtained in the sample with the highest dose of spirulina-25.

Studies show that with the increasing introduction of spirulina, the acidity of the drink increases disproportionately (Figure 7). When the content of spirulina is from 10 to 20%, the acidity of the finished product is acceptable. At introduction - 25% acidity in 5 hours makes 87-90 °T that indicates the overestimated acidity of a product.

According to the approved quality management system ISO 9001, we created a tasting commission, which conducted a point evaluation (from 1 to 10) of the developed sour milk drink (Fig. 8). Samples No. 1, 2 and 3 are similar in characteristics and meet the requirements for fermented milk drinks. In the fourth sample it was noted that the appearance and consistency have a heterogeneous structure, a clot with the release of serum drops, the taste and smell are herbaceous, greenish-gray color with spirulina. The overall score of the fourth sample is inflated. Due to the deterioration of organoleptic parameters, the study of sample №4 was suspended.

Food producers around the world are challenged with the task of improving the quality, biological value, safety, taste, and the assortment of certain types of food, including functional products.

Analysis of buttermilk processing technologies shows that its use for the production of sour milk drinks is relevant. In this context, the problem of full and rational use of buttermilk is considered topical. Therefore, the development of the latest techniques for the manufacture of fermented milk drinks based on buttermilk suitable for all functional population groups using natural prebiotic spirulina is considered quite a promising task. In particular, it is possible to produce a wide range of health, medical and dietary products based on dairy products.

Having analyzed buttermilk processing techniques, we concluded that the issue of full and rational use of the material is still actual. Therefore, the study related to the processing of buttermilk and the search for alternative ways of using it as a raw material for the manufacture of fermented milk drinks supplemented with prebiotics is worth the whistle.

The recipe of fermented milk drink developed by us based on buttermilk enriched with prebiotic spirulina can be used by dairy establishments.

Table 10 contains the recipe of the fermented milk drink based on buttermilk.

**Table 10** – The recipe of the fermented milk drink based on buttermilk enriched with pro- and prebiotic cultures

| Description | Used raw material, kg |
|---|---|
| Buttermilk | 920.0 |
| Granulated sugar | 50 |
| Starter | 20.0 |
| Spirulina | 10 |
| Total | 1000 |

The research results have shown that the subject drink is characterized by improved nutritional value and fit for human consumption.

## CONCLUSION

Functional dairy products are the main suppliers of probiotic microorganisms, help to restore the microflora of the human gastrointestinal tract. Strains of bifidobacteria and certain species of lactobacilli, such as *Lactobacillus acidophilus, Lactobacillus casei subsp. casei, Bifidobacterium* spp. (*B. adolescentis, B. animalis, B. bifidum, B. longum, B. breve*), belong to probiotic cultures. The peculiarity of our proposed application of bacterial leavens is that after high-temperature treatment of buttermilk in the mode (100 ±5 °C) with a holding time of 25 s was cooled to 30 ±2 °C.

One of the important components of our sour milk drink is spirulina, which is widely used to improve the taste and enrich it with protein. We have developed an innovative technology for combining the unique food green algae Spirulina platensis with secondary raw materials fermented by selected strains of probiotic crops. Experimental studies have shown that a loose clot with spirulina impairs the syneretic properties of the clot, as evidenced by centrifugation for 5 – 25 min clots obtained from buttermilk, which has undergone 2-stage pasteurization. The lowest amount of whey was obtained in the sample with the highest dose of spirulina – 25. The optimal ratios of components (buttermilk, sourdough, sugar and spirulina) for the preparation of the food composition were determined. The parameters of the technological process of product preparation are established (fermentation in tanks t = 30 ±2°C, τ = 6 – 8 h before clot formation).

The resulting fermented milk drink is a polydisperse colloidal system in which the buttermilk is a dispersion medium, and the proteins of buttermilk and spirulina are the dispersed phase. The results showed that under the same conditions of the coagulation process with increasing amount of spirulina added from 10 to 25% changes the acidity of the finished product. According to research, in our opinion, the most rational is the content of spirulina 10% for all defined indicators.

In the innovative development of the dairy industry, the technology of products using the plant extract of spirulina will be a new direction of enrichment of traditional fermented milk drinks to expand the range of functional products. The developed product can take the place of a functional sour milk drink in the system of healthy nutrition of the population.

The proposed technology for the production of sour milk drink from buttermilk and spirulina content allows to obtain a drink with improved technological properties and increased biological value.

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# DEVELOPMENT OF THE FOOD ACT OF THE SLOVAK REPUBLIC FROM 1995 TO 2021

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## ABSTRACT
Legislation in food is quite fragmented, it includes several regulations, amendments due to the rapid development both in the studied area and the influence of European law. It is a legal branch of a relatively hybrid nature. It contains agricultural legislation that intersects with environmental law. It also affects administrative, financial, and criminal law as a branch of public law. Nor can commercial and civil law as the basis of private law be circumvented here. The legal norm has undergone a relatively interesting development, which is characterized by several amendments. During the more than 25 years of validity of this legal norm, a total of more than twenty amendments. This certainly does not contribute to the stability of the legislation, the legal certainty of the entities concerned, or its clarity and system. The article provides an overview of the development of the legal regulation of the Food Act in Slovakia from 1995 to 2021.

**Keywords:** food low; Slovakia; food legislation; legal act; amendment

## INTRODUCTION
If we want to talk about the legislation in this legal norm, it is necessary to see it in a broader context. **Food Act No. 152/1995 Coll (NR SR, 1995a)** is a basic norm of food law in Slovakia. This is a legal sector of a relatively hybrid nature. Contains requirements related to agriculture and food production and the environment. It also affects administrative, financial, and criminal law as a branch of public law. Nor can commercial and civil law as the basis of private law be circumvented here. In addition, legislation in this area is characterized by the strong influence of European Union law. Overall, the lawyer can describe this branch of law as relatively fragmented, ie. including several decrees, amendments, which are caused both by rapid developments in the area under study and the mentioned impact of European law.

The characteristic of food law is the fact that it regulates the activities of various entities, which often have conflicting interests. On the one hand, it is food businesses operators, it is the European Union and the Member States that must protect food safety as part of their policies, and it is also consumers who have their requirements. Comprehensively, food law should be the result of the compromise claims of all these entities. The European Commission enforces EU food law by checking that Member States incorporate EU legislation into their national law and implement it correctly, while national agencies are responsible for ensuring that EU rules are respected **(European Union, 2021)**.

Legislation in the food industry must pursue the protection of public health and economic objectives must protect consumers, but also prevent abuse of economic position in the market between manufacturers and dealers. It is a fact that food law is a matter of everyday life that we all encounter. Naturally, as food is a necessary segment, we are entering the legal position of a consumer, so we are directly affected by these issues. Then, in the legal plane, it is necessary to consider the legal protection of the consumer, which has a special course and protect the weaker party in the event of civil procedure, and at the same time, it is necessary to take into account public administration in case of damage to a wider range of consumers. Although it should be mentioned here that our legal system does not yet include the possibility of filing a so-called class action. Although Directive of the European Parliament and of the Council No. 2020/1828 of 25 November 2020 **(EU, 2020)** by wildcard actions to protect collective interests of consumers are opening new space and how they are planning in the Czech Republic, the Slovak Ministry of Justice is not concerning such a change. The Slovak Ministry of Justice wants to wait until the European Directive enters into force (and it can be a tool for solving the dual quality of food).

## DISCUSSION

### Several aspects in the historical development of the Food Act No. 152/1995 Coll. Of the Slovak Republic

The need for the legal regulation of food properties and the conditions for their exchange and marketing arose here relatively soon. Already in Roman law, we find provisions on agricultural land, the prohibition of its crushing within the inheritance, or e.g., classification of food as consumable. At first, it was about individual products, e.g., food ingredients (e.g., salt, spices, mushrooms, flour milling). Later in the 19th century, more systematic legal foundations of food law were laid. In our legal field, until the disintegration of Austria-Hungary in 1918, Austrian law was valid for the Czechs. In contrast to the Czech law on the territory of Slovakia to form the first Czechoslovak Republic was true Hungarian common law.

In the Austrian part of the monarchy (where there was no general health law), was adopted law No. 89/1897 on trade in food and certain useful objects and Hungary this issue was regulated within the legal regulation of public health (§ 14 of Act No. XIV/1876), supplemented by a special act. No. XLVI/1895 on the prohibition of adulteration of crops, products, and needs. Based on them, several implementing regulations were gradually issued for individual products and cross-sectional issues (packaging, dyes, etc.). Special laws were issued prohibiting wine adulteration (Act No. 210 of 12 April 1907 and Act No. XLVII/1908) **(URL 1, 1995)**.

This legislation focused on food control, entrusted to a system of general bodies, and supplemented by special state or state-authorized institutes for technical testing of food. The legal regulation was supplemented by special regulations governing veterinary care and varietal law, general regulations on the protection of designations of origin, and, finally, regulations of a clear economic nature (e.g., making fixed milk prices for dairies by dairy treatment – Government regulation No. 76/1934), as and trade regulations.

To date, an important and valued step in Austrian law has been the adoption of the so-called Food Code by (*Codex Alimentaris Austriacus*). The standard was published in three volumes in 1911, 1912, and 1917 and unified for individual products' valid regulations, description of goods, its composition, testing, etc. During its translation into Czech and the current update in the 1930s (organized by the self-government of the food industry and officially confirmed by a government resolution of 23$^{rd}$ of June 1934), it was extended (then as the Czechoslovak Food Code) to the former Hungarian law. The legal nature of the code was multidirectional, it was intended for food control authorities and institutes for technical testing of food, it was a binding guide for the courts, it acted as an expert.

Current foods law was adopted by the Parliament of the Slovak Republic on the 27$^{th}$ of June 1995 with effect from 1$^{st}$ of January 1996 except for § 9 paragraph. 1 – food labeling and misleading advertising ban, which came into effect from 1 July 1996, is published in the Collection of Laws in part 51. The standard is designed as a special law governing the specific conditions of food production, handling, and circulation of food, including tasks and organization of food supervision in the interests of health and consumer protection in the food market. It is divided into seven sections and 32 paragraphs, which for the first time form a comprehensive legislative framework for the food Slovak Republic. At the same time, it also regulates the conditions to produce tobacco products and cosmetics. It sets out the basic obligations of food producers, conditions for their placing on the market, import, packaging of food, food labeling and prohibition of misleading advertising, storage conditions, transport, sale of food, tasks, and organization of food control authorities, powers of departments in authorizing laboratories performing testing and monitoring for state administration, etc. and subsequently, the section on the number of fines is based on these chapters according to which obligations the entrepreneur violates by law. The Food Act is only a framework regulation that regulates the basic conditions of food production, handling, and placing on the market. It pursues the objective of promoting and protecting human health and consumer protection. The Food Code of the Slovak Republic will be decisive, especially in its scope and focus, following § 3 of the Food Act, Food Codex of the Slovak Republic. The law radically intervened in the system of technical standards (replaced ordinary standards – common provisions), and in legal regulations in the field of hygiene, control, etc.

The theory recognizes the division of food law standards into horizontal or vertical standards, depending on their focus on either a particular type of food or on all foods across them. Thus, the horizontal rules set out the requirements for food quality (safety for human health, labeling requirements) in general. The Food Act itself can be included in this group. In the category of vertical regulations, we include standards that regulate requirements for a specific type of food, e.g., milk products. Food law shall aim at the protection of the interests of consumers and shall provide a basis for consumers to make informed choices to the foods they consume. It shall aim at the prevention of fraudulent or deceptive practices; the adulteration of food and any other practices which may mislead the consumer **(Ryan, 2016).**

### The European Union and food law

Governing food security is a global, complex problem **(Skovrad, 2011)**. The Slovak Republic is stable and long-term oriented towards integration into the European Union. The European Union combines health policy and consumer protection. The safety of goods, including food safety and the food warning system, the safety of services, which are also considered healthcare, have a high degree of priority.

Food law is also clearly defined by European standards. The basic standard is **Regulation (EC) No. 178/2002 (EU, 2002)** on the general principles and requirements of food law, which contains the definition of food law and lays down food law in force within the EU or at the national level. Fodo law in the EU must respect the Codex Alimentarius, as a complex of international quality standards and processes in the field of the food industry. The definition of food is present in **Regulation (EC) No. 178/2002 (EU, 2002).** Food is a substance or product intended for human consumption or in which it may be assumed give that person will consume it. Which is a very broad base. The regulation sets requirements for food quality and safety. It also contains a definition of food in a negative sense. It does not consider food, feed, live animals, tobacco products, medicinal products, etc. It does not apply

to the production of foodstuffs intended solely for personal consumption.

A further regulation, which can be included in the horizontal rules is for example a **Regulation (EC) No. 852/2004 (EU, 2004a)** on the hygiene of foodstuffs laying down hygiene requirements for the production, processing, and placing on the market of foodstuffs. The vertical norm here is e.g., **Regulation (EU) No. 1308/2013 (EU, 2013a)** on the common organization of the markets in agricultural products. This regulation is laying down requirements for milk products, wine, fresh fruits, etc. from wine grapes. It regulates legal names reserved for certain types of food, which must meet quality requirements. A similar example is a **Regulation (EC) No. 110/2008** of spirit drinks **(EU, 2008)**.

Harmonization in the field of food law intensified mainly at the beginning of the 21st century. At a time of free movement of people and goods, and therefore of food, within the European Union, it is inconceivable that there should be obstacles to their functioning. Harmonization of rules is intended to prevent the creation of barriers to food trade between the Member States. During this period, food standards have changed their nature from a directive to a regulation, which makes the standard more binding. Where a directive indicates only an objective and leaves the choice of its attainment to national law, the regulation must already be directly incorporated into the laws of the Member States.

European legislation in this area is based on the basic premise of the protection of human health (see Regulation No. 178/2002) and the "farm to fork" principle. Thus, the legislation should cover the whole process of food production up to final consumption. At the same time, associated goals such as good conditions for farmed animals, the quality of their feed. At the same time, the European Union emphasizes the protection of consumers who have the right to product information. From the 13$^{th}$ of December 2014, it entered into force new **Regulation (EU) No. 1169/2011 (EU, 2011a)**, which regulates the new labeling requirements and stipulates that voluntary information may not be provided to the detriment of mandatory particulars. Respect for the principles of fair trade in the food market is another pillar at the level of European regulation.

If we are talking about harmonization, the area of food law, which is regulated only by the primary law of the European Union, cannot be circumvented. The Treaty of Lisbon prohibits the restriction of the European Union's internal market based on national technical national standards. However, European secondary law (i.e., regulations, directives, decisions of EU institutions) does not regulate all food requirements. The unadjusted ones fall into the non-harmonized area and are corrected by the legislation of the Member States. Mainly in so-called vertical requirements, such as e.g., requirements for the quality of fatty products, there are no harmonized requirements set at the EU level. The principle of mutual recognition applies in the non-harmonized sphere. It was based on the European Court of Justice (currently the Court of Justice of the European Union) in the well-known Cassis de Dijon case (Case 120/78, Rewe-Bundesmonopolverwaltung fur Branntwein [1979] ECR 649) under French currant liqueur, the marketing of which was restricted regarding lower alcohol content in Germany due to non-compliance with German national regulations. The principle of mutual recognition thus means that if a product is placed on the Union market in one Member State following its legislation, it must also be recognized on the market of another Member State **(Čejka, 2014)**.

With the adoption of the Food Act, the conditions for food production, handling, and marketing in the Slovak Republic, as well as the tasks and organization of food supervision over compliance with this Act were established for the first time in the Slovak Republic. Based on empowering provisions in the Act, the acquis in the field of foodstuffs has been gradually implemented since 1996 by drafting horizontal and vertical legislation in the Slovak Republic in connection with EU Directives and Regulations and negotiations with the European Commission.

**Gradual development and changes in the Food Act No. 152/1995 Coll. Of the Slovakia Republic (NR SR, 1995a)**

From the point of view of a lawyer, the examined legal norm underwent a relatively interesting development, which is characterized by several amendments. During the more than 25 years of validity of this legal norm, a total of more than twenty amendments have been made. This certainly does not contribute to the stability of the legislation, the legal certainty of the entities concerned, or its clarity and system. So, let's try to approach the process of these changes gradually, although the study will certainly not be complete and exhaustive regarding the scope of the paper. The first change came a year after the law was passed.

**Act No. 290/1996 Coll. SR (NRSR, 1996a), which amends the Act of the National Council of the Slovak Republic No. 272/1994 Coll. SR (NRSR, 1994a) on the protection of human health, as amended by Act of the National Council of the Slovak Republic no. 222/1996 (NRSR, 1996b) Coll and amending certain other laws**

The amendment to the Act aimed to regulate the management of sources of ionizing radiation and to allow the qualitative and quantitative survey of environmental and occupational factors in terms of their possible impact on human health and to exclude these activities from the regime of **Act No. 455/1991 Coll. SR (NRSR, 1991)** on Trade Licensing (Trade Licensing Act) as amended. As stipulated by the new regulation “It is possible to use sources of ionizing radiation for irradiation of foodstuffs only with the permission of the Ministry of Health of the Slovak Republic. It is prohibited to place on the market foodstuffs accidentally irradiated or containing radionuclides from emergency areas. "

**Act No. 470/2000 Coll. SR also addressed the effects of ionizing radiation (NRSR, 2000)**.

The regulation of the protection of human health against the effects of ionizing radiation then contained in the Act of the National Council of the Slovak Republic no. 272/1994 Coll on the protection of human health, as amended by **Act of the National Council of the Slovak Republic No. 290/1996 Coll (NRSR, 1996a)**. He has not suited the current requirements for the protection of health, in addition, does not address la the issue of natural radioactivity and the issue of the use of ionizing radiation in medicine in healthcare. **Act of the National Council of the Slovak Republic No. 152/1995 Coll (NR SR, 1995a)** on foodstuffs has been amended as follows: "The addition of

radioactive substances in the production of food is prohibited. The import of food and ingredients for their production to which radioactive substances have been added is prohibited. Adding radioactive substances in the manufacture of cosmetic products and placing on the market of such cosmetic products".

**Act No. 553/2001 Coll (NRSR, 2001) on the abolition of certain state funds, on certain measures related to their abolition, and on the amendment of certain laws**

In § 28 par. 6 letter a) the words "State Health Fund" are replaced by the words "state budget". Until then, the fines imposed during the performance of food supervision by health protection authorities were the revenues of the State Health Fund - the amendment abolished the fund, and the fines became the revenues of the state budget.

**Act No. 23/2002 Coll (NRSR, 2002a) which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll on foodstuffs as amended and on the amendment of certain laws**

An extensive amendment came in 2002. The changes made eliminate non-compliance with EU legislation, for example in the use of terms, removed pre-market control (previously mandatory certification or mandatory conformity assessment before placing food on the market), food labeling was brought into line with **Directive No. 2000/13/EC (EU, 2000)**.

Other adjustments concerned the extension of the obligations of entrepreneurs, for example, the obligation to develop principles of good manufacturing practice, including the HACCP system following the Food Hygiene Directive, as well as the incorporation of new types of food, including labeling. All food businesses not related to primary production must comply not only with GHPs but also with procedures developed in the company based on the principles of the HACCP system **(Moran et al., 2017)**. Benefits and constraints, as well as identifying needs for tailoring a HACCP system to suit the needs of small and less developed businesses like restaurants and eating places have been analyzed and discussed in several articles **(Dzwolak, 2014, Fielding et al., 2011, Pichler et al., 2014)**.

This law also regulated the organization and scope, rights, and obligations of food supervisory authorities at all stages of the production process up to the sale of food in trade, which is in line with EU **Directive No. 89/397/EEC (EU, 1989)** on the official control of foodstuffs. An integrated body was created at the State Veterinary and Food Administration of the Slovak Republic.

The changes made were based on the White Paper on Food Safety of January 2000 as well as the proposal for a Regulation of the European Parliament and the Council of the European Union on the Food Act.

**Act No. 450/2002 Coll (NRSR, 2002b), Amending the Act of the National Council of the Slovak Republic No. 152/1995 Coll on Foodstuffs, as amended, and amending Act of the National Council of the Slovak Republic No. 277/1994 Coll (NRSR, 1994b) on health care as amended.**

In § 2 par. 1, in the third sentence, the words "are not" are replaced by the words "are also". Thus, foods according to the law are also dietary foods for special medical purposes as defined by a special regulation.

**Act No. 472/2003 Coll (NRSR, 2003a), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll on Foodstuffs, as amended, and on Amendments to Certain Acts.**

Changes based on where they need to incorporate into food law, certain provisions of **Regulation (EC) No. Regulation (EC) No. 178/2002 (EU, 2002)** of the European Parliament and of the Council of the 28th January 2002 establishing the general principles and requirements of Food Law, establishing the European Food Safety Authority, and laying down procedures in matters of food safety.

In the Food **Act No. 152/1995 Coll. SR (NR SR, 1995a)** some of the requirements of these articles have already been enshrined in the wording of later regulations, such as the requirements for food safety placed on the market (Articles 14 and 16), the liability of businesses and the system of official controls and the adoption of measures and penalties for infringements (Articles 17 and 19 of the Regulation), traceability (Article 18), which is not contrary to the Regulation.

Other proposed amendments, as well as proposed amendments to some laws, are based, for example, on the requirements of the Dublin Mission, the creation of conditions and redistribution of responsibilities for origin marking and geographical indications (§9a), food supervision reserves, and knowledge of the application of food law in practice. Further adjustments were required by the Food Act after the accession of the Slovak Republic to the EU in 2005 in connection with findings from the application of other articles of the Regulation, other forthcoming regulations such as the Hygiene Directive, official food, and feed control, etc., to resolve the possibility of laws directly to the EU Regulation and thus the reconstruction and scope of areas.

It is stipulated that the application of this law does not apply to areas related to the needs of private persons, such as animal husbandry, vegetable and fruit growing, etc. up to their processing into finished products and if it is carried out exclusively for private domestic consumption. The wording of what is food and what is not food is clarified. At present, as in some Member States and after consulting the Commission's representatives, tobacco and tobacco products have remained within the scope of this law.

It defines what is meant by a system for the rapid exchange of food information through notifications between the authorities concerned in cases where food is shown to be harmful to health or there is a reasonable suspicion that it may pose a serious risk to the health and safety of consumers.

The Rapid Alert System for Food and Feed is organized by the European Commission (DG XXIV. Consumer and Health Protection) as a form of rapid information to the Member States on food problems and risks. RASFF database provides information on each report recorded, including the type and date of notification, the reason for notification, the hazard(s), the nature of the product involved, the country of notification, and the country of origin **(European Food Safety Authority, 2010; Djekic, Jankovic and Rajkovic, 2017)**. The implementation of this system results from the commitments of the Slovak Republic in the accession process. The State Veterinary and Food Administration of the Slovak Republic is established

as a contact point. The principle of preventivity according to Art. 7 of the Regulation.

It provides for the redistribution of the competence of the Industrial Property Office of the Slovak Republic and the Ministry of Agriculture of the Slovak Republic in the administration of designations of origin and geographical indications of agricultural, **Regulation (EEC) No. 2081/92 (EU, 1992b)** requires an expert assessment of the product specification submitted by the applicants.

Professional assessment, as well as authorization of control bodies, falls fully within the competence of the Ministry of Agriculture of the Slovak Republic. The power of food control authorities to make copies or extracts from inspected documentation is added. The competence of the employees of the food supervisory authorities in the direct performance of control and the adoption of measures to eliminate the identified deficiencies, which are not covered by § 31 part 2 of the Act – general regulations on administrative proceedings.

In § 23, paragraphs 3 and 6 on the conditions for revoking the certificate of professional competence for the classification of carcasses according to § 6a and the control of food of plant origin at border crossings are added.

According to **Act No. 272/1994 Coll. SR (NRSR, 1994a)** on the protection of human health, as amended, control – health supervision in mass caterers is the responsibility of health protection authorities.

In the EU, however, regulations – Directives, Food Regulations also cover food in the provision of catering services. Inspection by health protection authorities must also be based on this Act and in particular the Food Codex and the interpretation of Art. 14 of the above-cited directive and not only regulations issued based on the Act on the Protection of Human Health.

Following the conclusions from the mission from Dublin, the responsibility of the Ministry of Defense of the Slovak Republic and the Ministry of Health of the Slovak Republic was redistributed within food law in individual areas (EU regulations or for national use) according to § 3 part 1, which stipulates the elaboration of the Food Codex and other generally binding legal regulations based on empowering provisions in the Act.

It specifies that the provisions of the Veterinary Care Act and the Health Protection Act do not apply to food supervision if it is carried out under this Act. As far as sampling and analysis are concerned, this procedure is not subject to the general rules on administrative procedure.

The EC maintains the maintenance of the food register as a barrier to the free movement of food. Based on this, the Decree of the Ministry of Justice of the Slovak Republic no. 240/2002 Coll. on the registration of manufactured and imported foodstuffs on the day of entry into force of the Treaty of Accession of the Slovak Republic to the EU.

It is further supplemented when the non-fulfillment of obligations is considered a misdemeanor or administrative offense.

A legal framework is being created for the establishment **(URL 2, 2003)**.

**Act No. 546/2004 Coll. SR (NRSR, 2004), which amends the Act of the Slovak National Council No. 30/1992 Coll. SR on the Slovak Chamber of Agriculture and Food, as amended by Act No. 448/2001 Coll. SR and amending certain laws**

The main objective of Act no. 546/2004 Coll. SR, Amending the Act of the Slovak National Council No. 30/1992 Coll. SR on the Slovak Chamber of Agriculture and Food, as amended by Act No. 448/2001 Coll. SR is to abolish compulsory membership in the Chamber, clarification of cooperation between the Chamber and the relevant state bodies, an extension of the provision of services for members of the Chamber. This novelization also touched on genetically modified foods and the so-called novel foods. Novel foods and genetically modified foods are also marked on the consumer packaging with data according to special regulations.

Food produced or put into circulation in a Member State of the European Community or originating in one of the countries of the European Free Trade Association which is also a party to the European Economic Area may not be refused for placing on the market in the Slovak Republic provided that the food complies with the regulations and production methods which are binding for the production or placing on the market of the food in one of these States and which guarantee their health safety.

The national authority for the administration of special regulations and contact with the European Food Safety Authority is the Ministry.

Testing at official food controls is carried out by laboratories authorized by the Ministry or the Ministry of Health. The ability to perform the test is demonstrated by an accreditation certificate. Other accredited laboratories may perform testing based on authorization from the Ministry or the Ministry of Health within the scope of the subject of accreditation.

**Act No. 195/2007 Coll. SR (NRSR, 2007), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR on foodstuffs as amended**

Act No. 195/2007 Coll lays down new requirements for the performance of official controls on foodstuffs, which are not addressed in the current legislation and are related to the adoption of **Regulation (EC) No. 882/2004**. It regulates at the national level the competence and tasks of state administration bodies for carrying out official food controls in practice following the procedures in the European Union valid from 1 January 2006. The aim of implementing Council **Regulation (EC) No. 882/2004 (EU, 2004b)** is to harmonize the performance of official food controls in the Slovak Republic. with the harmonized framework of general rules for the organization of inspections in the European Community.

**Act No. 318/2009 Coll. SR (NRSR, 2009), which amends the Act of the National Council of the Slovak Republic No. 270/1995 Coll. SR (NRSR, 1995b) on the state language of the Slovak Republic, as amended, and on amendments to certain laws**

In § 9 part 1, the words "in the state language" shall be replaced by the words "in the codified form of the state language". The aim of Act no. 318/2009 Coll. on the state language was to clearly define the position of the Slovak language in public life and to define the extent of the use of the Slovak language in public relations, but it also guarantees its protection and ensures its care.

**Act No. 114/2010 Coll. SR (NRSR, 2010) which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR on foodstuffs as amended and on**

**the amendment of Act No. 44/1988 Coll. ČSFR (ČSFR, 1988) on the protection and use of mineral wealth (the Mining Act), as amended**

The purpose of Act no. 114/2010 Coll. SR, it has been stricter to sanction those who do not comply with legal obligations by official food control authorities. Subsequently, because of these facts, it is desirable to legally establish the requirement of informing the public in the sense that the Ministry of Agriculture of the Slovak Republic and the Ministry of Health of the Slovak Republic within their competencies under this Act will inform the public about unsafe food, the risk to human health and the measures taken.

**Act No. 349/2011 Coll. SR (NRSR, 2011), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR on foodstuffs as amended**

The Ministry of Agriculture and Rural Development of the Slovak Republic prepared the above-mentioned novelization, especially regarding the significant dynamics in the adoption of legislation and its changes in the food area. When drafting the bill, the need for change or additions to the relevant provisions of Act no. 152/1995 Coll., In the implementation of which the bodies of official food control have encountered certain problems since the entry into force of the last amendment to the Act.

Among other things, the amendment amended the area of fines and other administrative offenses in a new way, introduced an information obligation for food business operators to place the results of inspections on a notice board, and deleted provisions that are already regulated by other directly applicable legal acts of the European Union. The subject of an amendment to Act no. 152/1995 Coll. SR. It was necessary to change the subject of the regulation and bring it in line with the current situation, given that a large area of food safety and quality is addressed by the currently directly applicable legal acts of the European Union. Terms that are governed by directly applicable EU acts are deleted. The regulation of tobacco products will be addressed comprehensively by the Tobacco Products Act.

Following the new concept, details on food safety, production, handling, and placing on the market will be regulated by regulations of the Government of the Slovak Republic, which will gradually repeal the Food Code. Health safety has also been regulated by a directly applicable legal act such as food safety.

Regarding the purpose of the submitted amendment, internet sales have also been added. § 6 paragraph 3 stipulates which foods the food business operator may place on the market, following directly applicable legal acts of the European Union. In § 6, a new paragraph 4 is inserted, which sets out the obligations of entities when placing food or nutritional supplements on the market through internet sales.

Established the early warning system and risk analysis. The Rapid Alert System for Food and Feed (RASFF) provides food and feed control authorities with an effective tool to exchange information about measures taken to respond to serious risks detected concerning food or feed **(EC - DG Health and Consumers, 2021)**. The new requirements in § 7 set out the obligations of the consignee of a food consignment and the consignee of a consignment of materials and articles intended to come into contact with food. Clarification of competencies and information towards the European Commission. It has been established which bodies carry out food controls within the scope of their competence.

The establishment of competencies of the Ministry of Agriculture and Rural Development of the Slovak Republic and the Ministry of Health of the Slovak Republic in the area of management and performance control the performance of state administration by official food control bodies, paragraph 2 specifies individual activities of the Ministry of Agriculture and Rural Development of the Slovak Republic and paragraph 3 specifies the activities of The Ministry of Health of the Slovak Republic. Both Ministry appoints one or more cooperating bodies, which cooperate with the authorities of other Member States as necessary. The role of the cooperating authorities is to assist and cooperate in the communication between the competent authorities, by sending and receiving requests for assistance. The Ministry of Agriculture and Rural Development of the Slovak Republic notifies the cooperating bodies to the European Commission and informs the European Commission and the other Member States of all relevant detailed data on the designated cooperating bodies and any changes to these data.

Regarding the aim of the amendment, a provision is added, the power of official control inspectors to perform control in another territorial district is extended to increase the effectiveness of official control and consumer protection. It is specified that laboratories must be accredited.

A new § 29a is inserted, which refers to the annex, which contains a list of legally binding acts of the European Union **(URL 3, 2011)**.

**Act No. 459/2012 Coll. SR (NRSR, 2012a), which amends Act No. 362/2011 Coll. SR (NRSR, 2011) on Medicinal Products and Medical Devices and on Amendments to Certain Acts, as amended by Act No. 244/2012 Coll. SR (NRSR, 2012b) and amending certain laws**

The aim of Act No. 244/2012 Coll. SR was the strengthening of the supervision of medicinal products for human use, market surveillance and sanctions for non-compliance with the relevant provisions, for marketing authorization holders of medicinal products for human use. The amendment to the Act amended the existing provisions on the production of medicinal products for human use, wholesale distribution of medicinal products for human use, and dispensing of medicinal products for human use following the **European Parliament and Council Directive 2011/62/EU (EU, 2011b)** to prevent the penetration of counterfeit medicines in the legal supply chain drug.

In § 31aa part 1, the words "31st December 2012 "are replaced by" 31st December 2014 ". The provisions of the Food Code remain valid and effective until the adoption of generally binding legislation in the relevant areas until 31st December 2014 at the latest.

**Act No. 42/2013 Coll. SR (NRSR, 2013), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR on Foodstuffs, as amended, and which supplements and amends certain laws**

The law aimed to achieve a high level of protection of human health and consumer protection in the market through new measures aimed at preventing the re-

importation of unsafe food and food unfit for human consumption from the Member States. Among other things, the Act expands the competencies of employees of the official food control body, the rates of fines have been adjusted, and it introduces a sanction for the municipality in the event of non-fulfillment of obligations under this Act.

The changes affected the Accredited Training Program, the minimum requirements for education and training, and the requirements for acquiring, maintaining, and demonstrating the required competence to perform tasks related to animal procedures.

**Act No. 36/2014 Coll (NRSR, 2014a), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll Food, as amended, for DPIs**

The purpose of the changes was to supplement the power to issue a decree on the labeling of foodstuffs with voluntary information, such as composition and origin (Slovak food) and their use for promotional and marketing purposes.
Another goal was to introduce measures to facilitate and simplify the feasibility of providing information on the percentage of sales of food produced in the Slovak Republic by turnover on food from the total supply of food sold and publishing a list of operators with this percentage.

The draft of the law regulated some ambiguities and inaccuracies of the **Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR (NRSR, 1995a)** on foodstuffs, as amended, on the obligations of the operator and the Ministry in submitting information and publishing the list. According to the current § 12a, § 28, part 2 letter q) and r) and § 30a par. 2 of the Act, the following problems were identified in their application:

• according to § 12a part 1 of the Act, it is not clear exactly to whom the obligation to provide information applies,
• according to § 12a part 2 of the Act, it is not clear in what form the list should be published,
• according to § 28, part 2 letter q) of the Act, a fine for breach of duty under § 12a part 1 of the Act is imposed by the body of official food control, in this case, the State Veterinary and Food Administration of the Slovak Republic, which, however, is not authorized by law to control the accuracy of the information provided,
• due to the ambiguity of § 12a part 1 of the Act, the forms of processing the list and the forms of its publication were also unclear.

By adjusting the publication of the list only for own establishments together, the publication of the list of other operators is prevented. In this way, the final consumer will have a clearer overview of the share of sales of food produced in the Slovak Republic by turnover in food from the total supply of food sold, broken down by individual branches of the food industry, which operator. It specifies the method of reporting the supply of food from another EU Member State.

The authorization of the Ministry to issue a generally binding legal regulation setting out details on the possibility of labeling food and agricultural products with voluntary claims and their use for promotional and marketing purposes, as well as the scope of effectiveness to which food and agricultural products are applied, is supplemented.

The obligation of the operator to submit information only for the Slovak Republic (originally for the first five countries of origin) and the obligation to publish this information in a visible place at the entrance to the establishment intended for the final consumer on its website is regulated.

The operator who will be obliged to submit information to the Ministry according to this draft law is precisely defined. For example, e.g., operators, with their retail establishments, whose main activity is food production or exclusive food distributors, i.e., those operators whose main activity is not the sale of food to the final consumer, but the production, preparation, or serving food.

By publishing a model of the half-yearly report on which the operator will send information to the Ministry, the methodology for submitting information will be unified.

By publishing the list of operators on the Ministry's website, an overall overview of the share of food sales produced in the Slovak Republic according to turnover on food from the total supply of food sold and broken down by individual branches of the food industry of obligatory operators. The proposed legislation prevents the publication of the list of other operators by other operators when entering the establishment intended for the final consumer, thus achieving a clearer overview of the share of total sales of food produced in the Slovak Republic by turnover of food from the total supply of food sold and individual branches of the food industry, the operator of which.

The competence to perform official food control of the bodies of the state veterinary and food administration is extended to foods marked with voluntary data.

The Ministry is authorized to carry out an inspection, within which it will examine the accuracy, completeness, and veracity of the information submitted by the operator according to § 12a part 1 letter a). Competence is supplemented due to the need for retrospective control and verification of submitted data by obligated persons. The findings are then the basis for the possible imposition of a fine, if the information required by law has not been sent, or if data have been provided that do not correspond to the facts, resp. otherwise unsatisfactory.

A new factual basis is established, in the fulfillment of which it is possible to impose a fine in the range from 100 euros to 100,000 euros if the liable person does not entrust the half-year statement in the manner and at the time provided by law, as provided for in § 12a par. 1 letter b).

The number of fines for the second and further breach of obligations under § 28 part 4. The authority of the Ministry to impose a fine shall be extended if the liable person has not sent the required data to the Ministry in the form of a half-yearly report in the time and form required by law or has provided false information **(URL, 4, 2014)**.

**Act No. 101/2014 Coll. SR (NRSR, 2014b), which amends Act No. 178/1998 Coll. SR (NRSR, 1998) on the conditions of sale of products and provision of services on marketplaces**

Requirements for the use of flavorings in foods for infants and young children and certain requirements for food fortification may be laid down in generally binding legislation issued by the Ministry of Health.

**Act No. 30/2015 Coll. SR (NRSR, 2015), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll (NRSR, 1995a) on foodstuffs as amended**

Ministry of Agriculture and Rural Development of the Slovak Republic the Ministry of Health of the Slovak

Republic shall regulate the areas specified by a generally binding legal regulation.

Requirements for food safety and their quality, hygiene must be observed by everyone who produces, handles, and places food on the market.

An operator who packages foodstuffs in connection with their production, handling, or placing on the market, other than the primary producer, is obliged to have a professional or other legally qualified people in the field of food or veterinary medicine.

The amendment affected the production of sprouts. The term "internet sales" is replaced throughout the text of the law by the words "distance selling"

**Act No. 376/2016 Coll. SR (NRSR, 2016), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR (NRSR, 1995a) on foodstuffs as amended and on the amendment of Act No. 39/2007 Coll. SR (NRSR, 2007) on veterinary care as amended**

The amendment regulates the possibility of using food after the date of minimum durability within the global trend to reduce the loss of food and food waste, including the determination of responsibility for the safety of these foods. Furthermore, the amendment imposes an obligation on legal entities and natural persons – entrepreneurs who perform food analysis to report to the ŠVPS SR a finding that the food is harmful to health.

After the date of minimum durability, only if it is safe, the operator may transfer the food free of charge to a person performing a public benefit activity (Charity Organization) free of charge.

A producer of small quantities of food, which sells its products directly to the final consumer or delivers them to a retail establishment in the Slovak Republic, is not obliged to label its products with nutrition information or include this information in the accompanying documents. A producer of small quantities of food is a producer under special regulations or a producer who employs a maximum of 15 people who carry out food production in an employment relationship, a similar employment relationship, or another employment relationship and whose annual turnover does not exceed EUR 700,000.

**Act No. 91/2019 Coll. SR (NRSR, 2019a) on Unreasonable Conditions in the Food Trade and on Amendments to Certain Acts.**

The amendment aimed to clarify the current provisions governing unfair terms, to incorporate new provisions governing unfair terms based on knowledge from the development of business relations between food suppliers and food buyers and on experience from application practice, to introduce a general unfair terms clause, violation of which can be sanctioned. the controller's streamlining of control by introducing a separate control process, enabling the submission of anonymous complaints drawing attention to possible violations of the law, application of the provisions of the law even if the contractual relations of the participants in the business relationship are governed by other laws than the Slovak Republic.

A detailed definition of unfair terms was necessary to protect those parties to the business relationship to those whose unfair terms are required, agreed upon, or applied. Unreasonable conditions are the result of the abuse of economic power by one of the parties to the business relationship. They are manifested by requiring, agreeing, or applying unilaterally advantageous business conditions, most often in the form of various monetary benefits or non-monetary benefits of the supplier beyond the agreed purchase price or in the form of discounts on the purchase price, which clearly benefits the customer and conversely, significantly weakens the supplier. Abuse of a stronger position in the business relationship and practices in the form of imposing unfair terms can seriously damage the food supply chain as such, which can have a subsequent negative impact on the whole economy as well as final consumers and can pose a serious risk to the food sovereignty of the Slovak Republic. Business relations are significantly distorted and the so-called factor of fear that causes a party with a weaker bargaining position not to fully exercise its rights and, out of fear of jeopardizing trade relations, prefers to accept the often unilaterally favorable terms of a counterparty with greater bargaining power.

In recent years, the issue of unfair trading practices (translated from the term "unfair trading practices" used at the European level) has begun to receive increasing attention. Analyzes and surveys have shown that unfair commercial practices are common in the food supply chain. Most Member States of the European Union have assessed their existence and application in trade relations as a risk to the proper functioning of the food supply chain and have introduced or plan to introduce legislation to eliminate them.

The issue of unfair trade practices (as part of the complex issue of strengthening the position of farmers in the food supply chain) was one of the priorities of the Slovak Presidency of the Council of the European Union in the second half of 2016. at the European level **(URL 5, 2019)**.

**Act No. 303/2019 Coll. SR (NRSR, 2019b), which amends the Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR (NRSR, 1995a) on foodstuffs as amended**

The Ministry of Agriculture and Rural Development of the Slovak Republic submitted an amendment based on the requirements of the European Union and the requirements that emerged from practice. The amendment was necessary to bring the current legislation into line with European Union food law. This need for harmonization arose in particular following the adoption of the new **Regulation (EU) No. 2017/625 (EU, 2017)** of the European Parliament and of the Council of 15$^{th}$ March 2017 on official controls and other official activities performed to ensure the application of food and feed law and animal health and animal welfare rules, on plant health and plant protection products, amending **Regulation (EC) No. 1907/2006 (EU, 2006a)** of the European Parliament and of the **Council 999/2001 (EU, 2001a), (ES) No. 396/2005 (EU, 2005), (ES) No. 1069/2009 (EU, 2009a), (ES) No. 110/2008 (EU, 2008)**.

This regulation has become mandatory throughout the range and directly applicable in the Slovak Republic as well as in the other Member States of the European Union of 14$^{th}$ December 2019 but in response to its application in practice will require amendment of the Act particularly in the area of competence and the tasks of bodies of state administration on the section of official food control.

On the basis of **Regulation (EU) 2017/625 (EU, 2017)** the amendment to the law empowers state veterinary and food authorities and public health authorities to designate border inspection posts for official controls on food entering the EU from third countries while being entitled to suspend or cancel border inspection posts if they do not meet the minimum requirements set out in **Regulation (EU) 2017/625 (EU, 2017)**.

In contrast to the previous legislation, the amendment to the Act extended the competencies of the official food control bodies of the Ministry of Health of the Slovak Republic, namely in the area of official control of food of plant origin in the places specified in the **Regulation (EU) No. 2017/625 (EU, 2017)** and the field of official control of agricultural products and foodstuffs with a protected designation of origin, a protected geographical indication, an indication of a traditional specialty guaranteed, an indication of optional quality terms or a geographical indication spirit drink in mass caterers.

The amendment to the Act also makes some legislative and technical amendments concerning the compliance of the Act with the **Regulation of the European Parliament and of the Council (EU) No. 609/2013** from 12 june 2013 **(EU, 2013b)** on food intended for infants and young children, food for special medical purposes and the total diet replacement for weight control which repealing **Directive No. 92/52/EEC (EU, 1992a), Directive Commission No. 96/8/EC (EU, 1996)**, **No. 1999/21/EC (EU, 1999)**, **2006/125/EC (EU, 2006b)** and **2006/141/EC (EU, 2006c) Directive** of the European Parliament and Council **Directive 2009/39/EC (EU, 2009a)** and **Regulation Commission (EC) No. 41/2009 (EU, 2009b)** and **(ES) No. 953/2009 (EU, 2009c)** in force as amended, and the Regulation of the European Parliament and of the Council **(EU) 2015/2283 (EU, 2015)** of 25th November 2015 on novel foods, which was amended Regulation of the European Parliament and Council **Regulation (EU) No. 1169/2011 (EU, 2011a)**, which was repealed Regulation of the European Parliament and Council **Regulation (EC) No. 258/97 (EU, 1997)** and **Commission Regulation (EC) No. 1852/2001 (EU, 2001b)**.

The amendment also removes some of the existing shortcomings in the law, which resulted from the application practice of official food control authorities. The shortcomings concerned the notification of the arrival of consignments imported by food business operators from third countries, the method of entrusting accredited official laboratories to perform the analysis of samples taken during official food control, and issuing official certificates by official food control authorities to food business operators producing food in the Slovak Republic. food on the third-country market.

To ensure the legal certainty of the addressees of the legal norm and to fulfill the informative function of footnotes, the draft law regulates footnotes due more consistently to their updating and obsolete footnotes to references to which there are no longer relevant references in the law and which they have not yet been omitted from the law **(URL 6, 2019)**.

**Act No. 198/2020 Coll. SR (NRSR, 2020) amending certain laws concerning the improvement of the business environment affected by measures to prevent the spread of dangerous contagious human disease COVID-19**

The provision deleting the suspensory effect of an appeal against a decision imposing a fine has been deleted, thus rendering the decision imposing a fine enforceable after it has been served on the addressee and the fine has become due on the expiry of the parity period. Re-granting the suspensory effect of an appeal as an appeal is of irreplaceable importance for the entities affected by the decision of the administrative body, as until the end of the appeal proceedings these entities are not obliged to fulfill the obligation imposed in the decision, the so-called pay the fine imposed.

This legislation repealing the Act. **No. 478/2019 Coll. SR (NRSR, 2019c)**, Amending the Act of the National Council of the Slovak Republic **No. 152/1995 Coll. SR (NRSR, 1995a)** on foodstuffs, as amended, which was to enter into force on 1 January 2024. This amendment to the Food Act, which has entered into force but not yet in force, contains, following the explanatory memorandum, legislation that is contrary to the principle of legal certainty (Article 1 (1) of the Constitution of the Slovak Republic) and is significant interference with contractual freedom. of the food business operator resulting from the principle of contractual freedom, i.e. donation should always be a voluntary and gratuitous fulfillment of the gift, not an obligation, as provided for in the amendment to the Food Act. For this reason, it is proposed to repeal this amendment to the Food Act **(URL 7, 2020)**.

**Law No. 83/2021 Coll. SR (NRSR, 2021), which amends Act No. 469/2003 Coll. SR (NRSR, 2003b) on Designations of Origin and Geographical Indications of Products and on Amendments to Certain Acts, as amended, and amending Act of the National Council of the Slovak Republic No. 152/1995 Coll. SR (NRSR, 1995a) on foodstuffs as amended**

In § 9a par. 2, the word "technical documentation" is replaced by the word's "specification". Before applying for protection of a designation of origin or geographical indication, the specification of an agricultural product or foodstuff and the specification of spirits applying for registration of a geographical indication and established geographical indications of spirits must be professionally assessed and approved by the Ministry of Agriculture and Rural Development of the Slovak Republic.

## CONCLUSION

The Food Act is the broadest standard that legislates all procedures and processes in the food industry to guarantee food safety for the consumer. The Food Act can also be called a competent standard, as it establishes a supervisory authority and sets all the rules for food throughout the production process. Current changes also bring a lot of considerations. Despite the socially and ecologically welcome idea, allowing food to be sold after the minimum durability period raises the question of the degree of risk to the life and health of the consumer, depending on the use-by date and the date of minimum durability. A reflection on the price of such foods would also be appropriate. The very significant reduction in the price of the consumer already informs that the food is not in the normal mode.

Act No. 152/1995 also raises questions in the field of criminal law. The provisions contain disproportionate punishment in violation of the EC requirement. The legislator abolishes exclusively the "disproportionate"

lower limit of the fine, for EUR 1,000,000, which the supervisory authority imposed according to § 28 section 8 and replaces it with the amount of EUR 50,000. For the first violation of the law, it is possible to impose an order of magnitude higher fine than for its repeated violation.

As can be seen, the legislation in this area is not final and there is a presumption of dynamic development in the future.

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# THE INHIBITION OF WINE MICROORGANISMS BY SILVER NANOPARTICLES

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## ABSTRACT

The presented work aimed to study the inhibition using nanoparticles produced by the green synthesis in selected acetic acid and lactic acid bacteria, which are related to viticulture. The degree of ability to eliminate silver particles produced by green syntheses was determined using the plate method on Petri dishes. This is done using two different approaches - the method of direct application of the solution to the surface of the inoculated medium (determination of inhibition zones) and the method of application using nanoparticles to the inoculated medium. *Gluconobacter oxydans* (CCM 3618) and A*cetobacter aceti* (CCM 3620T) were studied from acet acetic bacteria. The lactic acid bacteria were *Lactobacillus brevis* (CCM 1815) and *Pediococcus damnosus* (CCM 2465). The application of silver nanoparticles was always in concentrations of 0, 0.0625, 0.125, 0.25, 0.5, and 1 $g.L^{-1}$. All applied concentrations of silver nanoparticles showed an inhibitory effect on the monitored microorganisms. Silver particles could be used in wine technology for their antibacterial effects, mainly to inhibit microorganisms during vinification, as a substitute for sulfur dioxide.

**Keywords:** acetic acid bacteria; lactic acid bacteria; inhibition; silver particles; green synthesis

## INTRODUCTION

Silver nanoparticles, or silver-based materials and preparations, are already well known for their proven antimicrobial activity and are currently commonly used in practice across various branches of human activity. Silver is effective in treating burns, severe chronic osteomyelitis, urinary tract infections, and central venous catheter infections **(Feng et al., 2000)**.

In recent years, they have also been the subject of research in viticulture, in connection with their possible use in the inhibition of undesirable microorganisms, and thus as a means of reducing the need for sulfur dioxide **(Moreno-Arribas and Sualdea, 2016)**.

Traditionally, silver nanoparticles have only been prepared by physical and chemical methods. Chemical synthesis often leads to toxic effects caused by the rest of the chemicals on the surface of the nanoparticles, which significantly limits their use for medical and food purposes **(Parashar, Saxena and Srivastava, 2009 )**. In recent years, silver nanoparticles have also been prepared using so-called green synthesis. The production of nanoparticles using green synthesis is a new, cheap, fast, and gentle method for synthetic production. Green synthesis can be used to modify the surface of the obtained silver nanoparticles by biomolecules from the extracts used **(Forough and Farhadi, 2010)**.

From the ranks of bacteria, in wine technology, we encounter mainly bacteria of acetic and lactic fermentation. Acetic acit bacteria, in contrast to lactic acid bacteria, are classified in enology primarily as undesirable representatives of microorganisms. However, lactic acid bacteria can also cause many wine diseases. Representatives of both mentioned groups of bacteria occur not only on grapes but also in wine-growing areas and on all wine-growing equipment, from where they can spread and subsequently contaminate the wine. It is therefore important to keep the winery clean at all times. The use of silver particles in winemaking technology could be one of the options for sanitation in the cellar, but also a possible use in the actual production of wine.

**Izquierdo-Cañas et al. (2012)** and **Ebelashvili et al. (2014)** focused on the possible influence of nanosilver in terms of significant analytical parameters of the resulting wine. They equally concluded that red wines treated with the tested silver particles and wines prepared with $SO_2$ did not show significant differences in this respect. The same was true of the white wines observed in the work of the first-mentioned **Izquierdo-Cañas et al. (2012)**.

The aim of this work is the production of nanoparticles by green synthesis using grape seed extract and the study of the inhibitory effect of these nanoparticles on bacterial cultures of acetic acid and lactic acid bacteria.

### Scientific hypothesis

Silver particles have antimicrobial properties, they are usually prepared by a synthetic route. The production of nanoparticles by green synthesis is a possible gentle alternative to the production of nanoparticles by chemical synthesis. A grape seed extract has high levels of antioxidant components. Its incorporation into the process of production of silver nanoparticles should increase the antimicrobial effects of silver nanoparticles. The possible use of nanoparticles prepared in this way is in the food industry, as they show lower toxicity. In viticulture, silver-based particles could be used to eliminate unwanted acetic acid and lactic acid bacteria.

## MATERIAL AND METHODOLOGY

### Production of grape seed extract

#### Samples

This extract was made from seeds of the Cerason variety. (Merlot × Seibel 13 666).

#### Chemicals

75% Ethyl alcohol (Lach-Ner, s.r.o, Czech Republic) was used for extraction.

#### Instruments

Grinder (IKA MF 10 basic, Merci, Czech Republic).

Centrifuge (Hettich MIKRO 220, Hettich, Czech Republic).

Lyophilizer (Heto PowerDry PL 3000, Trigon-plus, Czech Republic).

For vacuum evaporation was used IKA RV 10 digital V-C (IKA Werke, Staufen im Breisgau, Germany).

#### Laboratory Methods

The antioxidant activity was determined by the DPPH method, the content of polyphenolic compounds was determined by the Folin-Ciocalte method, and selected antioxidant components were determined by HPLC-UV/VIS.

The antioxidant activity value was 12.436 $g.L^{-1}$ gallic acid equivalent and the value of total polyphenolic compounds 8.568 $g.L^{-1}$ gallic acid equivalent. The values of the individual components are shown in Table 1 below.

**Table 1** Values of the contents of individual antioxidant components.

| Compound | Value ( $mg.L^{-1}$ ) |
|---|---|
| Gallic acid | 19.9 ±0.435 |
| Caffeic acid | 1.8 ±0.124 |
| Koutaric acid | 0.431 ±0.00602 |
| Ferulic acid | 0.26 ±0.0163 |
| Fertaric acid | 0.41 ±0.0135 |
| Catechin | 0.393 ±0.0126 |
| Epicatechin | 136.9 ±1.569 |
| Trans-piceid | 0.786 ±0.0568 |
| Trans-piceatannol | 0.0238 ±0.00165 |
| Rutin | 1.62 ±0.040 |
| QuercetinGlukoside | 0.428 ±0.0172 |
| Quercitrin | 0.135 ±0.00986 |
| Myricetin | 0.155 ±0.00650 |

#### Description of the Experiment

The seeds have undergone sorting, purification, subsequent drying, and grinding. Next, these seeds were dried in an oven at 55 °C for 12 hours. After drying and cooling to room temperature, the seeds were ground with a laboratory grinder. The seeds were then extracted in 75% ethanol for 120 hours in the following ratio: 10 parts of ethanol were added to 1 part of ground seeds. After complete extraction, the resulting sample was divided into 25 mL tubes. Subsequently, centrifugation was performed at 4 °C and RCF 25 000 x g. Subsequently, the sample was transferred to a distillation flask of a vacuum evaporator. In this step, the excess ethanol was evaporated and evaporated in vacuo at 90 °C. Such an extract was then transferred to Petri dishes and lyophilized at -55 °C.

### Production of nanoparticles using green synthesis

#### Chemicals

Distilled water.

99.8% Silver nitrate (ETC GmbH, Germany).

99.9% Methanol Honeywell, France)

#### Biological Material

From the acetic fermentation bacteria, one representative of the acetic fermentation from the genus *Gluconobacter* and one from the genus *Acetobacter* was selected for experimental work. The first species was *Gluconobacter oxydans* CCM 3618 and the second *Acetobacter aceti* CCM $3620^{T}$.

In the case of lactic acid bacteria, there was one heterofermentative representative of the genus *Lactobacillus* and one homofermentative representative of the genus *Pediococcus*. The first bacterium was *Lactobacillus brevis* CCM 1815 and the second *Pediococcus damnosus* CCM 2465.

All the mentioned species of bacteria come from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University in Brno.

#### Instruments

Magnetic stirrer with heating (STAR LAB, United Kingdom).

Centrifuge Hettich MIKRO 220, (Hettich, Czech Republic).

#### Description of the Experiment

The next step was to homogenize the seeds extract to a fine powder in a mortar. The material thus prepared was used to prepare the extract itself. This material was then mixed with distilled water at 80 °C. This extraction was carried out with stirring for 24 hours. A 0.1 M solution of silver nitrate was added to the filtered and cooled extract in a ratio of 1:1. Subsequently, the solution prepared in this way was stirred on a magnetic stirrer with heating at room temperature (22 °C) for 24 hours. During the formation of the silver particles, the color gradually changed from a solution from bright red to the resulting gray-green solution.

These particles were precipitated in a 1:1 ratio (precipitant and AgNPs solution) with the aid of an organic solvent - methanol. The solution was precipitated for 10 minutes with stirring. The next step was centrifugation for 30 minutes, 14,000 g and at 5 °C. After centrifugation, the tubes were removed from the apparatus, in which the nanoparticles were already visibly precipitated and deposited. Next, the supernatant was pipetted very carefully and the open tubes were dried at 60 °C for 24 hours. Already dried and cooled silver particles were weighed and diluted with distilled water to a weight concentration of 1 $g.L^{-1}$. The tube with the resulting suspension had to be homogenized in ultrasound

three times for 15 minutes at room temperature because this homogenization increases the surface area of the produced particles and thus also increases the antibacterial activity of the product.

### Application of test substance to the inoculated nutrient medium

**Chemicals**

Homogenized silver particles.
Distilled water.
Mueller-Hinton agar (HiMedia Laboratories, USA).

**Description of the Experiment**

10 μL of a solution of homogenized silver particles and 10 μL of distilled water as control were applied to the inoculated medium. Five different concentrations (the sixth was a control variant with distilled water) of silver particles were applied to one petri dish, namely 0, 0.0625, 0.125, 0.25, 0.5, and 1 $g.L^{-1}$ . After 48 hours, the Petri dishes were removed from the incubators and the resulting inhibition zones were measured separately, to the nearest 1 mm. All experiments were performed in three independent replicates, from which the average inhibition zones were then calculated.

### Application of the test substance directly to the inoculum

**Chemicals**

Homogenized silver particles.
Distilled water.
Mueller-Hinton agar (HiMedia Laboratories, USA).

**Description of the Experiment**

By applying the solution directly to the inoculum, the inhibitory properties of various homogenized concentrations of silver particles were tested, with concentrations of 0, 0.0625, 0.125, 0.25, 0.5, and 1 $g.L^{-1}$. First, a suspension of a broth mixture (250 μL) with bacteria with a degree of turbidity of 1 McF and a concentration of silver particles (50 μL) was prepared. After the preparation of these suspensions, this inoculum was applied to the surface of the culture medium. The sixth suspension was a control, replacing 50 μL of the homogenized particle solution with 50 μL of distilled water. The inoculated Petri dishes were again left in the laminar box for 10 minutes to allow the inoculum to be completely absorbed into the culture medium. Subsequently, these samples were transferred to an incubator for 48 hours. After 48 hours, the percentage of the inhibited area was determined, when the entire area of the Petri dish was divided into identically large divisions. Subsequently, the sections on which bacterial growth was inhibited were counted, and the ratio of inhibition to total area was determined.

**Sample preparation:**

### Inoculation of microorganisms

**Chemicals**

Nutrient Broth No. 1 (SIGMA ALDRICH, Germany).

**Description of the Experiment**

The bacterial culture in the form of gelatin disks was first transferred with a sterile needle to an Erlenmeyer flask together with sterile Nutrient Broth No. 1, all flasks being covered with a special laboratory foil – PARAFILM® M (SIGMA ALDRICH, Germany). The flasks covered in this way were placed in a shaker incubator. This cultivation was carried out for 24 hours at temperatures. Subsequently, the bacterial suspension was diluted with sterile Nutrient Broth No. 1 with a degree of turbidity of 1 McF. Next, 100 μL of the bacterial suspension prepared in this way was pipetted and spread with a sterile glass loop on the prepared culture medium in a disposable petri dish. After 10 minutes, when all the inoculum was absorbed into the culture medium, subsequent application of silver particles with concentrations of 0, 0.0625, 0.125, 0.25, 0.5, and 1 $g.L^{-1}$ took place.

**Number of samples analyzed:**

During the course of the research, a total of four species of wine bacteria were analyzed for susceptibility to the test substance.

**Number of repeated analyses:**

A total of two methods were selected for this research to test the sensitivity of the selected bacteria to the test substance. These were the Application of the test substance to the inoculated nutrient medium and the Application of the test substance directly to the inoculum

**Number of experiment replication:**

Each method for testing the sensitivity of a given bacteria to the test substance was repeated three times. From the results, averages were calculated and used for this research. The results were also statistically processed.

### Statistical Analysis

An analysis of variance was performed (single–factor ANOVA), which showed significant differences in antibacterial effects between given concentrations on selected species of microorganisms. Statistical results are significant at $p = 0.05$. Subsequently, a post-hoc test (Tukey HSD test) was performed for each concentration. All statistical evaluation is available from the author.

## RESULTS AND DISCUSSION

The standard diffusion disc method was first and foremost tested for testing silver particles, but this method of application appeared to be highly inappropriate as the particles were not released from the disk into the agar. After that, the solution was tested directly into the medium, unfortunately, the automatic pipette failed to exert a strong enough pressure to inject the test substance into the rigid agar. The syringe with the needle was able to inject the solution into the medium, but soon after the needle was ed, all the test substances flowed uncontrollably to the surface of the agar. Also, injection with a needle did not allow a sufficiently accurate measurement of the applied volume. Finally, two methods were chosen: A) the method of direct application of the solution to the surface of the inoculated medium (determination of inhibition zones) and B) the method of application of the solution to the inoculated medium.

This chapter is therefore divided into two parts. Thanks to two different methods, it was possible to better interpret the results of the microorganisms studied.

### Inhibition of bacterial growth by application of test substance to the inoculated nutrient medium

This method is characterized by sufficient diffusion of the sample with agar. Many optimizations were carried out, with the greatest stability after several tests, due to the highest surface tension, a drop of 10 μL. The applied

concentrations and sizes of inhibition zones are given in table 2 below, the average size of the zone of inhibition being rounded to one decimal place.

In Figure 1 we can see the inhibition zones produced by different concentrations of silver particles, where 0 $g.L^{-1}$ is marked as F, 0.0625 $g.L^{-1}$ as E, 0.125 $g.L^{-1}$ as D, 0.25 $g.L^{-1}$ as C, 0.5 $g.L^{-1}$ as B, and 1 $g.L^{-1}$ as A. The same concentrations were applied to all bacteria. The zones were used to demonstrate the inhibitory capabilities of the silver particles tested against all the bacteria studied, as even a concentration of 0.125 $g.L^{-1}$ was always able to limit the growth of bacteria.

At lower concentrations (less than 0.25 $g.L^{-1}$), approximately half of the experiments experienced a slight increase in resistant colonies within the range of the applied substance. In lactic bacteria, in cases of testing of silver particles diluted to a mass concentration of 0.0625 $g.L^{-1}$ , almost no inhibition occurred and, if so, the inhibited area has always been increased by resistant colonies. From this point of view, we can say that the concentration of 0.0625 $g.L^{-1}$ appears insufficient to limit the growth of lactic acid bacteria. Acetic bacteria at a concession of 0.0625 $g.L^{-1}$ had already been colony-wide, but the inhibition zone values were embroidered (2.7 mm for *G. oxydans* and 3.7 mm for *A. aceti).*

In Figure 2 we can see the size of the inhibition zones formed after application of the homogenized silver particles, which were diluted to a mass concentration of 1 $g.L^{-1}$ measured in representatives of the acetic acid species *Acetobacter aceti*. In this bacterium, the size of the inhibition zones ranges from 16.2 mm to 19.2 mm. In contrast, the smallest growth restriction was observed in *Glukonobacter oxydans*. In this bacterium, the size of the measured inhibition zones ranged from 10.5 mm to 18.2 mm.

In Figure 3 we can see the sizes of the inhibition zones formed after the application of the homogenized silver particles, which were diluted to a mass concentration of 0.5 $g.L^{-1}$ . vinegar bacteria of the species *Acetobacter aceti*. In this bacterium, the size of the inhibition zones ranged from 12.3 mm to 17.5 mm. In contrast, the smallest growth restriction was observed in *Glukonobacter oxydans*. In this bacterium, the size of the measured inhibition zones ranged from 7.5 mm to 13.1 mm.

Figure 4 shows the sizes of the inhibition zones formed after the application of the homogenized silver particles, which were diluted to a mass concentration of 0.25 $g.L^{-1}$. representatives of acetic bacteria of the species *Acetobacter aceti*. In this bacterium, the size of the inhibition zones ranged from 7.8 mm to 10.8 mm. On the contrary, the smallest growth restriction was recorded this time in the lactic acid bacterium *Lactobacillus brevis*. In this bacterium, the size of the inhibition zones ranged from 5.9 mm to 8.8 mm.

In Figure 5 we can see the sizes of the inhibition zones formed after the application of homogenized silver particles, which were diluted to a mass concentration of 0.125 $g.L^{-1}$. All representatives of lactic and acetic fermentation bacteria showed some sensitivity to this lower concentration of the applied substance, while the largest inhibited areas were also measured in the representative of acetic bacteria of the species *Acetobacter aceti*. In this bacterium, the sizes of the inhibition zones ranged from 4.9 mm to 7.8 mm. On the contrary, the smallest growth restriction was recorded in this case in the lactic acid bacterium *Pediococcus damnosus*. In this bacterium, the sizes of the inhibition zones ranged from 3.2 mm to 6.1 mm.

Figure 6 shows the sizes of the inhibition zones formed after the application of homogenized silver particles, which were diluted to a mass concentration of 0.0625 $g.L^{-1}$. In some cases, all representatives of lactic and acetic fermentation bacteria showed some sensitivity to this lower concentration of the applied substance, while the largest inhibited areas were also measured in the representative of acetic bacteria of the species *Acetobacter aceti*. In this bacterium, the sizes of the inhibition zones ranged from 2.2 mm to 5.1 mm. On the contrary, the smallest growth restriction was recorded this time in the lactic acid bacterium *Lactobacillus brevis*. However, there was no inhibition of lactic acid bacteria in about half of the testing. These were the *Lactobacillus brevis* and *Pediococcus damnosus* species. In these bacteria, the sizes of the inhibition zones ranged from 0 mm to 6.3 mm.

Distilled water was used as a control and showed no inhibitory properties in either case.

## Growth inhibition by applying nanoparticles directly to the inoculum

The antimicrobial properties of silver particles against both lactic and acetic fermentation bacteria have also been confirmed by this method. The results of the application of different concentrations of homogenized silver nanoparticles are given in Table 3 below.

In Figure 7 we can see the inhibitory effects of different concentrations of the tested silver particles against individual bacteria by applying the test substance directly into the inoculum. *L. brevis* exhibits a high sensitivity to high concentrations of the applied substance in this method. At the highest concentration (1 $g.L^{-1}$), the inhibited area was 95%. When this concentration was reduced to 0.5 $g.L^{-1}$, there was a slight increase in lactic acid bacteria, which is also true for a concentration of 0.25 $g.L^{-1}$. Although *P. damnosus* exhibits some sensitivity to the test substance, this method of application of silver particles directly into the inoculate in the bacterium does not show as much inhibition as in *L. brevis*. At a high concentration of the applied substance (1 $g.L^{-1}$), growth was limited (44%), but this was only about half the inhibition of previous bacteria. When these particles were diluted to 0.125 $g.L^{-1}$, the inhibited area was only around 10%. The concentration of 0.0625 $g.L^{-1}$ no longer showed any ability to limit the growth of these lactic acid bacteria. *G. oxydans* show considerable sensitivity to high concentrations of the applied substance in this method. At the highest concentration (1 $g.L^{-1}$), the inhibited area was 92 %. By reducing this concentration to 0.5 $g.L^{-1}$, the number of colonies increased proportionally to 46%. At further dilution, the inhibited area decreased considerably, with a concentration of 0.625 $g.L^{-1}$ only to a small extent (15%) inhibited. *A. aceti* also exhibits considerable sensitivity to high concentrations of the applied substance in this method. At the highest concentration (1 $g.L^{-1}$), the inhibited area was 94%. When this concentration was reduced to 0.5 $g.L^{-1}$, there was no significant increase in acetic fermentation bacteria, such as *G. oxydans.*

**Table 2** Size of inhibition zones (expressed in mm) resulting from different mass concentrations of silver nanoparticles in the *L. brevis*, *P. damnosus*, *G. oxydans*, and *A. aceti* bacteria studied.

| Concentration ( g.L$^{-1}$) | *L. Brevis* | *P. damnosus* | *G. oxydans* | *A. aceti* |
|---|---|---|---|---|
| **1** | 15.3 ±1.3 | 13 ±1 | 14.3 ±1.7 | 17.7 ±0.7 |
| **0.5** | 10.7 ±1.3 | 12 ±1 | 10.3 ±1.3 | 13 ±1 |
| **0.25** | 7.3 ±0.7 | 7.7 ±1.3 | 8 ±1 | 9.3 ±0.7 |
| **0.125** | 5 ±1 | 4.7 ±0.7 | 5.7 ±0.7 | 6.3 ±0.7 |
| **0.0625** | 2 ±2 | 2 ±2 | 2.7 ±0.7 | 3.7 ±0.7 |
| **0 (Dest. $H_20$)** | 0 ±0 | 0 ±0 | 0 ±0 | 0 ±0 |

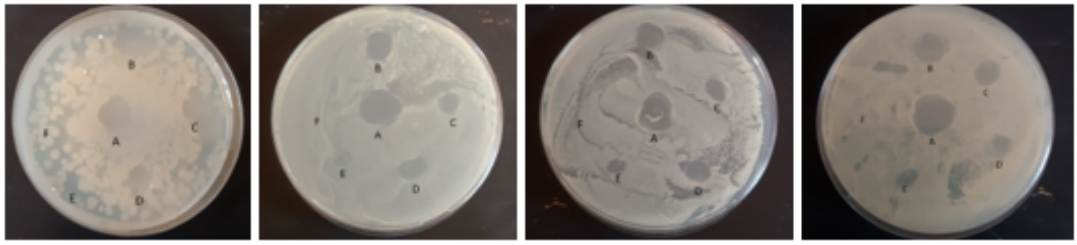

**Figure 1** Photographs of inhibition of micro-organisms by silver nanoparticles at concentrations 0 (F), 0.0625 (E), 0.125 (D), 0.25 (C), 0.5 (B), and 1 g.L$^{-1}$ (A) in micro-organisms (sorted from left to right) *L. brevis*, *P. damnosus*, *G. oxydans* and *A. aceti*.

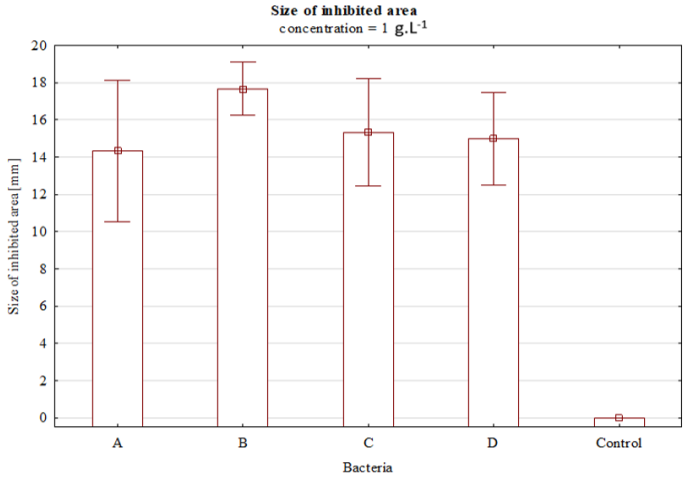


**Figure 2** Statistical expression of the measured values of the test substance diluted to 1 g.L$^{-1}$.

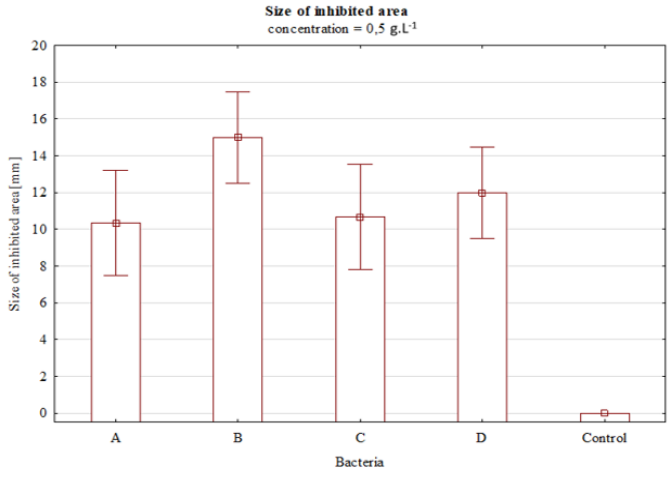


**Figure 3** Statistical expression of the measured values of the test substance diluted to 0.5 g.L$^{-1}$.

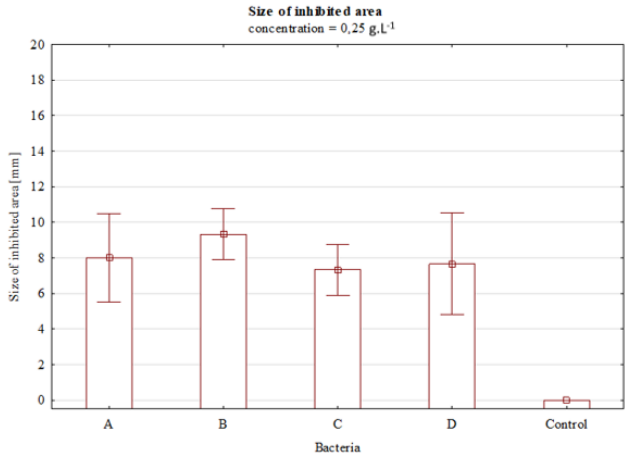


**Figure 4** Statistical expression of the measured values of the test substance diluted to 0.25 g.L$^{-1}$.

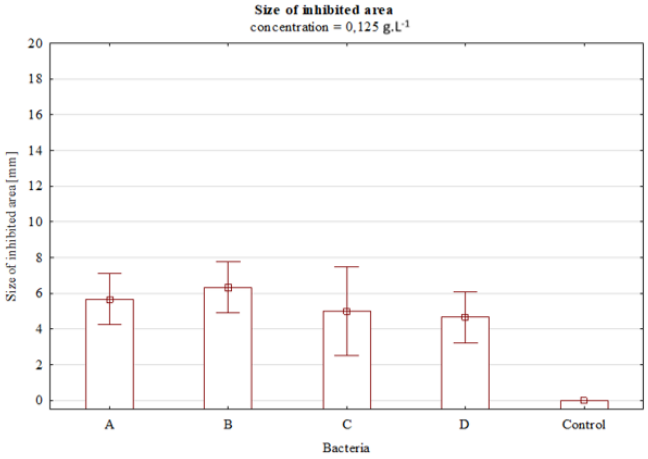


**Figure 5** Statistical expression of the measured values of the test substance diluted to 0.125 g.L$^{-1}$.

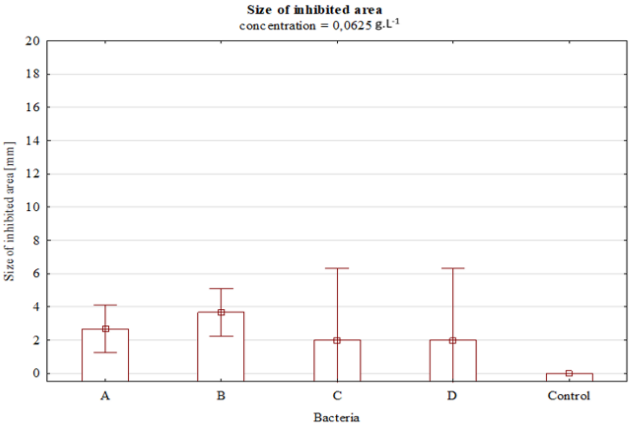


**Figure 6** Statistical expression of the measured values of the test substance diluted to 0.0625 g.L$^{-1}$.

**Table 3** Size of inhibited area (expressed in %) concentrations of silver particles in the *L. brevis*, *P. damnosus*, *G. oxydans*, and *A. aceti* bacteria studied.

| **Concentration (g.L$^{-1}$ )** | ***L. brevis*** | ***P. damnosus*** | ***G. oxydans*** | ***A. aceti*** |
|---|---|---|---|---|
| **1** | 95% | 44% | 92% | 94% |
| **0.5** | 74% | 28% | 46% | 89% |
| **0.25** | 61% | 18% | 41% | 43% |
| **0.125** | 41% | 12% | 32% | 40% |
| **0.0625** | 8% | 0% | 15% | 10% |
| **0 (Dest. $H_20$)** | 0% | 0% | 0% | 0% |

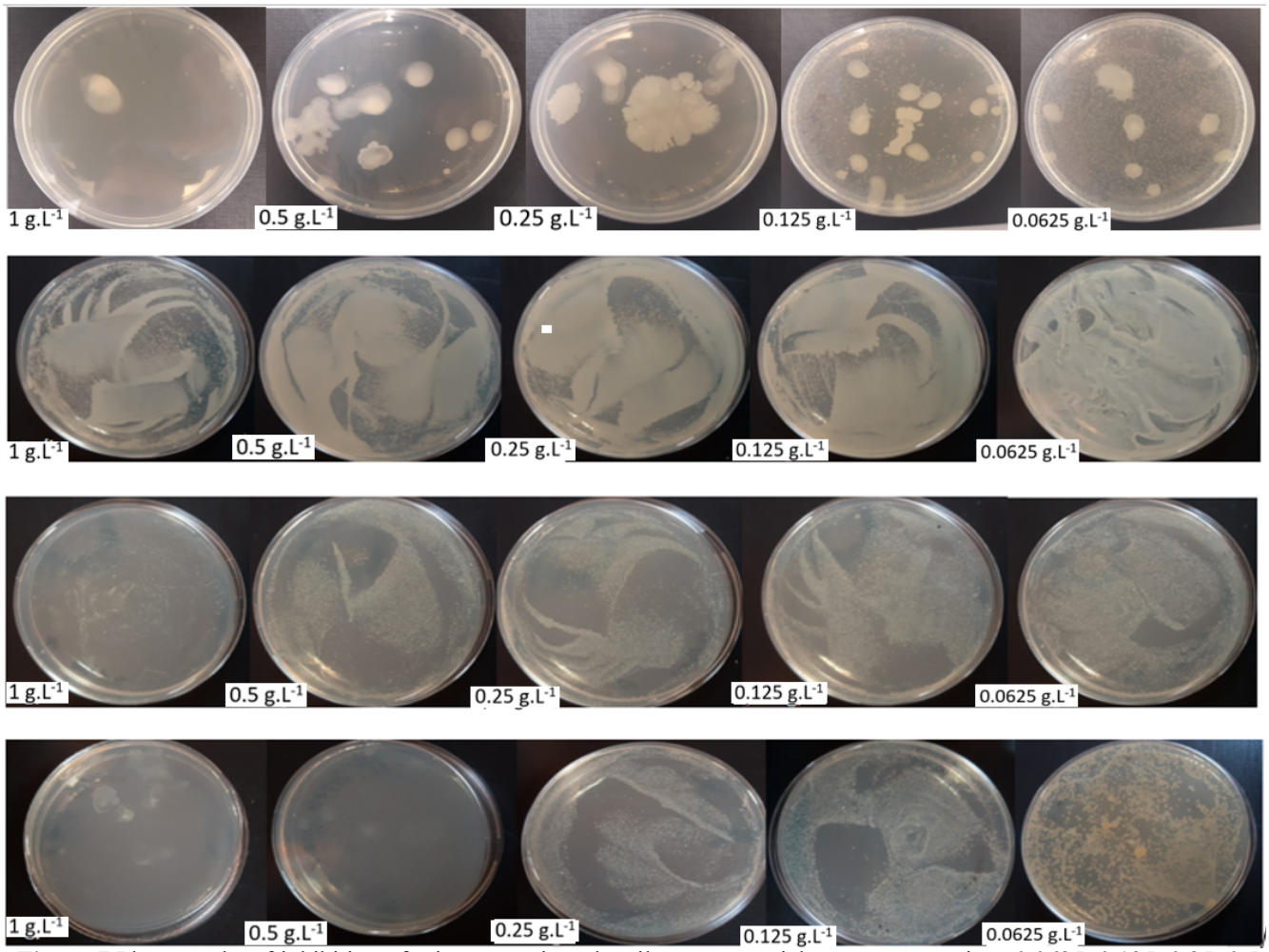


**Figure 7** Photographs of inhibition of micro-organisms by silver nanoparticles at concentrations 0.0625, 0.125, 0.25, 0.5, and 1 g.L$^{-1}$ in micro-organisms (sorted from top to bottom) *L. brevis*, *P. damnosus*, *G. oxydans*, and *A. aceti*.

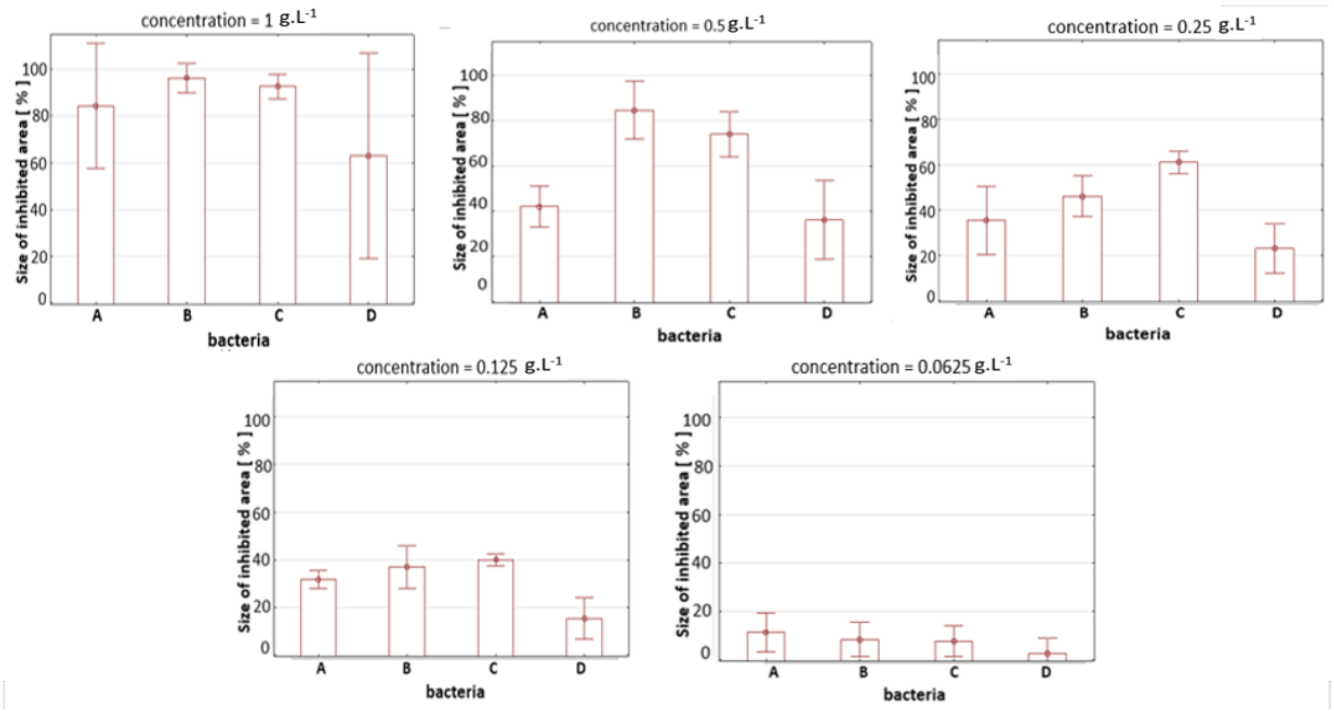


**Figure 8** Growth inhibition by applying nanoparticles directly to the inoculum. The following graphs show how decreasing concentrations of silver particles affect the growth of test bacteria. A= *Gluconobacter oxydans*, B= *Acetobacter aceti*, C = *Lactobacillus brevis*, D = *Pediococcus damnosus.*

However, with further dilution, the inhibited area decreased considerably. At a concentration of 0.25 $g.L^{-1}$, the size of this area was only 43%. When the applied substance was diluted to 0.0625 $g.L^{-1}$, growth was limited only slightly.

In Figure 8 we can see the inhibitory abilities of homogenized silver particles in all bacteria tested. In this method, the acetic acid species *Acetobacter aceti* showed the highest sensitivity to the test substance, and, conversely, the growth of the lactic acid bacterium *Lactobacillus brevis* was least restricted in this way.

Distilled water was used as a control in all experiments and showed no inhibitory properties against the monitored bacterial strains.

The antimicrobial effects of silver have been known for thousands of years. In ancient times, silver containers were used to store food. Silver coins were inserted into the milk to extend the shelf life of the product. Even the use of silver cutlery was supposed to lead to disease prevention. During the 15th century, bowls of silver powder appeared on the tables of privileged families, and a small amount of ingested powder was supposed to lead to a lower probability of infection with various diseases, which led to the coloration of the blood turning gray-blue. This is the source of the so-called blue blood. Today, silver, due to its properties, is used in many fields **(Večeřová, 2016)**.

Antibacterial activity of silver particles has been shown in several types of research, but it is difficult to compare their results, since there are not yet standard protocols for evaluating the antimicrobial activity of silver particles, and there is also the problem that scientists use different methods to determine the antimicrobial activity of silver particles **(Zarei, Jamnejad and Khajehali, 2014)**.

A very important aspect that determines the degree of antibacterial properties is the resulting size of the particles produced, where we attribute better inhibitory properties to smaller units of particles **(Espinosa-Cristóbal et al., 2009)**.

The antimicrobial action of silver particles, specifically in the form of a commercially available 1% colloidal silver preparation, was also tested by **Garde-Cerdán et al. (2014)**. They investigated the effects of this substance both during vinification and subsequently during wine storage. They then concluded the experiment by also confirming the sufficient antibacterial effects of silver. Wines treated with the product did not show any of the possible signs of undesirable microbial activity **(Garde-Cerdán et al., 2014; Moreno-Arribas and Sualdea, 2016)**.

**García-Ruíz et al. (2015)** researched the sensitivity of different types of acetic and milk bacteria to silver nanoparticles. In this research, antibacterial abilities were tested on two types of acetic bacteria and a total of eighteen strains of *Lactobacillus plantarum, Lactobacillus casei, Pediococcus pentosaceus, and Oenococcus oeni.* Of the acetic bacteria, it was *a* species of *Acetobacter aceti and Gluconobacter oxydans*. Identical representatives of acetic bacteria were chosen for research in this work as well. The results of the research demonstrated the good inhibitory abilities of silver nanoparticles to selected representatives of acetic and milk bacteria. The effect of silver nanoparticles was to some extent comparable to the antimicrobial effect of a commonly used device, potassium dissimite.

Silver particles can largely limit the growth of bacteria in wine, which could certainly lead to an overall reduction in the sulfur dioxide dosage in wine production. However, the facts about silver particles used in this way in the actual production of wine are very important. Through these conditions, we can mention the study of **Ebelashvili et al. (2014)** which carried out research involving precisely testing the inhibitory properties of silver particles against acet acetic and lactic aceti bacteria in the production of red wine. According to its findings, the rate of growth limitation in both acetic and lactic acid bacteria was substantially comparable to that of sulfur dioxide. This work also provided foran appropriate dose of silver particles at different stages of red wine production, with a dose of 0.4 $mg.L^{-1}$ silver particles are recommended in this study before the start of alcohol fermentation and a dose of 0.6 $mg.L^{-1}$ should be administered after fermentation **(Ebelashvili et al., 2014)**.

From earlier research, we can also mention the extensive work of Izquierdo-Cañas and Co. from 2012, which also examined the possibilities of replacing sulfur dioxide with silver particles in the production of red and white wines. The results of this work largely coincide with the previous work of **Ebelashvili et al. (2014)**. In both types of research, they concluded that in the case of red wines, there were no significant differences in the wine into which silver particles were applied compared to wines treated with the classical method using sulfur dioxide, both from an organoleptic and analytical point of view. There was a slight difference in results for white wines, as wines treated only with silver particles had higher wine oxidation, which is understandable because sulfur dioxide acts as an antioxidant in wine **(Izquierdo-Cañas et al., 2012; Ebelashvili et al., 2014)**.

**Gil-Sánchez et al. (2016)** also found in their work that silver nanoparticles have strong inhibitory properties against lactic acid bacteria. Their experiment was conducted in 50 ml samples of Airén wine variety. All samples treated with the tested silver nanoparticles showed complete inhibition of the original lactic acid bacteria populations, while the control (untreated sample) contained the same amount of bacteria as at the start of the experiment. It is also important to note to mention that the applied nanoparticles were able to inhibit the growth of the bacteria studied even slightly more effectively and faster than sulphur dioxide.

From a laboratory point of view, we can compare the results of this work with the **El-Fadly et al. (2016)** study, which concluded that G – bacteria, as acetic bacteria, show greater sensitivity to silver particles than G + bacteria, or lactic acid bacteria. This finding is to some extent consistent with the results of this thesis, as it was found through the disc diffusion method that silver particles were able to inhibit acetic bacteria to a greater extent than lactic acid bacteria.

But there are also studies **(Devi and Bhimba, 2014)** that show the opposite effect, i.e. a stronger inhibitory effect against gram-positive bacteria, or at least identical or not so different **(Shoo-Hwan et al., 2011)**. Thus, the findings are consistent with the fact that the exact mechanism of inhibitory effects against specific genera, species, or strains of bacteria is still not fully understood and very likely varies within different bacterial groups.

Through the above-mentioned results of two types of research, we could conclude that the treatment of wines with silver particles is more suitable for red wines since these wines are not so much susceptible to higher oxygen content.

Many studies have shown the antimicrobial activity of silver particles against various species of pathogens such as bacteria, viruses, fungi, yeast, etc. **(Panyala, Peña-Méndez and Havel, 2008)**.

A study by **Yoon et al. (2008)** shows the bactericidal effects of silver particles on two representatives of bacteria. These were *Escherichia coli* and *Bacillus subtilis*. These bacteria also showed some sensitivity to silver particles. In this study, it was found that $G^+$ bacteria Bacillus subtilis had a higher sensitivity to silver particles than $G^-$ Escherichia coli. These results are mild contradict the results in this presented work. On the contrary, we found a greater sensitivity of $G^-$ acetic acid bacteria to silver particles than in $G^+$ lactic acid bacteria **(Yoon et al., 2008)**. These data suggest that it is still not the exact mechanism of the antibacterial effect of silver particles against various species of bacteria.

An interesting finding regarding the use of antimicrobial effects comes from the study of **El-Rafie et al. (2010)**, where silver particles were applied to various types of fabrics. Was determined the efficacy and durability of the inhibitory effects of silver particles against *Staphylococcus aureus* and *Escherichia coli*. In this study, a high reduction in the number of both bacteria was found on fabrics treated with silver particles compared to ordinary fabrics. The high ability to reduce the growth of both bacteria was demonstrated even after several works cycles **(El-Rafie et al., 2010)**.

The issue is the consumption of silver particles in the wine itself. It has been shown that the topical and total use of colloidal silver can lead to adverse effects in this respect in the form of allergic reactions in sensitive individuals, or even irreversible grey-blue discoloration of the skin and eyes (so-called argyria) due to the deposition of silver and its salts in the body's tissues. Therefore, the internal use of colloidal silver has been banned within the EU since 1. January 2010. However, due to the low concentrations required to inhibit bacteria, it is currently the subject of intensive research to remove this barrier **(Večeřová, 2016)**. The possible future application of silver in viticulture naturally depends on it.

## CONCLUSION

The results show that silver particles can eliminate to a sufficient extent both genera of the bacteria studied. The plot method found that acetic fermentation bacteria showed slightly higher sensitivity to the silver particles tested than dairy fermentation bacteria. However, even a low concentration of silver particles (0.125 $g.L^{-1}$) has always been able to limit the growth of all kinds of bacteria tested in the case of the plate method. Silver particles could be used in wine technology to inhibit microorganisms during vinification. Further use of these particles could be the surface treatment of materials in wine technology, which would simplify sanitary work during wine production.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This article does not contain any studies that would require an ethical statement.

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# DEVELOPMENT OF A TWO-LEVEL CONTROL SYSTEM FOR THE ANALYSIS OF THE COMPOSITION OF MEAT PRODUCTS

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## ABSTRACT

Because of the increased demand for processed meat, there is an urgent need to introduce specific identification methods. Strategies such as molecular genetics and the physical condition of meat are used to quickly explore multi-component products. However, a single methodology does not always unambiguously classify a product as counterfeit. In laboratory practice, as a rule, screening techniques are rarely used in the first stage, followed by arbitration. This work aimed to study individual methodologies using artificially falsified meat samples as examples and to identify their composition based on muscle tissue. For the experiments, the three most common types of raw meat were selected: pork, beef, and chicken. The calculation of the content of muscle tissue was carried out according to the BEFFE method. The study of muscle protein was carried out by ICA, ELISA, PCR, microstructural analysis, and mass spectrometric identification. In this connection, we proposed a multilevel control system for multicomponent meat products. Both classical methodologies, such as calculation by prescription bookmarks (BEFFE) and microstructural analysis, and approaches of highly sensitive methodologies, such as identification of muscle tissue by marker peptides (LC/MS-MRM) and semi-quantitative PCR analysis, were evaluated.

**Keywords:** biomarker; LC-MS/MS; PCR; species specificity

## INTRODUCTION

Meat is a highly nutritious food that most consumers love. The variety and quality of meat, as well as its delicacy, depending on the type of meat. According to national and international regulations, all ingredients must be marked (on the label) and be traceable within the enterprise to protect the information integrity of food products. Counterfeiting, unregistered ingredients, and contamination of any food product, whether intentionally or by gross negligence, may violate both international rules and religious laws.

Counterfeiting is a crucial health and/or ethical concern, involving specific food allergies, religion, fraud, and malicious marketing practices, in addition to economic, legal, and economic concerns **(Moore, Spink and Lipp, 2012; Ali et al., 2012)**. Over the past two decades, many researchers have sought to develop analytical methods for the species identification of meat or meat products, which were based predominantly on the assessment of either DNA or protein **(Calvo, Zaragoza and Osta, 2001; He et al., 2015; Floren et al., 2015)**.

Molecular genetic methods of analysis, despite the significant progress in this methodology, must still be regarded as confirmatory (arbitration) methods, implemented in specialized laboratories for samples taken based on the results of the preliminary screening. Polymerase chain reaction (PCR) is a worldwide recognized arbitration method of species identification and personal identification. However, because of the high costs of the equipment and the required qualifications of the personnel, the method cannot be used in small laboratories. Therefore, the feasibility of its use in the food industry depends on a reduction in instrumental and operating costs.

Immunoassay methods allow simple and quick screening directly at the sampling site and do not require special training. Immunochromatographic analysis (ICA), based on the use of multi membrane composites – test strips on which all the immunoreagents necessary for the specific binding of the compound to be detected are initially applied – allows for rapid testing of multicomponent samples with minimal sample preparation. To obtain quantitative data, the microplate enzyme-linked immunosorbent assay (ELISA) is the optimal methodological solution.

In thermally processed foods, there may be some detection problems due to interfering compounds and the degradation of nucleic acids and proteins. Hydrolysis and denaturation are especially severe in foods that have an acidic pH (e.g., lemon juice or vinegar) and/or are exposed to strong heat effects (e.g., during prolonged cooking or sterilization). Thus, for the most complex food matrices, with different ingredients mixed or subjected to severe heat treatment, the need for reliable, sensitive, and selective methods continues to be high **(Chernukha et al., 2019)**.

### Scientific hypothesis

It seemed important to develop an assessment system for controlling the composition of meat products, aimed at identifying cases of violation of established recipes using a set of methods for a two-level system of screening and arbitration control. The aim of this work was therefore to develop a two-level control system, using raw chicken products in ready-made mixtures with other types of meat as examples.

## MATERIAL AND METHODOLOGY

### Samples

The samples consisted of raw chicken (boneless breasts: *m. pectoralis major*), pork (*m. latissimus dorsi*), and beef (thigh). Pork, beef, and chicken underwent a trimming process to separate muscle tissue from fat and connective tissue. Losses during trimming were as follows: pork 19%, beef 7%, and chicken 23% (including bones).

The calculation of muscle tissue content (Table 1) was carried out according to BEFFE (bindegewebseiweißfreies Fleischeiweiß, i.e., meat proteins that do not contain connective tissue) **(Federal Ministry of Food and Agriculture, 2021)**.

The meat was minced twice in a Hurakan HKN-12SC meat grinder (China). All equipment was thoroughly rinsed after each sample. Minced meat was weighed on a balance with an error of 0.01 g to obtain mixtures of the prescribed formulations (Table 1). Further grinding until homogeneity was achieved was carried out on a Fimar CUCL5V240050T laboratory cutter (Italy) at a knife rotation speed of 2800 rpm for 3 min. A homogeneous mixture of minced meat was packed in vacuum bags of 250 ±12 g each (Figure 1). Then the samples were sealed and transported on a Webomatic Easy Pack vacuum packer (Germany).

The packaged mixtures in vacuum bags were placed in a Kerres Jet-Smoke Maxi 3000 universal thermal chamber (Austria). A medium heating mode was used: 80 °C, humidity 100%, for 45 min until reaching 72 °C in the center of the piece. After reaching the desired temperature, the samples were sprayed with tap water for 30 min to a temperature of 35 °C (Figure 1). Then the samples were cooled to a temperature of 6 ±2 °C.

### Chemicals

All reagents used were of U.S.P. purity or higher. All solvents, including water, were used with the LC/MS label.

### Laboratory Methods

Immunochromatographic analysis; Linked immunosorbent assay – Homemade laboratory methods

PCR analysis – **Kurbakov et al. (2019)**.

Microstructural analysis - Homemade laboratory methods

LC-MS/MS – **Khvostov, Vostrikova and Chernukha (2020)**.

### Immunochromatographic analysis

The analysis was carried out using pre-made test strips, consisting of multi membrane composites with applied immunoreagents and components labeled with colloidal gold. Sample preparation included grinding and extraction: 15-min incubation in phosphate buffer with detergent. For thermostable biomarkers, such as troponin, a reduction in the effect of the matrix on the assay results was achieved by introducing a 3-min incubation step for the extracts in a bath of boiling water. The test strips were vertically immersed in the test sample, and after 15 min the result (staining of the test area) was checked visually or with a scanner.

**Table 1** Muscle tissue content in the experimental mixtures.

| No | Mixture | Processing type | Pork (90% [w/w] muscle tissue), % (w/w) | Chicken (97% [w/w] muscle tissue), % (w/w)[1] | Beef (97% [w/w] muscle tissue), % (w/w) |
|---|---|---|---|---|---|
| **1.1** | pork + chicken | minced meat mixtures | 95.00 | 5.00 | |
| **1.2** | | | 90.00 | 10.00 | |
| **1.3** | | | 80.00 | 20.00 | |
| **1.4** | | | 60.00 | 40.00 | |
| **1.5** | chicken skin + pork + chicken | | 90.00 | 5.00 + skin 5.00 | |
| **1.6** | | | 70.00 | 17.00 + skin 17.00 | |
| **1.1b** | pork + chicken | mixtures cooked to a core temperature of 72 °C | 95.00 | 5.00 | |
| **1.2b** | | | 90.00 | 10.00 | |
| **1.3b** | | | 80.00 | 20.00 | |
| **1.4b** | | | 60.00 | 40.00 | |
| **1.5b** | chicken skin + pork + chicken | | 90.00 | 5.00 + skin 5.00 | |
| **1.6b** | | | 70.00 | 17.00 + skin 17.00 | |
| **2.1** | pork + chicken + beef | minced meat mixtures | 71.25 | 5.00 | 23.75 |
| **2.2** | | | 67.50 | 10.00 | 22.50 |
| **2.3** | | | 60.00 | 20.00 | 20.00 |
| **2.4** | | | 45.00 | 40.00 | 15.00 |
| **2.1b** | pork + chicken + beef | mixtures cooked to a core temperature of 72 °C | 71.25 | 5.00 | 23.75 |
| **2.2b** | | | 67.50 | 10.00 | 22.50 |
| **2.3b** | | | 60.00 | 20.00 | 20.00 |
| **2.4b** | | | 45.00 | 40.00 | 15.00 |



1 2

**Figure 1** Cooking samples in a heat chamber: (1) raw and (2) after cooking.

**Linked immunosorbent assay**

The analysis was carried out on microplates with immobilized antibodies for the biomarker. The test sample, biotinylated specific antibodies, streptavidin-peroxidase conjugate, and peroxidase substrate mixture (3,3',5,5'-tetramethylbenzidine + hydrogen peroxide) were sequentially incubated in the microplate wells. After each stage, the plate was washed with phosphate buffer and detergent. The immune complexes formed were recorded by the optical density of the oxidized chromogenic substrate at 450 nm.

**PCR analysis**

For DNA isolation, 50 mg of food products and control samples were taken. Then lysis and purification with chloroform were carried out using the reagents of the Sorb-GMO-B Kit (Syntol, Moscow, Russia) according to the manufacturer's instructions. Further DNA isolation was performed at the MagNA Pure LC 2.0 isolation station (Roche) using the MagNa Pure LC DNA Isolation Kit II (Tissue) (Roche, Mannheim, Germany) **(Kurbakov et al., 2019)**. Real-time PCR was performed on an ANK-32 amplifier (Syntol, Moscow, Russia). The reaction mixture (Syntol, Moscow, Russia), with a volume of 30 μL, contained primers with a concentration of 300 nM, a probe with a concentration of 150 nM, 2.5 mM $MgCl_2$, dNTP with a concentration of 0.25 mM each, SynTaq polymerase with a concentration of 2.5 activity units, and 5 μL of isolated DNA. The PCR reaction mode was as follows: preliminary denaturation at 95 °C, 7 min, and 35 amplification cycles (60 °C for 40 s and 95 °C for 15 s).

**Microstructural analysis**

In general, the preparation of histological samples included the following stages: sampling, fixation, preparation for cutting thin slices, microtomation, staining, and the interpretation of the micrographs to identify the signs of the meat product components. Properly prepared thin slices, stained to achieve the highest contrast of the analyzed components, were examined under a light microscope. First, small lens magnifications were used (10x, 20x), and then large ones (40x, 60x).

**Selection of marker peptides by LC-MS/MS**

Proteins were extracted from the samples and digested with trypsin based on previously published studies **(Khvostov, Vostrikova and Chernukha, 2020)**, and peptide mixtures were analyzed by HPLC-MS.

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 with the following 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, and 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)**.

**Description of the Experiment**

**Sample preparation:** 20
**Number of samples analyzed:** 20
**Number of repeated analyses:** 3
**Number of experiment replication:** 4

## Statistical analysis

STATISTICA 10.0 software (Dell Software, USA) was used for statistical analysis. Significant differences were verified by using a two-way analysis of variance (ANOVA), $p < 0.05$. Differences were analyzed and reliably determined in histological and PCR studies in determining species identification for ingredients of 5% (w/w) and above. Data were extracted from bioprograms in Microsoft Excel (USA).

## RESULTS AND DISCUSSION

**Immunochemical methods**

The choice of a molecular identifier that is recognized by antibodies is of fundamental importance. Such a biomarker should be unique for the controlled types of raw meat products, be characterized by a stable high content of muscle tissue, and have differences in the structure of antigenic determinants in different animals. In addition,

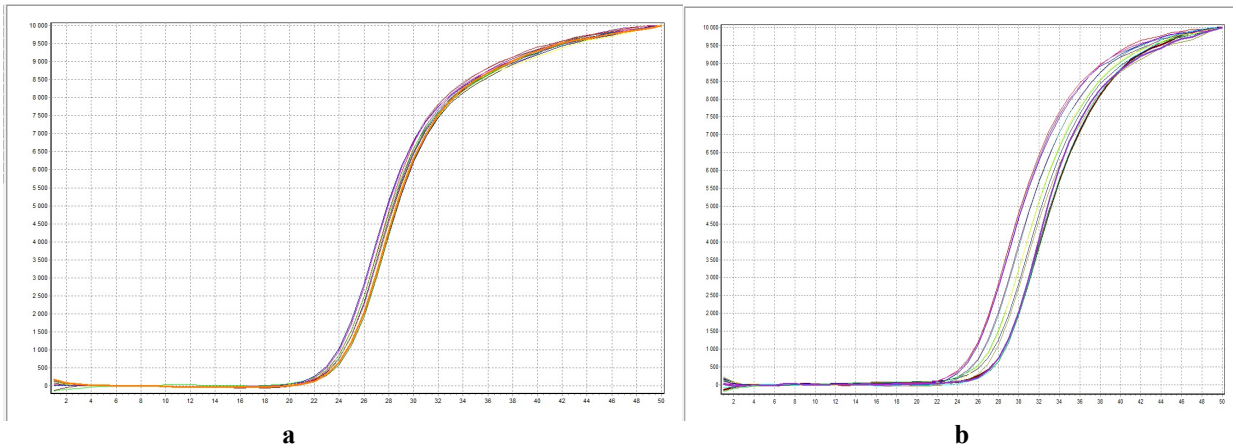
stability or at least preservation of the antigenic properties of the marker is required during various types of processing to which the raw meat products are subjected. To select the most promising biomarkers, we used the data of the proteomic analysis of muscle tissue proteins of different animal species and accompanying meat product components, obtained in joint studies by the Research Center of Biotechnology RAS and the V. M. Gorbatov FRC for Food Systems of the RAS **(Vostrikova et al., 2017)**. Taking into account the above criteria, skeletal troponin I (sTnI), myoglobin, and immunoglobulins were selected as biomarkers.

***Immunochromatographic systems***

Based on the selected immunoreagents, an immunochromatographic test system was developed for the detection of sTnI from mammalian muscle tissues (detection limit 25 ng.mL$^{-1}$) **(Zvereva et al., 2020a)**. The test system allows one to quantify the content of beef in minced chicken, starting with a 1% additive. Because of the high stability of the biomarker, the system retains its effectiveness for food products subjected to heat treatment (in particular, different types of sausages). The total analysis time is 20 min.

**Table 2** The content of muscle tissue measured by PCR in the test mixtures.

| No | Mixture | Processing type | Pork (90% [w/w] muscle tissue), % (w/w) | Chicken (97% [w/w] muscle tissue), % (w/w)[1] | Beef (97% [w/w] muscle tissue), % (w/w) |
|---|---|---|---|---|---|
| **1.1** | pork + chicken | minced meat mixtures | 94.59 | 5.41 | |
| **1.2** | | | 88.31 | 11.69 | |
| **1.3** | | | 80.16 | 19.84 | |
| **1.4** | | | 61.88 | 38.12 | |
| **1.5** | chicken skin + pork + chicken | | 88.15 | 11.85 | |
| **1.6** | | | 81.77 | 18.23 | |
| **1.1b** | pork + chicken | mixtures cooked to a core temperature of 72 °C | 94.00 | 6.07 | |
| **1.2b** | | | 90.00 | 9.84 | |
| **1.3b** | | | 85.00 | 14.76 | |
| **1.4b** | | | 60.50 | 39.50 | |
| **1.5b** | chicken skin + pork + chicken | | 89.00 | 10.77 | |
| **1.6b** | | | 84.00 | 15.63 | |
| **2.1** | pork + chicken + beef | minced meat mixtures | 78.50 | 4.53 | 16.97 |
| **2.2** | | | 77.26 | 7.90 | 14.90 |
| **2.3** | | | 57.31 | 21.00 | 22.00 |
| **2.4** | | | 51.75 | 34.00 | 14.00 |
| **2.1b** | pork + chicken + beef | mixtures cooked to a core temperature of 72 °C | 63.34 | 8.55 | 28.11 |
| **2.2b** | | | 65.10 | 13.60 | 21.30 |
| **2.3b** | | | 57.65 | 19.00 | 23.00 |
| **2.4b** | | | 52.15 | 32.85 | 15.00 |



**a** **b**

**Figure 2** Amplification curves for Samples 1.1–1.4: (a) a reference gene common to all animal DNA and (b) a species-specific chicken gene.

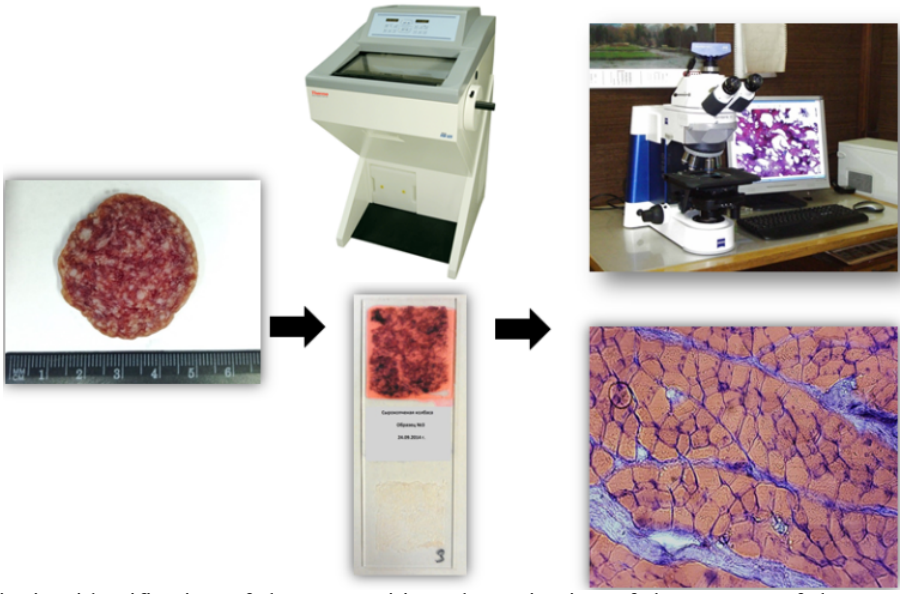
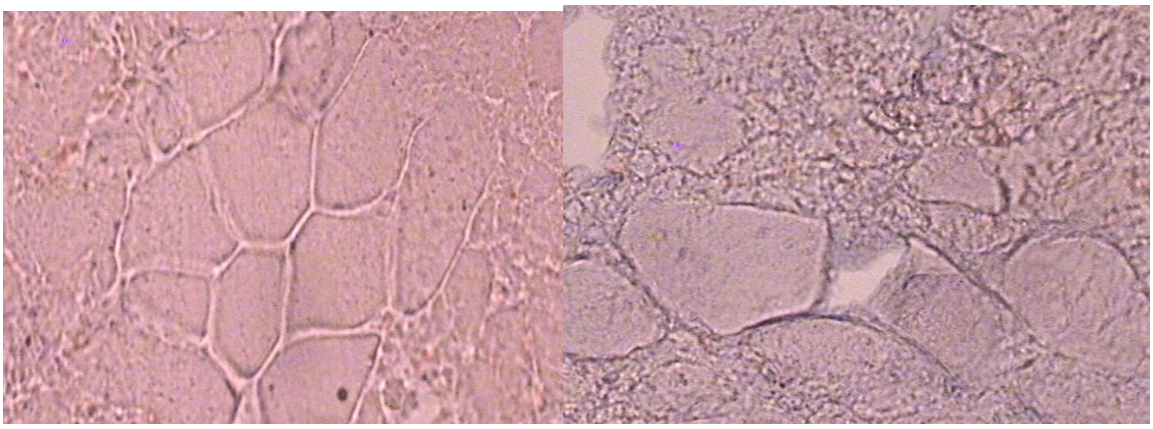
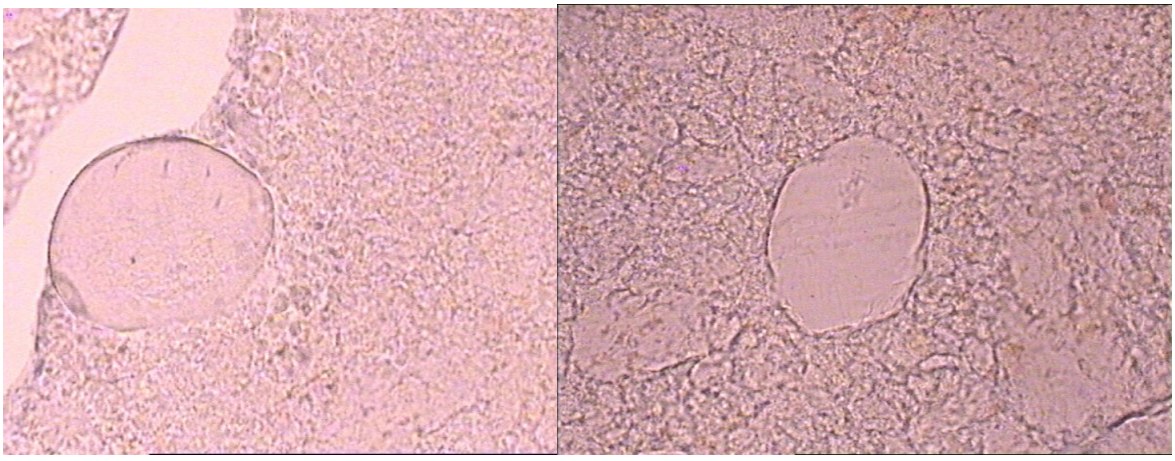


**Figure 3** Quantitative identification of the composition: determination of the amount of the component in volume percentages on the Carl Zeiss image analysis system.



**a** **b**

**Figure 4** Microstructure of minced meat containing 5% poultry meat: (a) fragments of muscle tissue (pork) before heat treatment and (b) fragments of muscle tissue (pork) after heat treatment (x340).



**a** **b**

**Figure 5** Microstructure of minced meat containing 5% of poultry meat: (a) fragments of muscle tissue (poultry) before heat treatment and (b) fragments of muscle tissue (poultry) after heat treatment (x340).

Myoglobin, despite its lower thermal stability compared to sTnI, retains its antigenic properties during heat and enzymatic treatment, so it is an effective alternative biomarker for evaluating various types of raw meat products. We implemented an immunochromatographic test system for the specific detection of porcine myoglobin **(Zvereva et al., 2020b)**. ICA of myoglobin was characterized by a detection limit of 5 ng.mL$^{-1}$ and a test

**Table 3** Accuracy of the real-time PCR method for identifying finished meat products.

| Type of raw meat | Processing type | Mean, % | CV, % |
|---|---|---|---|
| pork | minced meat mixtures | 103.2 | 7.3 |
| | boiled mix | 98.5 | 5.1 |
| chicken | minced meat mixtures | 100.3 | 13.1 |
| | boiled mix | 111.8 | 29.5 |
| beef | minced meat mixtures | 85.5 | 20.4 |
| | boiled mix | 110.1 | 13.4 |

duration of 15 min. The test system was capable of detecting 0.01% pork additives in ground beef. High reproducibility of ICA results was shown; the RSD of the measured signal intensity was not more than 14%.

Detection of immunoglobulins, which are present in the plasma of all vertebrates and can easily be extracted from muscles, is also an effective approach for identifying sources and assessing the total content of raw meat products. The detection limit of pig immunoglobulin G in the optimized ICA was 0.5 ng.mL$^{-1}$, which corresponds to a detection of up to 0.1% pork. With a different set of immunoreagents, the selective detection of chicken immunoglobulin Y is provided **(Hendrickson et al., 2021)**. The developed test system allows detecting 0.063% – 0.125% of chicken meat in samples consisting of one type of meat or a mixture of different types of meat (beef, pork, lamb, and rabbit).

***Immunoassay systems***

For the quantitative determination of sTnI, a sandwich ELISA technique was proposed, including sample preparation (protein extraction with 50 mM K-phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.5 M KCl in combination with heat treatment) **(Zvereva et al., 2015)**. The method is characterized by a detection limit for sTnI of 4.8 ng.mL$^{-1}$ at a concentration range of 8.7 to 52 ng.mL$^{-1}$. The measurement error in this range does not exceed 6.7%. It has been shown that the developed technique, because of the use of antibodies of a certain specificity, enables the detection of sTnI in samples of mammalian meat (beef, pork, lamb, horse meat) and in samples obtained after technological heat treatment, making it possible to assess the content of mammalian muscle tissue in various types of meat products. At the same time, the developed system does not detect sTnI present in poultry meat (chicken, turkey, duck).

Several complete sets of test systems for the determination of porcine myoglobin in the sandwich ELISA format have been proposed. The method is characterized by a detection limit for myoglobin in the range of 2 to 23 ng.mL$^{-1}$, depending on the combination of immunoreagents used. A method is proposed for the preparation of samples of raw meat products that makes it possible to extract myoglobin quickly (within 5 min) and efficiently.

**Assessment of the identification signs of meat products based on PCR data**

Over the years, PCR has established itself as the most promising method for determining the presence of a species in meat, because of its high sensitivity and specificity, as well as its fast processing time and low costs. DNA is naturally stable at high temperatures, at high pressures, and during chemical processing. DNA-based methods rely on the identification of the sequence of certain DNA segments of a particular tissue or animal **(Cai et al., 2014; He et al., 2015; Floren et al., 2015)**. In the category of DNA-based methods, PCR is the most used, simple, effective, sensitive, and specific method that can identify the species of origin presented under various processing conditions **(Bottero and Dalmasso, 2011)**. During this study, experimental characterization of the main types of raw meat products was carried out by the PCR method for all formulations (Table 2). The main component of Formulation 1 was pork, with a mass fraction of 60% – 95% of muscle tissue. The analysis was carried out for two thermal states of laboratory mixtures: chilled raw meat and meat after heat treatment in a heat chamber. Formulation 2 consisted of pork (45% – 72% w/w) with beef (15% – 24% w/w) and chicken (5% – 40% w/w) additives.

The reaction to the positive control (PC) must be as follows: the threshold cycle Ct (FAM) for the PC must consist of fewer than 20 cycles. Obtaining more than 20 threshold cycles for the PC indicated a deterioration in the quality of the reagents or an improper preparation for the reaction. The reaction to the negative control (NC) must be negative. If a positive reaction to the NC was obtained, the results of the determination of the samples were considered invalid. The results of the analysis of the test samples were used for analysis only when all the above conditions for the control samples were met. The amplification curves of the formulations under study are shown in Figures 2a and 2b. The result of the test samples was considered positive if the difference ΔCt (FAM) of the average values of the threshold cycle of the species-specific reaction of the test sample and the standard sample (100%) did not exceed 13 cycles at a signal flare-up level of at least 25%. If there was no reaction or if the difference ΔCt (FAM) of the threshold cycle of the test sample and the standard sample exceeded 13 cycles, the result was considered negative. The accuracy of the data on the type of raw materials for finished meat products is presented in Table 3. The average values of the accuracy of the PCR method are presented in percentages. During the statistical analysis, threshold values were established for three types of raw meat in the range of 5% to 30% deviation for the percentage of raw materials specified at the preparation stage ($p$ <0.05) included in the formulation. An indicator of less than 10% deviation in accuracy was set for pork, where the percentage in the finished formulation was not less than 57.5% ($p$ <0.05). For chicken, this indicator was 13.1% and 29.5% for raw ($p$ <0.05) and boiled product ($p$ <0.05), respectively. This is because during cooking, the amount of extracted DNA increases. Reducing the error limits is possible only with the use of standard samples that

have undergone heat treatment. For beef, acceptable error limits were obtained that did not exceed 30%.

**Histology**

In the initial stage, a semi-quantitative assessment was used according to the following classification of the occurrence of the component:

- in a predominant amount (the component is predominant in the entire volume of the test sample);
- in a sufficient amount (the component in the sample is more than half of the volume);
-in an average amount (the component in the sample is about half of the volume);
- in a moderate amount (the component in the sample is less than half of its volume);
- in a small amount (the component is evenly distributed at least in a small amount in each section of the sample);
- in some cases (the component is detected in single fields of view or sample sections).

Quantification was performed when nonconformity to labeling or product technology was identified (Figure 3). For this, studies were carried out on a computer image analyzer according to the program proposed by the manufacturer, adapted for histological studies. Automatic morphometry, in contrast to manual morphometry, provides data of higher accuracy, which in turn depends on the methods used to prepare the sample for examination and to stain the specimen.

***Histological evaluation of samples of one mixture***

Minced meat, containing poultry and pork, is a fine-grained homogeneous mass, including a small number of fragments of muscle, connective tissue, and fat, distributed in the mass of the sample in the form of small droplets. In cross-sections, fragments of muscle tissue of various species differ in the location of the nuclei in the muscle fiber and in the shape of the fiber itself. Muscle fibers of poultry are characterized by a clear round shape and a localization of nuclei throughout the fiber, in contrast to fragments of pig muscle tissue, which have a predominantly polygonal shape and a peripheral arrangement of nuclei (Figure 4 a, b). The indicated morphological differences make it possible to identify the presence of poultry meat in minced meat even in an amount of 5% of the mass of the minced meat. As studies have shown, heat treatment does not have any effect on the shape of muscle fibers, which makes it possible to identify poultry meat in minced meat after heat treatment (Figure 5 a, b).

***Histological evaluation of samples of two mixtures***

Minced meat, containing poultry, pork, and beef, is a fine-grained homogeneous mass, including a small number of fragments of muscle, connective tissue, and fat, distributed in the mass of the sample in the form of small droplets. Fragments of muscle tissue of various species, including beef, differ from poultry meat by the same microstructural characteristics by which pork differs from poultry. These fragments are characterized by a polygonal cross-sectional shape and a peripheral arrangement of nuclei. Muscle fibers of beef differ from pork by a smaller diameter in cross-sections and by a pronounced transverse striation in longitudinal sections. In cross-sections, poultry is identified by the shape of the muscle fiber and the arrangement of the nuclei typical of poultry. After heat treatment, the muscle tissue of poultry retains round-shaped muscle fibers, which makes it possible to identify poultry meat in minced meat when the content of poultry meat is from 5% to 40% ($p$ <0.05).

**Marker identification of research objects**

DNA hybridization and PCR methods, optical fiber chemiluminescence, species-specific protein recognition by immunoassays (ELISA), and HPLC/MS for peptide detection **(Khvostov et al., 2019; Montowska and Fornal, 2017)** were used to assess the authenticity of meat. Therefore, in the next stage of this study, the method for comparing species-specific peptides for the identification of chicken meat was adapted **(Khvostov, Vostrikova and Chernukha, 2020)**. It has been demonstrated that this approach for the detection of peptides is more effective than PCR and ELISA for determining the origin of products subjected to strong heat treatment or acid-base extraction **(Grundy et al., 2016)**.

In this study, we used the Skyline program **(Skyline software, 2021)**, which is theoretically capable of cleaving proteins and making a list of SRMs for each peptide (Table 4). Protein analysis was performed using biomodelling. The peptides presented in a recent review **(Stachniuk et al., 2019)** were selected for the comparison of potential biomarkers. These included more than 20 heat-resistant chicken markers from 2010 to 2019. This large number of markers is due to chicken being the most consumed meat in the world **(Escriba-Perez et al., 2017)**. Chicken meat is often used as a substitute for more expensive types of meat. Table 4 lists proteins such as creatine kinase M, myosin, β-enolase, M-proteins, and pyruvate kinase. They are a source of thermostable peptides.

For myosin chains, nine heat-resistant peptides were analyzed. The myosin family is the most representative peptide family in white meat because of its role in muscle contraction **(Pan et al., 2018)**. Two markers can be distinguished in Figure 4. The first marker is the DQGTFEDFVEGLR peptide, which showed a signal value of (10 – 30) * $10^3$ cps. These findings agree with studies by **Sentandreu et al. (2010)** and **Wang et al. (2018)**, where only this peptide was found. The second marker is the VAGAALPCAPAVK peptide **(Montowska and Fornal, 2017; Montowska and Fornal, 2019)**, for which the transitions are shown in Figure 7. Its signal value was (10 − 110) * $10^3$ cps, which was the highest value among all analyzed peptides. This new finding may shift the focus to VAGAALPCAPAVK.

The peptides SAMLQLAVTEIEK **(Montowska and Fornal, 2017)**, DLFDPVIQDR **(Li et al., 2018)**, LSVEALNSLEGEFK, and LAMQEFMVLPVGAASFHDAMR **(Claydon et al., 2015)** showed a signal value of (10 – 90) * $10^3$ cps. However, the spread ($p$ <0.05) was more than 50% (Figure 6). This can affect results during enzymatic hydrolysis and sample preparation. The analysis results will not be able to pass the convergence threshold of 25%.

**Development of a two-level control system for the composition of meat products**

The development of improved methodologies based on mass spectrometry, ELISA, and ICA with the ability to quantify specific proteins in complex biological matrices and to determine the level of protein expression is a promising direction.

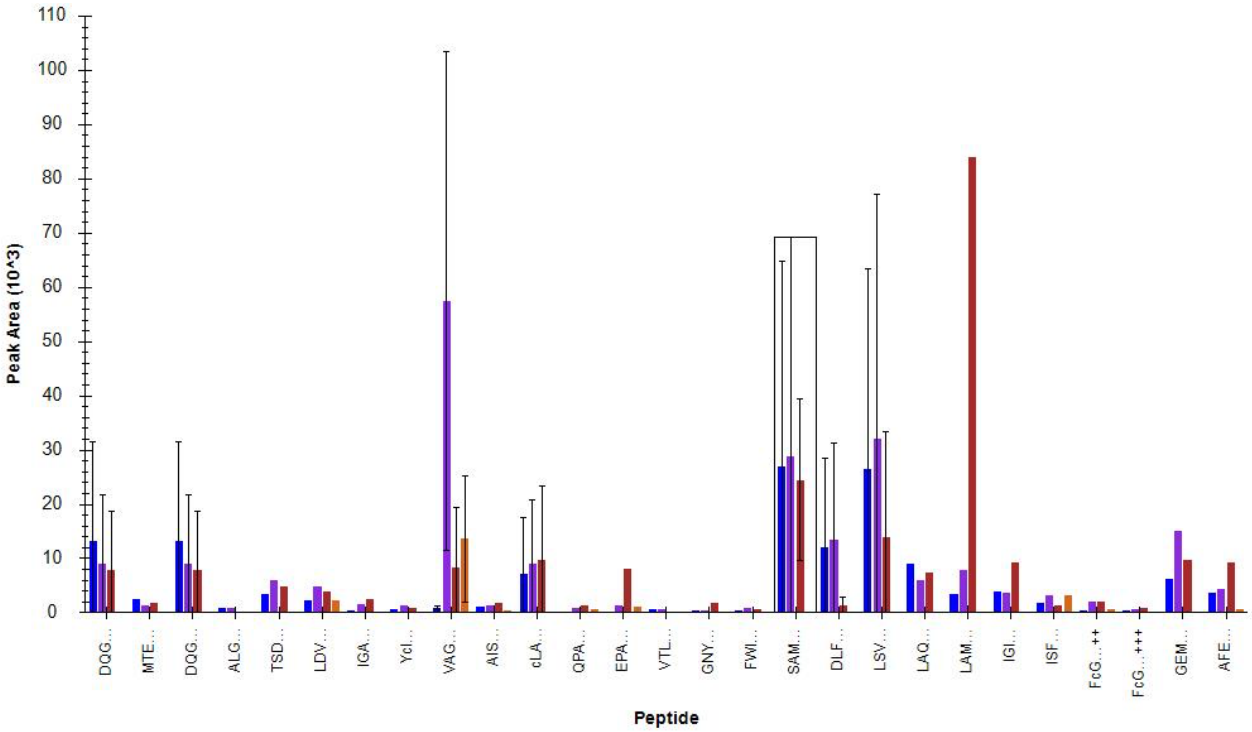



**Figure 6** Comparison of the areas of chicken marker peptides for all MRMs of the studied samples.

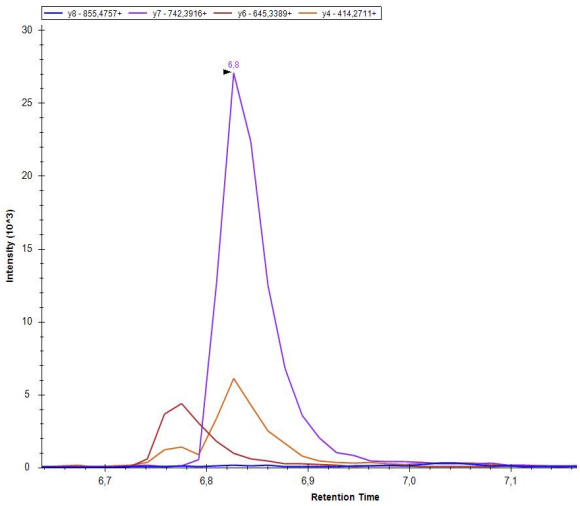



**Figure 7** Retention time, transitions, and daughter ion mass spectrum for the species-specific peptide VAGAALPCAPAVK.

In the past decade, there has been a rapid introduction of ELISA into the practice of food laboratories because of technical improvements in this method and the need for fast, sensitive, specific, and simple methods. The determination of species-specific proteins by ELISA is carried out using various test systems: systems for species-specific serum albumin and troponin I and systems for thermostable glycoproteins. Such test systems are designed for ruminants, pork, horse meat, small cattle, and poultry (chicken, turkey) **(Thienes et al., 2019)**. At the same time, there is a need to determine the composition of structureless meat products that have been exposed to heat and that may contain additional animal ingredients. Systems based on a single methodology are, as a rule, still not able to determine the content of these products. This paper presents a model for the development of a two-level control system for determining the origin of raw materials in meat products.

It seemed interesting to create an assessment system for controlling the composition of meat products, aimed at identifying cases of violation of established recipes using a two-level system of screening and arbitration. Table 5 presents a comparative assessment of the methodologies used, assessed to identify the muscle proteins in raw meat and products made from it. The table also includes two-dimensional electrophoresis. In the comparative study of this article, prototypes of this methodology were not considered, but we studied it in detail. Some aspects of this methodology were used in practice, and its performance was validated and confirmed by mass spectrometry **(Vostrikova et al., 2017)**

Thus, a review of existing methodological approaches and their experimental confirmation revealed the absence of one specific method that would solve such an urgent problem as the quantitative determination of undeclared components in

meat products. According to the prospects for inclusion in the system of multilevel control of the composition of meat products, ICA and/or ELISA (low-cost methods, with a high level of reliability) is recommended as a screening method, and LC-MS as an arbitration (confirming) identification method ($p$ <0.05) (Figure 8). When analyzing the complementarity of the considered methods, it is worth using two or three methods together:

- ELISA/ICA: within the framework of production control laboratories, in the context of rapid decision-making;
- ELISA/MS: within the framework of confirmatory (arbitration) control, as the most highly reliable method;
- ICA/2D electrophoresis biomarker identification / MS: within the framework of confirmatory (controversial) control, as the most demonstrative confirmation.

When creating a multilevel control system for the composition of meat products, it is proposed to use the principle of a decision tree, as shown in Figure 5.

**Table 4** Identification characteristics of heat-stable chicken peptide markers for the LC-MS/MS methods.

| Protein | Marker peptide sequence | Parent ion (*m/z*), product ions (*m/z*) | Collision energy (V) | References* |
|---|---|---|---|---|
| Myosin light chain 1 | MTEEEVEELMK | 684.3 → 1006.5, 877.4, 748.4 | 22.2 | **Wang et al. (2018)** |
| | DQGTFEDFVEGLR | 756.9 →1111.5, 964.5, 835.4 | 24.5 | **Sentandreu et al. (2010), Wang et al. (2018)** |
| Myosin light chain 3 | ALGQNPTNAEINK | 685.4 → 886.5, 789.4, 688.4 | 22.2 | **Sentandreu et al. (2010)** |
| | TSDVDSVFFIR | 643.3 → 1097.6, 883.5, 582.3 | 20.9 | **Montowska and Fornal (2017, 2019)** |
| | LDVPISGEPAPTVTWK | 855.5 → 1085.6, 1028.5, 899.5, 731.4 | 27.5 | **Montowska and Fornal (2017, 2019)** |
| Myosin-binding protein C | IGAGGVDGYLVEWCR | 826.4 →1353.6, 1197.5, 650.3 | 26.6 | **Montowska and Fornal (2017), Fornal and Montowska (2019)** |
| | YCITVTNPVGEDSATLHVR | 1066.5 → 1280.7, 1183.6, 783.5 | 34.1 | **Montowska and Fornal (2017), Fornal and Montowska (2019)** |
| | VAGAALPCAPAVK | 612.8 → 855.5, 742.4, 645.3, 414.3 | 20.0 | **Montowska and Fornal (2017, 2019)** |
| Myosin-binding protein H | AISASGTSDPATLEQPVLIR | 1013.5 → 1236.7, 1139.7, 1068.6, 597.4 | 32.4 | **Montowska and Fornal (2017)** |
| Pyruvate kinase | CLAAALIVMTESGR | 746.4 →1005.5, 892.5, 779.4 | 24.1 | **Wang et al. (2018)** |
| | QPAHDAWAEDVDLR | 811.9 → 1494.7, 1074.5, 1003.5, 817.4 | 26.2 | **Li et al. (2018)** |
| Pyruvate kinase (P00548.2) | EPADAMAAGAVEASFK | 782.9 → 1152.6, 950.5, 879.5, 950.5, 808.4 | 25.3 | **Montowska and Fornal (2017, 2019), Montowska and Fornal (2019)** |
| M-protein | GNYTFEIFSDK | 660.8 → 986.5, 885.4, 738.4 | 21.5 | **Wang et al. (2018)** |
| | FWIQAESLSPNSTYR | 899.9 → 1153.5, 1024.5, 937.5, 737.4 | 28.9 | |
| Troponin I | SAMLQLAVTEIEK | 716.9 → 902.5, 789.4, 718.4 | 23.2 | **Montowska and Fornal (2017)** |
| Creatine kinase M-type | DLFDPVIQDR | 609.3 → 842.5, 727.4, 630.4 | 19.9 | **Li et al. (2018)** |
| | LSVEALNSLEGEFK | 768.4 → 1036.5, 923.4, 809.4 | 24.8 | **Claydon et al. (2015)** |
| β-enolase | LAQSHGWGVMVSHR | 782.9 → 1028.5, 971.5, 785.4 | 25.3 | **Li et al. (2018)** |
| | LAMQEFMVLPVGAASFHDAMR | 1161.0 → 1371.6, 1258.6, 1161.5 | 37.0 | **Claydon et al. (2015)** |

Note: * Only the peptide sequence provided in the review article by **Stachniuk et al. (2019)**. The MRM transitions and collision energy metrics were selected anew.

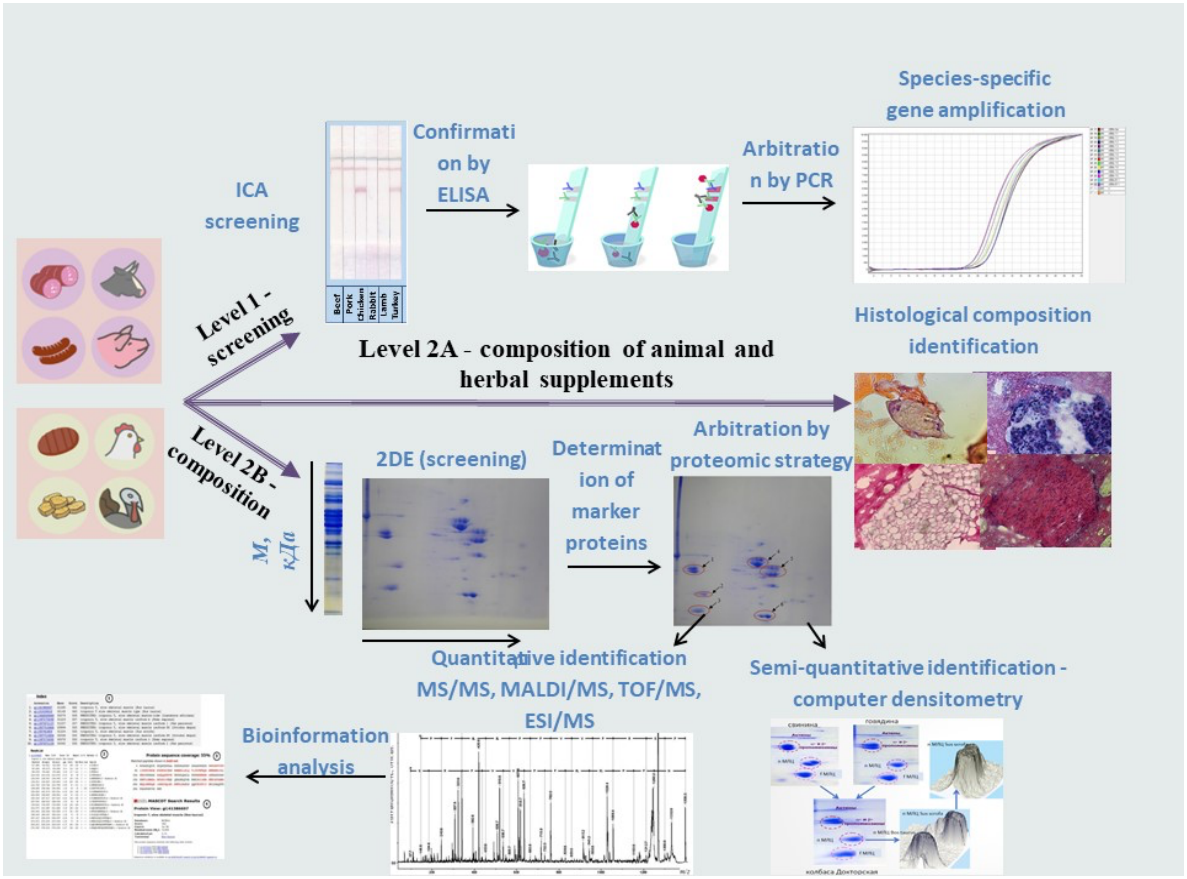



**Figure 8** A two-level control system for the composition of meat products.

**Table 5** Comparative assessment of screening and arbitration control methods.

| Method | Time per sample / confidence level | Cost level for reagents (kit) * | Cost level** | Inclusion in the system of multilevel control | Reference |
|---|---|---|---|---|---|
| **Histology** | 48h/80% | low cost | costly | no | **Khvylya, Pchelkina and Burlakova (2012)** |
| **Real-time PCR** | 5h/99% | medium cost | costly | yes | **Cai et al. (2014), He et al. (2015), Floren et al. (2015)** |
| **2-DE (screening)** | 24h/80% | costly | medium cost | no | **Akhremko and Vasilevskaya (2020, 2021)** |
| **2-DE (biomarker identification)** | 48h/95% | costly | costly | yes | **Akhremko and Vasilevskaya (2020, 2021)** |
| **2-DE (densitometric quantity confirmation)** | 48h/85% | costly | costly | yes | **Akhremko and Vasilevskaya (2020, 2021)** |
| **LC/MS-MS** | 5h/99% | costly | costly | yes | **Kulikovskii et al. (2019)** |
| **ELISA** | 3.5h/95% | low cost | medium cost | yes | **Zvereva et al. (2015)** |
| **Lateral flow immunosensor** | 0.3h/45% | low cost | low cost | yes | **Zvereva et al. (2020a, 2020b), Hendrickson et al. (2021)** |

Note: * Level of costs for reagents (kit): low cost (less than 500 €), medium cost (500–1000 €), costly (more than 1000 €); ** The level of costs for auxiliary equipment and measuring instruments: low cost (less than 1000 €), medium cost (1000–10000 €), costly (more than 10000 €).

The primary task for the execution and final selection of methods that will be included in the multilevel control system is the development of standards for the products under study and a description of the result of the methods used (see Table 5). Similar approaches are applicable to all meat products that contain proteins of muscle origin. Thus, the proposed methodology can have a wide range of applications. Applied proteomics and immunodetection aimed at finding biomarkers, the composition of products of plant and animal origin, and the identification signs of the authenticity of products require a completely different approach from that of classical protein analysis, which requires a more detailed study or complete mapping of the sequence of a known protein.

## CONCLUSION

Based on the economic feasibility (costs, research time, and reliability) of the methodology, ELISA and ICA were proposed as screening methods. The advantage of troponins, as thermostable biomarkers of muscle tissue, has been shown in the identification of meat. This made it possible to distinguish between the muscle tissues of mammals (beef, pork, lamb, horse) and poultry (chicken, turkey, duck). With ELISA, the evaluation time could be reduced to 30 – 40 min.

Using a video-digital recording of the intensity of staining of the analytical zone of the test strip in the ICA, this analysis can be transformed from a qualitative into quantitative analysis, using portable optical detectors, such as video cameras of serial communication devices, as well as standard office scanners. The standard deviation of the measurements of the biomarker concentration did not exceed 10%.

Highly sensitive methodologies, such as identifying the amount of muscle tissue by a marker peptide, were also evaluated. Monitoring of multiple reactions of specific peptides by mass spectrometry (MPM-MS) was accepted as an arbitration method and proposed for confirmatory analyses. Proteins from meat were extracted using trypsin, the mixture of peptides was analyzed by high-performance liquid chromatography using QQQ-MS, and candidate proteins were characterized using Skyline software. The choice of species-specific proteins and their peptides (more than six amino acids) was based on the high content in muscles, in terms of the signal-to-noise ratio, in the absence of missing fragments. Myoglobin and lactate dehydrogenase were chosen as biomarker proteins. A simplified regulation scheme is proposed for action: screening and arbitration control. It is recommended to introduce such use of the various methodologies into the sphere of state regulation at the level of control bodies.

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The authors declare no conflict of interest.

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This article does not contain any studies that would require an ethical statement.

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# THE EFFECT OF CARBON DIOXIDE ON THE QUALITY OF THE MUSHROOMS

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## ABSTRACT

Mushrooms' quality may be significantly changing depending on their type, strain, growing cycle, packing, cooling, postharvest handling (PHH), and conditions of storage. This work aimed to define the influence of the type and mushrooms' strain, the regime of the PHH by carbon dioxide on their preservation (marketability, loss of weight (LW)), changes in the chemical substances, and physiological activity (intensity respiration (IR) and heat release (HR)). Mushrooms *Agaricus bisporus* (AB) (strains ІБК-25 and ІБК-15) and *Pleurotus ostreatus* (PO) (strains НК-35 and Amycel 3000) were used for testing. Three regimes of treatment by $CO_2$ with a concentration of 20% were applied: 2 h; 12 h and 22 h. The control was the mushrooms without treatment by $CO_2$. Changes in the chemical substances such as dry matters (DM), protein nitrogen (PN), and ascorbic acid (AA) in the researched mushrooms were observed. The best result of mushroom preservation was provided by the regime of $CO_2$ treatment during 12 h. The yield of marketable AB was 94.9% (IBK-25) and 94.2% (IBK-15) comparison to control 93.5%, and 92.5%, respectively. The regime of PHH 2 h almost has no influence but 22 h harmed this indicator. PHH of mushrooms by carbon dioxide was promoted to preserve the DM and increasing concentration of $CO_2$ was supplied better results. Thus, DM at the end of storage in the AB of strain IBK-25 depend on the regime were 8.5, 8.6, and 8.4%, against – 8.3% in the control variant. Significant quantitative changes in the PN and AA as a result of treatment by $CO_2$ were not established. PHH also affected the IR and HR. The increased duration of treatment by $CO_2$ inhibited the intensity of physiological processes in the mushrooms. But, as in previous cases, the best result was provided PHH by 20% $CO_2$ during 12 h. Similar trends of treatments effect by carbon dioxide were observed in the mushrooms of PO.

**Keywords:** mushrooms; *Agaricus bisporus*; *Pleurotus ostreatus*; postharvest handling; carbon dioxide

## INTRODUCTION

Mushroom growing is an ecologically clean and waste-free production **(Varoquaux et al., 1999)**. There are whole mushroom industries were created in France, England, Holland, Germany, and the USA **(Yoo et al., 2005)**. The world leaders in mushroom growing are China, the USA, and France **(Zhang et al., 2014)**. The industrial production of cultivated mushrooms is one of the most perspective industries of agriculture in Ukraine. The main types of mushrooms that are officially allowed to grow in our country are AB and PO **(Dubinina and Timofeeva, 2009; Nesterenko, 2011)**. Mushroom is a rich source of good quality proteins **(Simahina, 2008)**, having most of the essential amino acids, fatty acids, minerals, and vitamins with low calories **(Mattila et al., 2002; Lee et al., 2011)**. Consumption of mushrooms has a positive effect on the human body. Their components enhance immunity, have hepatoprotective, antitumor, antidiabetic, cardiac effects, help reduce the level of "harmful" cholesterol, improve the functional state of certain organs and systems of the body (including nervous, sexual) **(Wasser, 2002; Lindequist, Niedermeyer and Julich, 2005; Patel and Goyal, 2012; Kalač, 2013)**.

The shelf life of the fresh mushrooms is limited to 1 – 3 days at ambient temperature and it may be increased up to 4 – 7 days at the 4 ºC. Browning and texture changes are the main processes that responsible for the loss of the sensory quality of mushrooms. The free water high level in the fruiting bodies of mushrooms promotes their high IR and causes dehydration and metabolic activity.

The IR of the harvested mushrooms is high in comparison to the other horticultural crops and this main cause their shorter postharvest life. Many short-term storage measures are followed to retard the deterioration in quality at the level of mushroom grower till it reaches the consumer. By following proper packing, cooling, PHH, and transportation, the shelf life of mushrooms can be extended **(Kumar et al., 2014)**.

Good results in extending the shelf life of mushrooms have been obtained as a result application of the Controlled Atmosphere Storage (CAS), Modified Atmosphere Packaging (MAP), and Modified Humidity Packaging

(MHP) **(Barron et al., 2002; Kim et al., 2006; Mahajan et al., 2008; Jiang et al., 2010)**.

In the case of CAS, the product is stored in cold storage chamber where created atmospheric composition with certain relative humidity (RH) and concentration of $O_2$ and $CO_2$ that is maintained constant throughout storage. CAS reduces IR of mushrooms, their texture changes, and brown discoloration (enzymatic browning) as a result shelf life is extended **(Djekic et al., 2017; Park et al., 2020)**. A major disadvantage of CAS is the significant cost of equipment and its maintenance.

MAP is a method of storage when fresh product is in a sealed package and is creating a modified atmosphere by respiratory gas exchange, namely oxygen intake and carbon dioxide evolution **(Zhang, Pu, and Sun, 2018; Zalewska et al., 2018)**. Equilibrium concentrations of $O_2$ and $CO_2$ are consequently established in the case the rate of gas permeation through the packaging material equals respiratory gas exchange **(Gantner et al., 2017; Gholami, Ahmadi and Farris, 2017)**. The balance of the gas exchange depends on product weight, temperature, the respiration rate of a certain product, permeability $O_2$ and $CO_2$ through packaging material, free volume in the package, and film area. MAP helps to extend the shelf life and keep quality as a result creation of a corresponding atmosphere around the products that are packaged in plastic films **(Ozturk, Havsut and Yildiz, 2021; Vunduk et al., 2021)**. There are two methods of creating a modified atmosphere: active and passive modifications. The product is only sealed in a polymeric package at the passive modification. The atmosphere is modified as a result of the fresh product breathing and permeation of gases into the package. It takes a long time to reach the steady-state conditions within the package in passive modification. In the case of the active modification, air initially compulsorily is pushed into the package. Therefore, a stable state of the atmosphere is reached after packaging quickly **(Li et al., 2014; Han Lyn et al., 2020)**. MAP of mushrooms has been shown successfully to delay senescence and keep quality after harvest.

### Modified Humidity Packaging

The many polymeric films used for fresh product packaging have lower water vapor transmission rates compared to rates of breathing of this product. As a result, the packages creating saturated conditions of water vapor. The high relative humidity in the package (HRHP) can be cause condensation of water vapor within a package and promote microbial growth. It is may be increasing or decreasing the spoilage depending on the products, their breathing coefficients, and water potentials. There are two possible ways for reaching the desired HRHP: perforation of the package **(Dhalsamant et al., 2015)** and use of in-package water-absorbing compounds like calcium chloride that can be keeping the required RH **(Villaescusa and Gil, 2003)**. Combination MAP with MHP promotes improving the shelf-life of fresh mushrooms. The best result for keeping mushrooms obtained at the HRHP of 87 – 90% during the storage.

The disadvantages of MAP and MHP are the need for special packaging materials, water-absorbing compounds, and packaging equipment that increases the cost of products. Besides, as a result, MAP storage takes place excessive accumulation of CO2 that may be damage the cell membrane and physiological injuries to the mushrooms, such as severe enzymatic browning and loss of firmness **(Varoquaux et al., 1999)**.

The perspective method of PHH of mushrooms and extended their shelf life is the treatment by carbon dioxide with high concentration during a short time.

The effect of this influence depends on the stage developmental of mushrooms, the concentration of $CO_2$, and the time of exposure **(Fonseca, Oliveira and Brecht, 2002; Li et al., 2013)**.

This work aimed to investigate the effect of the short-term treatment by high concentrations of carbon dioxide on the mushrooms' quality and their physiological activity.

### Scientific hypothesis

Mushrooms are products with a high level of water in the fruiting bodies (more than 90%). That explains high IR and metabolic activity to comparison other horticultural crops and causer loss of the sensory quality of mushrooms (browning and texture changes). Mushrooms have a large loss of moisture as a result of evaporation, especially at the storage in the reduced RH. The shelf life of mushrooms depends on their type, packing, regimes, and ways of cooling, technologies of PHH, and conditions of transportation.

## MATERIAL AND METHODOLOGY

**Samples**

Materials of the study were mushrooms of AB (strains IBK-25 and IBK-15) and PO (strains HK-35 and Amycel 3000) from the collection of Institute of Botany after name M.G. Xolodnogo of National Academy Science of Ukraine. These strains are widespread, suitable to grow all year, and have universal purposes. Mushrooms AB and PO were supplied by the Trynchuk Mushroom Farm (Kyiv region, Fastiv district, Borova village) and transported to the National University of Life and Environmental Sciences of Ukraine (Kyiv, Ukraine) at 2 ±2 °C and 90% ±1 RH.

**Chemicals**

Barium Hydroxide (brand LLC "Khimlaborreaktiv", producer NVP LLC Alfarus, Ukrain, clean for analysis), Hydrochloric Acid (brand LLC "Khimlaborreaktiv", producer NVP LLC Alfarus, Ukrain, chemically pure), and Tillman's reagent (2,6-Dichlorophenolindophenolate Sodium, brand Himtest Ukraine, Ukrain) were used in the analysis.

**Animals and Biological Material:**

No animals and biological materials were used for the studies

**Instruments**

The four cold chambers KH–6U with volume 6 $m^3$ were used for the mushrooms treatment by $CO_2$ and their following storage. The required concentration of $CO_2$ was created by the rotameter of the brand RM-2.5 GUZ (GK PriborMarket, Russia). The gaseous medium in the chamber was stirred by fans.

Concentration CO2 in the chambers was monitoring by a VTI-2 gas analyzer (Thermal Engineering Institute, Russia). The temperature and RH in the chamber were controlled daily. The air temperature was measured by alcohol thermometer TLC-5 (PJSC "Glass Device", Ukraine, I accuracy class) with the division price of 0.5 °C

but RH – by August psychrometers (Lab Time, Ukraine, I accuracy class), daily.

Samples were weighted by laboratory scales ADG2200C (AXIS) from the company "Scales of AXIS Ukraine" with the 2nd class of accuracy.

**Laboratory Methods**

For chemical analyses, only the first wave mushrooms were used. In mushrooms, before storage and after 6 days were determined DM, PN, and AA.

Content of DM was performed by the weighted method **(DSTU 7804, 2015; Skaletska, Podpryatov and Zavadska, 2014)**.

Content of protein nitrogen (PN) was performed by the state standard of Ukraine **DSTU 4923 (2008)**.

Content of AA – restoring the Tillman's reagent, by extraction acid solution of mushrooms sample followed by filtration of the resulting substrate by the titrimetric method according to the state standard of Ukraine **DSTU 7803 (2015)**.

**Description of the Experiment**

The scheme of researches is presented in **Figure 1**.

**Sample preparation:** Mushrooms that were used in the investigations were harvested at the peak of fruiting of the first, second, and third waves.

Mushrooms were harvested manually, immediately placed in plastic boxes with the volume of 5 kg where they were stored. Every sample before storage was weighed, numbered, and added a label with indication weight, temperature, time of the start storage, and repeatability.

***PHH of mushrooms by $CO_2$***

Regimes PHH of mushrooms of AB and PO: 20% $CO_2$ during 2 h; 20% $CO_2$ during 12 h and 20% $CO_2$ during 22 h. The control was the mushrooms without treatment by carbon dioxide. The temperature of the product storage was 1 °C. Time after mushrooms' harvesting to the PHH by $CO_2$ did not exceed 3 h.

***Density of carpophore***

The density of carpophore (DC) of fruiting bodies of AB mushrooms before and after storage was determined by the formula:

$$DC = \frac{m_c}{d_h} \quad (1)$$

Where:

DC – density of carpophore ($g.cm^{-1}$); $m_c$ – weight of carpophore (g); $d_h$ – diameter of mushroom hat (cm).

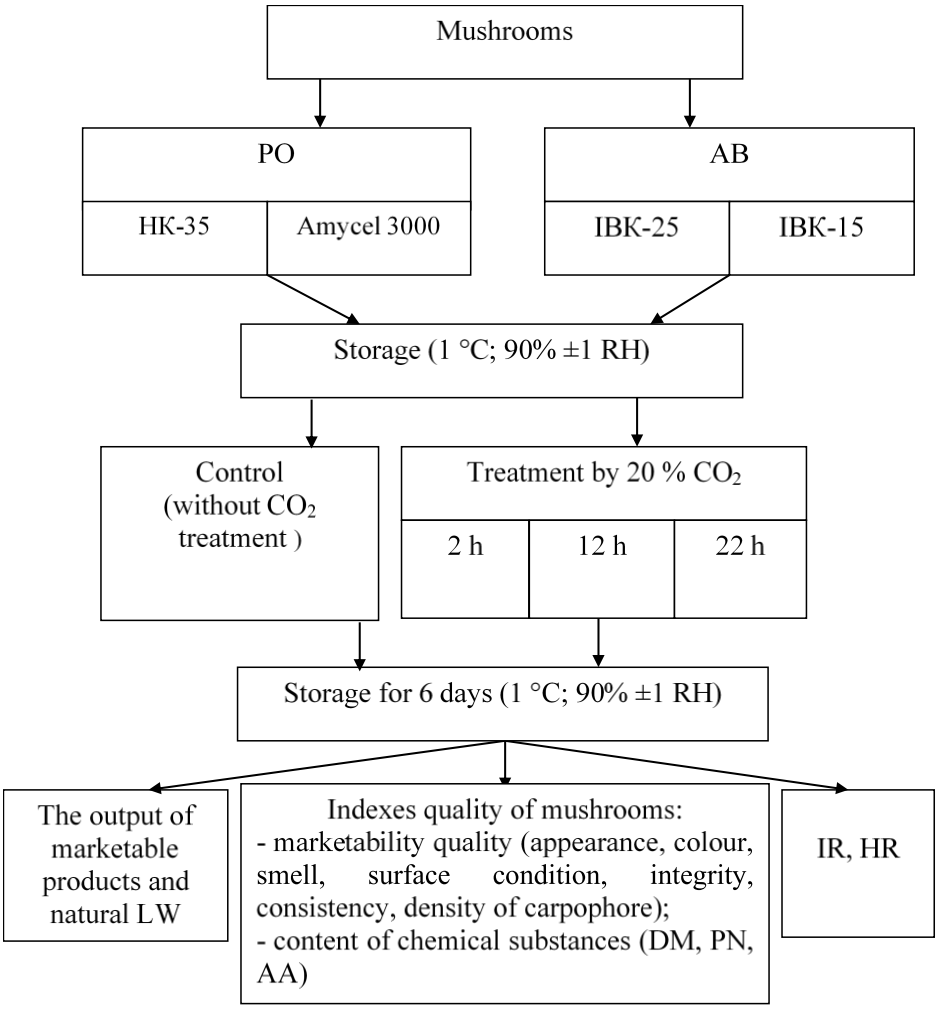


**Figure 1** Scheme of researches the influence of treatment by $CO_2$ on the quality and physiological activity of mushrooms.

***Loss of weight***

LW was determined by the method of fixed samples. Samples were weighed before storage and every day. The calculation LW was performed by the formula:

$$\mathrm{LW} = \frac{W_{bs} - W_{eds}}{W_{eds}} \cdot 100\% \qquad (2)$$

Where:

LW – loss of weight (g); $W_{bs}$ – the weight of the sample before storage (g); $W_{eds}$ – the weight of a sample of every day of storage (g).

***Intensity of respiration***

The IR of mushrooms was determined experimentally in desiccators every day during the storage. This method is based on the absorption of $CO_2$ by solutions of alkalis ($Ba(OH)_2$) with known concentration and followed by the determination of the amount of alkali that did not react with acid for titration (HCl). Simultaneously, the alkali was titrated from a desiccator without mushrooms (control). IR was determined by the formula:

$$\mathrm{IR} = \frac{(Q_c - Q_e) \cdot a \cdot 480000 \cdot T}{w_m \cdot b \cdot t} \qquad (3)$$

Where:

IR – the intensity of respiration ($mL.kg^{-1}.h^{-1}$ for $CO_2$); $Q_c$ – quantity of acid which was used for control titration (mL); $Q_e$ – quantity of acid which was used for titration in the experiment (mL); $w_m$ – *the* weight of the mushrooms (g); t – time duration of the product in the desiccator (min); T – correction to a titter of the 0.1 N alkali; a – average between atmospheric pressure at the start and end of the experiment; b – average between the temperature at the start and end of the experiment.

***Heat release***

The amount of HR by mushrooms was determined by the amount of $CO_2$ released during aerobic respiration:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + 674 \text{ kcal } (2824 \text{ kJ}) \quad (4)$$

For each g of $CO_2$ that was released during respiration spent 2,553 kcal or 10.69 kJ of heat. The amount of carbon dioxide expressed by $mL.kg^{-1}.h^{-1}$ but heat – $kJ.kg^{-1}.h^{-1}$. Thus the final formula has the following form:

$$HR = IR \cdot 10.69 / 1000 \cdot 24, \qquad (5)$$

Where:

HR – heat released from the product ($kJ \cdot kg^{-1} \cdot d^{-1}$); IR – intensity respiration ($mL.kg^{-1}.h^{-1}$ for $CO_2$).

**Number of samples analyzed:** The weight of samples for investigations was up to 4 kg. The average sample for chemical analysis was 20 mushrooms fruit bodies with average weight.

**Number of repeated analyses:** 3

**Number of experiment replication:** Every variant was replied three times. Mushrooms were stored for 6 days. The marketable quality, the chemical composition of mushrooms, the yield of marketable products, and natural LW were determined before and after the storage. The intensity of physiological processes was monitored daily.

## Statistical Analysis

The experiment was established as completely randomized designs with three repetitions in 2017 – 2019 years. The data are reported as mean values ± standard deviation (*SD*). As the statistical analysis software was used Microsoft Excel version 2016.

## RESULTS AND DISCUSSION

Many publications report that reduced $O_2$ and elevated $CO_2$ concentration have beneficial effects on the shelf life of mushrooms **(Lin et al., 2017; Iqbal et al., 2009; Singh et al., 2010; Simón, González-Fandos and Tobar, 2005; Hosseini and Moradinezhad, 2018)**.

The atmospheres with a high concentration of $CO_2$ can potentially reduce respiration rate, production of ethylene, sensitivity, decay, and physiological changes in the mushrooms **(Farber et al., 2003).**

The IR depends on the product, its developmental stage, $CO_2$ concentration, and time of exposure **(Fonseca, Oliveira and Brecht, 2002)**. $CO_2$ has significant and direct antimicrobial activity. Aerobic bacteria, such as Pseudomonas, are inhibited by moderate to high levels of $CO_2$ (10-20%) **(Farber et al., 2003; Lee et al., 1995)**. A combination of 3% $O_2$ and 10% $CO_2$ is reported to extend the storage life of mushrooms up to 12 to 15 days at 0 °C **(Cantwell and Suslow, 2002)**. Another report says that 10 to 15% $CO_2$ concentration reduces cap opening, browning, and stripe elongation **(Zhang, Pu and Sun, 2018)**.

The low level of $O_2$ and very high $CO_2$ in the atmosphere may cause the development of off-flavors, veil opening, and stripe elongation **(Farber et al., 2003; Lee et al., 1995)**.

Besides, the excessive level of $CO_2$ can cause cell membrane damage and physiological damages to the product, such as strong enzymatic browning and loss of mushroom density **(Briones et al., 1992)**.

So, low concentrations of $CO_2$ did not allow obtaining the desired effect but high has negatively affected the quality and shelf life of the mushrooms.

To establish the optimal regime of AB and PO mushrooms treatment by carbon dioxide, we were kept them in sealed chambers with 20% $CO_2$ in the environment for 2, 12, and 22 h, and then stored with temperature 1 °C for 6 days in the normal atmosphere.

The influence of parameters and regimes of the PHH on the quality of mushrooms was determined by organoleptic and chemical indexes. Organoleptic evaluation of AB and PO showed that PHH by 20% $CO_2$ for 2 h had almost no effect on changes in color, smell, and consistency of fruiting bodies. Prolonged treatment by carbon dioxide during 22 h significantly deteriorated the color of mushrooms (Figure 2). In our opinion, prolonged contact of mushroom tissues with high concentrations of $CO_2$ has caused respiratory disorders and inhibited the biochemical processes associated with oxygen deficiency. This caused the death of individual cells, enzymatic browning, and a decrease in the fruit density of mushrooms, which corresponding with the results obtained by other researchers **(Briones et al., 1992)**. Besides, products had a slight characteristic smell of carbon dioxide.

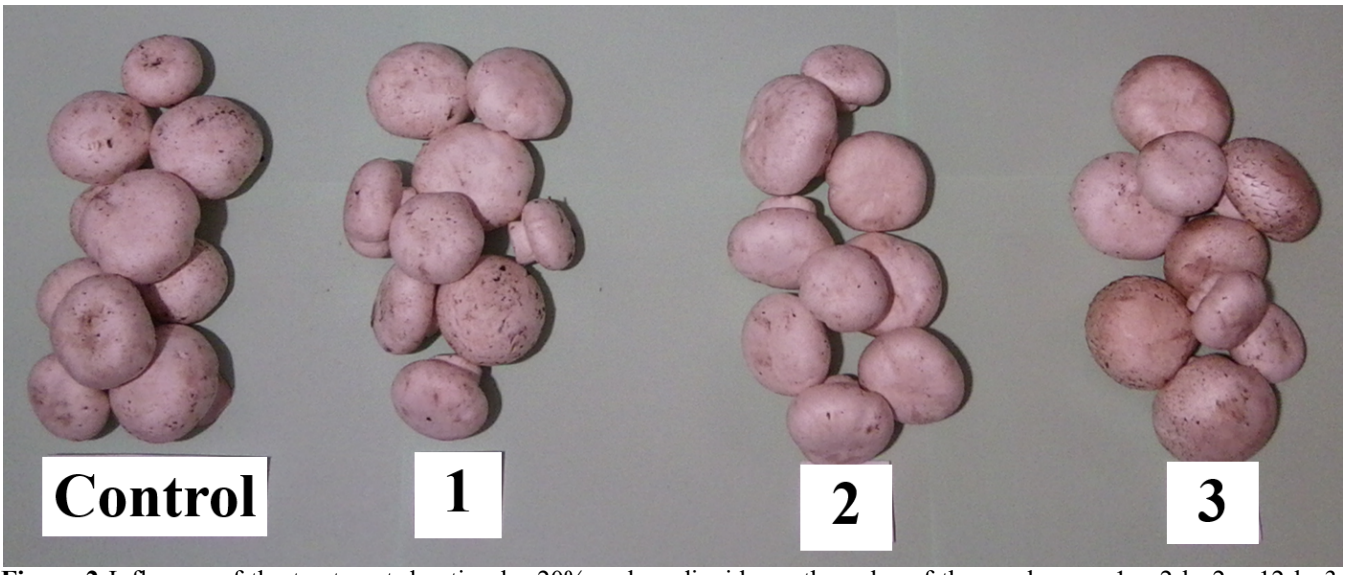


**Figure 2** Influence of the treatment duration by 20% carbon dioxide on the color of the mushroom: 1 – 2 h; 2 – 12 h; 3 – 22 h.

**Table 1** Preservation of mushrooms AB depending on the regimes of PHH by $CO_2$.

| The regime of treatment, % $CO_2$ – h | Output non-commodity mushrooms, % | | | | Output of the commodity mushrooms, % | | | | DC, g.cm$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| | FGC* | SGC** | TGC*** | average | FGC* | SGC** | TGC*** | Average | |
| **Strain IBK-25** | | | | | | | | | |
| control | 2.0 ±0.08 | 2.0 ±0.02 | 1.5 ±0.03 | 1.8 ±0.05 | 93.0 ±0.51 | 93.1 ±0.61 | 93.9 ±0.55 | 93.5 ±0.36 | 5.58 ±0.21 |
| 20% – 2 h | 1.8 ±0.05 | 2.0 ±0.04 | 1.7 ±0.02 | 1.8 ±0.05 | 93.3 ±0.47 | 93.3 ±0.47 | 93.7 ±0.43 | 93.5 ±0.41 | 5.60 ±0.18 |
| 20% – 12 h | 1.2 ±0.05 | 1.0 ±0.02 | 1.0 ±0.03 | 1.0 ±0.02 | 94.5 ±0.6 | 94.9 ±0.52 | 95.0 ±0.61 | 94.9 ±0.47 | 5.65 ±0.22 |
| 20% – 22 h | 4.0 ±0.18 | 4.8 ±0.22 | 4.5 ±0.18 | 4.4 ±0.24 | 91.9 ±0.38 | 91.3 ±0.47 | 91.5 ±0.37 | 91.6 ±0.31 | 5.42 ±0.21 |
| **Strain IBK-15** | | | | | | | | | |
| control | 2.5 ±0.06 | 2.9 ±0.01 | 2.2 ±0.03 | 2.5 ±0.12 | 92.3 ±0.52 | 92.0 ±0.69 | 93.0 ±0.61 | 92.5 ±0.51 | 5.48 ±0.24 |
| 20% – 2 h | 2.5 ±0.09 | 3.1 ±0.04 | 2.0 ±0.04 | 2.5 ±0.18 | 92.4 ±0.68 | 92.0 ±0.8 | 93.3 ±0.44 | 92.6 ±0.47 | 5.51 ±0.27 |
| 20% – 12 h | 1.9 ±0.1 | 1.5 ±0.06 | 1.0 ±0.01 | 1.5 ±0.03 | 93.7 ±0.6 | 94.2 ±0.51 | 94.9 ±0.52 | 94.2 ±0.64 | 5.56 ±0.19 |
| 20% – 22 h | 5.1 ±0.04 | 5.4 ±0.18 | 4.9 ±0.16 | 5.1 ±0.11 | 90.6 ±0.47 | 90.5 ±0.44 | 91.0 ±0.71 | 90.7 ±0.41 | 5.37 ±0.16 |

Note: * – The first growing cycle; ** – The second growing cycle; *** – The third growing cycle. Values are means ± standard deviation, $p \leq 0.05$.

A positive effect on the organoleptic characteristics of the mushrooms was established in the variant of treatment by 20% $CO_2$ for 12 h. There was no foreign smell; the fruiting bodies remained whole and dense. As can be seen in Figure 2, the mushrooms of AB had advantages over control in appearance at this regime.

However, it should be noted that photos do not fully demonstrate changes in the appearance of mushrooms. The difference is more clearly visible in the organoleptic evaluation products in the boxes. The better appearance of mushrooms at this regime, compared with the control, can be explained by inhibition of the activity of the enzyme tyrosine as a result of $CO_2$ treatment. As a consequence, the formation of melanins slows down, which causes enzymatic browning of AB. A similar result in terms of inhibition of the enzyme tyrosine activity as a result of treatment by high concentrations of $CO_2$ was obtained in the investigations of straw mushrooms (*Volvariella volvacea*) **(Jamjumroon et al., 2013; Jamjumroon et al., 2012)**.

PO in contrast to the AB did not change in appearance at different regimes of $CO_2$ treatment.

Preservation of AB and PO depending on the regime of PHH was determined by the results of studies of three cycles of cultivation (repetitions). The highest yield of marketable products was observed at the regime of treatment by 20% $CO_2$ within 12 h (Table 1).

For mushrooms AB of the IBK-25 strain, on average, was 94.9% that is 1.4% more than the control variant (93.5%). For strain IBK-15, this index was 94.2% (in the control variant – 92.5%). The duration of treatment during 2 h did not significantly affect the output of marketable fruit bodies. Depending on the growing cycle, they were 93.3 – 93.7%, while in the control variant – 93.0 – 93.9%.

Negatively affect had a variant of treatment by $CO_2$ for 22 h on the yield of marketable products. On average, during three growing cycles, their quantity decreased relative to control by 1.8-1.9%, depending on the strain of the AB. The regime of treatment by 20% $CO_2$ during 2 h had little effect on the natural LW (Table 2) and quantity of non-marketable fruiting bodies of mushrooms. Such a short-term treatment cannot significantly change the vital processes that take place in the mushrooms after harvest. The treatment by carbon dioxide also affected the

**Table 2** Natural LW of mushrooms AB depending on the regimes of PHH by $CO_2$.

| The regime of treatment, % $CO_2$ – h | Natural LW, % | | | |
|---|---|---|---|---|
| | FGC* | SGC** | TGC*** | average |
| **Strain IBK-25** | | | | |
| control | 5.0 ±0.2 | 4.9 ±0.14 | 4.6 ±0.2 | 4.8 ±0.15 | 2.0 ±0.08 |
| 20% – 2 h | 4.9 ±0.1 | 4.7 ±0.18 | 4.6 ±0.14 | 4.7 ±0.21 | 1.8 ±0.05 |
| 20% – 12 h | 4.3 ±0.11 | 4.1 ±0.1 | 4.0 ±0.17 | 4.1 ±0.17 | 1.2 ±0.05 |
| 20% – 22 h | 4.1 ±0.12 | 3.9 ±0.15 | 4.0 ±0.12 | 4.0 ±0.12 | 4.0 ±0.18 |
| **Strain IBK-15** | | | | |
| control | 5.2 ±0.14 | 5.1 ±0.19 | 4.8 ±0.1 | 5.0 ±0.17 | 2.5 ±0.06 |
| 20% – 2 h | 5.1 ±0.11 | 4.9 ±0.12 | 4.7 ±0.15 | 4.9 ±0.12 | 2.5 ±0.09 |
| 20% – 12 h | 4.4 ±0.18 | 4.3 ±0.16 | 4.1 ±0.09 | 4.3 ±0.1 | 1.9 ±0.1 |
| 20% – 22 h | 4.3 ±0.1 | 4.1 ±0.11 | 4.1 ±0.12 | 4.2 ±0.19 | 5.1 ±0.04 |

Note: * – The first growing cycle; ** – The second growing cycle; *** – The third growing cycle. Values are means ± standard deviation, $p \leq 0.05$.

**Table 3** Natural LW of mushrooms PO depending on the regimes of PHH by $CO_2$.

| The regime of treatment, % $CO_2$ – h | Natural LW, % | | | |
|---|---|---|---|---|
| | FGC* | SGC** | TGC*** | average |
| **Strain HK-35** | | | | |
| control | 6.1 ±0.15 | 6.0 ±0.11 | 5.7 ±0.12 | 5.9 ±0.17 |
| 20% – 2 h | 5.9 ±0.11 | 5.8 ±0.17 | 5.7 ±0.1 | 5.8 ±0.1 |
| 20% – 12 h | 5.4 ±0.12 | 5.2 ±0.14 | 5.1 ±0.1 | 5.2 ±0.14 |
| 20% – 22 h | 5.2 ±0.15 | 5.0 ±0.14 | 5.1 ±0.14 | 5.1 ±0.12 |
| **Strain Amycel 3000** | | | | |
| control | 5.5 ±0.15 | 5.4 ±0.18 | 5.1 ±0.21 | 5.3 ±0.11 |
| 20% – 2 h | 5.3 ±0.1 | 5.3 ±0.14 | 5.2 ±0.17 | 5.3 ±0.15 |
| 20% – 12 h | 4.9 ±0.1 | 4.7 ±0.12 | 4.6 ±0.14 | 4.7 ±0.2 |
| 20% – 22 h | 4.7 ±0.2 | 4.5 ±0.11 | 4.6 ±0.17 | 4.6 ±0.13 |

Note: * – The first growing cycle; ** – The second growing cycle; *** – The third growing cycle. Values are means ± standard deviation, $p \leq 0.05$.

DC of mushrooms (Table 1). There best values were observed for treatment during 12 h (5.56 and 5.65 $g.cm^{-1}$), and in the control variant – 5.48 and 5.58 $g.cm^{-1}$, respectively.

The regime of the mushrooms treatment during 22 h had positive effects on the natural LW (Table 2). In this regime, a natural loss is slightly lower (4.0 – 4.2% depending on the strain), compared with the control (4.8 – 5.0%), but at the same time in the experimental version significantly increases the quantity of non-marketable fruiting bodies (from 1.8 – 2.5 to 4.4 – 5.1%, respectively).

The fruiting bodies of AB of the strain IBK-15 have larger fruiting bodies compared to the strain IBK-25, respectively, and a larger area of moisture evaporation. This, in turn, affected the natural LW during storage (4.2 – 5.0% for strain IBK-15) against 4.0 – 4.8% for strain IBK-25, depending on the regime of PHH.

The tendencies concerning the influence of PHH of 20% $CO_2$ on the natural LW, quantity of a commodity, and non-commodity fruit bodies in the AB were characteristic and for PO (Table 3). There was a slight increase in the yield of marketable products – by 0.6 – 0.9% relative to the control for the twelve-hour treatment by carbon dioxide. This is due to the breaking of vital processes in mushrooms as a result of $CO_2$ treatment. The total LW during storage of PO mushrooms in these conditions was formed mainly due to natural losses. For strain NK-35 their value was 5.2%, for Amycel 3000 – 4.7%. The quantity of non-commodity fruit bodies was 0.5 and 1.5%, respectively (Table 4). PO of strain Amycel 3000 had a higher number of non-marketable fruiting bodies that explain the botanical features of the formation of their clusters. The clusters of this strain had a large number of small fruit bodies, which after storage for 6 days dried up or became watery.

The treatment OP by $CO_2$ during 22 h harmed the quantity of non-marketable products.

Their numbers increased up to 3.4 and 3.6%, compared with controls – 0.8 and 1.8%, depending on the strain. Some mushrooms had brown spots. In our opinion, the reason for this is the burns of the tissues of the mushrooms by carbonic acid that was formed by the interaction of condensate, which appeared as a result of respiration of products and carbon dioxide of high concentration.

Natural losses of mushrooms during storage were formed due to the evaporation of moisture and loss of chemical substances (Tables 5 and 6).

The postharvest handling by carbon dioxide during the storage of AB mushrooms contributes to the preservation of DM. It explains by the inhibition effects of $CO_2$ on the respiration processes and development of the mushroom's fruiting body that decreased sugar consumption. Thus, at the end of storage for the mushroom of strain IBK-25, the amount of DM in the fruiting bodies of the experimental variants were 8.5, 8.6, and 8.4%, while in the control variant – 8.3%.

**Table 4** Preservation of mushrooms PO depending on the regimes of PHH by $CO_2$.

| The regime of treatment, % $CO_2$ – h | Output non-commodity mushrooms, % | | | | Output of the commodity mushrooms, % | | | |
|---|---|---|---|---|---|---|---|---|
| | FGC* | SGC** | TGC*** | average | FGC* | SGC** | TGC*** | average |
| **Strain HK-35** | | | | | | | | |
| control | 0.9 ±0.01 | 0.9 ±0.01 | 0.5 ±0.02 | 0.8 ±0.03 | 93.0 ±0.41 | 93.1 ±0.36 | 93.8 ±0.5 | 93.4 ±0.47 |
| 20% – 2 h | 0.7 ±0.01 | 0.9 ±0.01 | 0.7 ±0.03 | 0.8 ±0.02 | 93.4 ±0.3 | 93.3 ±0.32 | 93.6 ±0.62 | 93.4 ±0.35 |
| 20% – 12 h | 0.5 ±0.02 | 0.6 ±0.02 | 0.4 ±0.01 | 0.5 ±0.02 | 94.1 ±0.28 | 94.2 ±0.21 | 94.5 ±0.41 | 94.3 ±0.4 |
| 20% – 22 h | 3.0 ±0.01 | 3.7 ±0.03 | 3.4 ±0.02 | 3.4 ±0.04 | 91.8 ±0.21 | 91.3 ±0.37 | 91.5 ±0.64 | 91.5 ±0.62 |
| **Strain Amycel 3000** | | | | | | | | |
| control | 1.3 ±0.01 | 2.5 ±0.01 | 1.6 ±0.01 | 1.8 ±0.03 | 93.2 ±0.4 | 92.1 ±0.5 | 93.3 ±0.28 | 92.9 ±0.24 |
| 20% – 2 h | 1.6 ±0.01 | 2.0 ±0.02 | 1.6 ±0.01 | 1.7 ±0.02 | 93.1 ±0.38 | 92.0 ±0.2 | 93.2 ±0.33 | 92.8 ±0.3 |
| 20% – 12 h | 1.2 ±0.01 | 1.9 ±0.01 | 1.5 ±0.02 | 1.5 ±0.02 | 93.9 ±0.5 | 92.7 ±0.3 | 93.9 ±0.3 | 93.5 ±0.2 |
| 20% – 22 h | 3.5 ±0.04 | 4.2± 0.03 | 3.1 ±0.02 | 3.6 ±0.02 | 91.8 ±0.33 | 91.3 ±0.3 | 92.3 ±0.3 | 91.8 ±0.2 |

Note: * – The first growing cycle; ** – The second growing cycle; *** – The third growing cycle. Values are means ± standard deviation, $p \leq 0.05$.

**Table 5** The changes in the chemical substances of mushrooms AB depend on the regimes of PHH by $CO_2$.

| The regime of treatment, % $CO_2$ – h | DM, % | | PN, % | | AA, mg% | |
|---|---|---|---|---|---|---|
| | before storage | after storage | before storage | after storage | before storage | after storage |
| **Strain IBK-25** | | | | | | |
| control | 9.0 ±0.11 | 8.3 ±0.08 | 3.7 ±0.02 | 3.7 ±0.01 | 7.5 ±0.05 | 6.4 ±0.03 |
| 20% – 2 h | 9.0 ±0.11 | 8.5 ±0.07 | 3.7 ±0.02 | 3.7 ±0.02 | 7.5 ±0.05 | 6.5 ±0.01 |
| 20% – 12 h | 9.0 ±0.11 | 8.6 ±0.04 | 3.7 ±0.02 | 3.7 ±0.01 | 7.5 ±0.05 | 6.4 ±0.02 |
| 20% – 22 h | 9.0 ±0.11 | 8.4 ±0.04 | 3.7 ±0.02 | 3.5 ±0.01 | 7.5 ±0.05 | 6.4 ±0.02 |
| **Strain IBK-15** | | | | | | |
| control | 8.6 ±0.14 | 7.8 ±0.11 | 3.5 ±0.00 | 3.5 ±0.02 | 7.4 ±0.03 | 6.3 ±0.01 |
| 20% – 2 h | 8.6 ±0.14 | 7.9 ±0.14 | 3.5 ±0.00 | 3.5 ±0.01 | 7.4 ±0.03 | 6.4 ±0.03 |
| 20% – 12 h | 8.6 ±0.14 | 8.0 ±0.17 | 3.5 ±0.00 | 3.5 ±0.01 | 7.4 ±0.03 | 6.3 ±0.02 |
| 20% – 22 h | 8.6 ±0.14 | 7.8 ±0.12 | 3.5 ±0.00 | 3.4 ±0.01 | 7.4 ±0.03 | 6.2 ±0.02 |

Note: Values are means ± standard deviation. $p \leq 0.05$.

**Table 6** The changes in the chemical substances of mushrooms OP depend on the regimes of PHH by $CO_2$.

| The regime of treatment, % $CO_2$ – h | DM, % | | PN, % | | AA, mg% | |
|---|---|---|---|---|---|---|
| | before storage | after storage | before storage | after storage | before storage | after storage |
| **Strain HK-35** | | | | | | |
| control | 12.5 ±0.21 | 11.4 ±0.24 | 3.3 ±0.01 | 3.3 ±0.01 | 10.5 ±0.24 | 9.2 ±0.17 |
| 20% – 2 h | 12.5 ±0.21 | 11.5 ±0.18 | 3.3 ±0.01 | 3.2 ±0.02 | 10.5 ±0.24 | 9.3 ±0.1 |
| 20% – 12 h | 12.5 ±0.21 | 11.7 ±0.14 | 3.3 ±0.01 | 3.3 ±0.02 | 10.5 ±0.24 | 9.5 ±0.13 |
| 20% – 22 h | 12.5 ±0.21 | 11.3 ±0.11 | 3.3 ±0.01 | 3.1 ±0.02 | 10.5 ±0.24 | 9.5 ±0.08 |
| **Strain Amycel 3000** | | | | | | |
| control | 12.3 ±0.18 | 11.2 ±0.04 | 3.3 ±0.02 | 3.3 ±0.01 | 10.6 ±0.2 | 9.2 ±0.12 |
| 20% – 2 h | 12.3 ±0.18 | 11.3 ±0.08 | 3.3 ±0.02 | 3.3 ±0.01 | 10.6 ±0.2 | 9.2 ±0.14 |
| 20% – 12 h | 12.3 ±0.18 | 11.5 ±0.01 | 3.3 ±0.02 | 3.3 ±0.01 | 10.6 ±0.2 | 9.6 ±0.04 |
| 20% – 22 h | 12.3 ±0.18 | 11.1 ±0.03 | 3.3 ±0.02 | 3.2 ±0.02 | 10.6 ±0.2 | 9.6 ±0.08 |

Note: Values are means ± standard deviation, $p \leq 0.05$.

Carbon dioxide in most cases did not significantly affect the changes in the amount of PN and AA compared to the control. Only after treatment duration by 22 hours, the amount of PN partially changed.

For strain IBK-25 its amount was 3.5% for the experimental variant and 3.7% for the control; for strain IBK-15 – 3.4 and 3.5%, respectively. This may be due to oxygen deficiency and protein breakdown. Similar trends in the effect of postharvest handling by carbon dioxide on the content of chemical substances were observed for OP mushrooms. The amount of DM in mushrooms of strain NK-35 during the storage period decreased from 12.5 to 11.3 – 11.7% depending on the exposure and in the control variant – up to 11.4%.

Most DM was preserved in fruiting bodies after twelve hours of treatment: in strain NK-35 – 11.7% and in the strain Amycel 3000 – 11.5%, that it is significantly more than the control variant.

There are similar to the AB in PO mushrooms wasn't observed significant changes in the quantity AA during the treatment by $CO_2$.

**Table 7** IR of mushrooms AB depends on the regime of PHH by carbon dioxide, $mL \cdot kg^{-1} \cdot h^{-1}$ for $CO_2$.

| Regime PHH, $\%CO_2$ – h | Before storage | Duration of storage, days 1 | 2 | 3 | 4 | 5 | 6 | average |
|---|---|---|---|---|---|---|---|---|
| **Strain IBK-25** | | | | | | | | |
| control | 4.5 ±0.2 | 9.2 ±0.34 | 11.3 ±0.33 | 5.3 ±0.19 | 3.4 ±0.03 | 3.0 ±0.04 | 2.5 ±0.02 | 5.6 ±0.08 |
| 20% – 2 h | 4.3 ±0.17 | 8.7 ±0.27 | 10.5 ±0.21 | 5.0 ±0.12 | 3.2 ±0.01 | 2.7 ±0.01 | 2.3 ±0.01 | 5.2 ±0.04 |
| 20% – 12 h | 4.3 ±0.21 | 8.0 ±0.21 | 9.2 ±0.21 | 4.5 ±0.12 | 3.0 ±0.01 | 2.5 ±0.02 | 2.2 ±0.03 | 4.8 ±0.05 |
| 20% – 22 h | 4.4 ±0.08 | 7.5 ±0.21 | 8.1 ±0.28 | 4.0 ±0.08 | 2.7 ±0.02 | 2.5 ±0.01 | 2.1 ±0.01 | 4.5 ±0.05 |
| **Strain IBK-15** | | | | | | | | |
| control | 4.2 ±0.2 | 9.2 ±0.22 | 11.0 ±0.28 | 5.3 ±0.12 | 3.5 ±0.01 | 3.3 ±0.02 | 2.6 ±0.01 | 5.6 ±0.05 |
| 20% – 2 h | 4.3 ±0.11 | 8.6 ±0.15 | 10.5 ±0.21 | 4.9 ±0.11 | 3.4 ±0.01 | 2.8 ±0.01 | 2.3 ±0.02 | 5.3 ±0.12 |
| 20% – 12 h | 4.0 ±0.14 | 7.8 ±0.1 | 8.9 ±0.14 | 4.1 ±0.05 | 3.0 ±0.02 | 2.6 ±0.02 | 2.4 ±0.01 | 4.7 ±0.09 |
| 20% – 22 h | 4.4 ±0.11 | 7.2 ±0.17 | 7.9 ±0.11 | 3.8 ±0.05 | 2.6 ±0.01 | 2.5 ±0.02 | 2.0 ±0.01 | 4.3 ±0.05 |

Note: Values are means ± standard deviation, $p \leq 0.05$.

**Table 8** IR of mushrooms OP depend on the regime of PHH by carbon dioxide, $mL \cdot kg^{-1} \cdot h^{-1}$ for $CO_2$.

| Regime PHH, $\%CO_2$ – h | Before storage | Duration of storage, days 1 | 2 | 3 | 4 | 5 | 6 | average |
|---|---|---|---|---|---|---|---|---|
| **Strain HK-35** | | | | | | | | |
| control | 6.0 ±0.24 | 11.1 ±0.21 | 12.5 ±0.15 | 7.1 ±0.04 | 4.0 ±0.01 | 3.4 ±0.02 | 2.9 ±0.01 | 6.7 ±0.03 |
| 20% – 2 h | 6.3 ±0.28 | 9.2 ±0.17 | 11.1 ±0.11 | 6.5 ±0.02 | 3.7 ±0.01 | 3.0 ±0.01 | 2.8 ±0.00 | 6.1 ±0.03 |
| 20% – 12 h | 6.2 ±0.22 | 8.1 ±0.11 | 10.4 ±0.12 | 5.8 ±0.03 | 3.6 ±0.01 | 3.0 ±0.02 | 2.6 ±0.01 | 5.7 ±0.04 |
| 20% – 22 h | 6.5 ±0.21 | 7.4 ±0.04 | 9.9 ±0.11 | 5.6 ±0.02 | 3.1 ±0.02 | 2.4 ±0.02 | 2.3 ±0.01 | 5.3 ±0.04 |
| **Strain Amycel 3000** | | | | | | | | |
| control | 5.7 ±0.17 | 9.8 ±0.2 | 13.7 ±0.05 | 8.4 ±0.03 | 4.5 ±0.01 | 3.5 ±0.00 | 3.2 ±0.01 | 7.0 ±0.21 |
| 20% – 2 h | 6.0 ±0.13 | 8.8 ±0.18 | 10.7 ±0.02 | 8.0 ±0.03 | 4.0 ±0.01 | 3.1 ±0.00 | 3.0 ±0.01 | 6.2 ±0.14 |
| 20% – 12 h | 6.0 ±0.12 | 7.5 ±0.12 | 9.6 ±0.04 | 7.2 ±0.01 | 4.0 ±0.02 | 2.7 ±0.00 | 2.4 ±0.01 | 5.6 ±0.11 |
| 20% – 22 h | 5.6 ±0.12 | 6.8 ±0.08 | 8.3 ±0.03 | 6.8 ±0.02 | 3.2 ±0.01 | 2.7 ±0.02 | 2.2 ±0.01 | 5.1 ±0.12 |

Note: Values are means ± standard deviation, $p \leq 0.05$.

**Table 9** HR of mushrooms of AB after PHH by $CO_2$, $kJ.kg^{-1}.day^{-1}$.

| Regime PHH, $\%CO_2$ – h | Before storage | Duration of storage, days 1 | 2 | 3 | 4 | 5 | 6 | average |
|---|---|---|---|---|---|---|---|---|
| **Strain IBK-25** | | | | | | | | |
| control | 1.2 ±0.2 | 2.4 ±0.34 | 2.9 ±0.33 | 1.4 ±0.19 | 0.9 ±0.03 | 0.8 ±0.04 | 0.6 ±0.02 | 1.4 ±0.08 |
| 20% – 2 h | 1.1 ±0.17 | 2.2 ±0.27 | 2.7 ±0.21 | 1.3 ±0.12 | 0.8 ±0.01 | 0.7 ±0.01 | 0.6 ±0.01 | 1.3 ±0.04 |
| 20% – 12 h | 1.1 ±0.21 | 2.1 ±0.21 | 2.4 ±0.21 | 1.2 ±0.12 | 0.8 ±0.01 | 0.6 ±0.02 | 0.6 ±0.03 | 1.2 ±0.05 |
| 20% – 22 h | 1.1 ±0.08 | 1.9 ±0.21 | 2.1 ±0.28 | 1.0 ±0.08 | 0.7 ±0.02 | 0.6 ±0.01 | 0.5 ±0.01 | 1.1 ±0.05 |
| **Strain IBK-15** | | | | | | | | |
| control | 1.1 ±0.2 | 2.4 ±0.22 | 2.8 ±0.28 | 1.4 ±0.12 | 0.9 ±0.01 | 0.8 ±0.02 | 0.7 ±0.01 | 1.4 ±0.05 |
| 20% – 2 h | 1.1 ±0.11 | 2.2 ±0.15 | 2.7 ±0.21 | 1.3 ±0.11 | 0.9 ±0.01 | 0.7 ±0.01 | 0.6 ±0.02 | 1.3 ±0.12 |
| 20% – 12 h | 1.0 ±0.14 | 2.0 ±0.1 | 2.3 ±0.14 | 1.1 ±0.05 | 0.8 ±0.02 | 0.7 ±0.02 | 0.6 ±0.01 | 1.2 ±0.09 |
| 20% – 22 h | 1.1 ±0.11 | 1.8 ±0.17 | 2.0 ±0.11 | 1.0 ±0.05 | 0.7 ±0.01 | 0.6 ±0.02 | 0.5 ±0.01 | 1.1 ±0.05 |

Note: Values are means ± standard deviation, $p \leq 0.05$.

**Table 10** HR of mushrooms of PO after PHH by $CO_2$, $kJ \cdot kg^{-1} \cdot day^{-1}$.

| Regime PHH, $\%CO_2$ – h | Before storage | Duration of storage, days 1 | 2 | 3 | 4 | 5 | 6 | average |
|---|---|---|---|---|---|---|---|---|
| **Strain HK-35** | | | | | | | | |
| control | 1.5 ±0.24 | 2.8 ±0.21 | 3.2 ±0.15 | 1.8 ±0.04 | 1.0 ±0.01 | 0.9 ±0.02 | 0.7 ±0.01 | 1.7 ±0.03 |
| 20% – 2 h | 1.6 ±0.28 | 2.4 ±0.17 | 2.8 ±0.11 | 1.7 ±0.02 | 0.9 ±0.01 | 0.8 ±0.01 | 0.7 ±0.00 | 1.6 ±0.03 |
| 20% – 12 h | 1.6 ±0.22 | 2.1 ±0.11 | 2.7 ±0.12 | 1.5 ±0.03 | 0.9 ±0.01 | 0.8 ±0.02 | 0.7 ±0.01 | 1.5 ±0.04 |
| 20% – 22 h | 1.7 ±0.21 | 1.9 ±0.04 | 2.5 ±0.11 | 1.4 ±0.02 | 0.8 ±0.02 | 0.6 ±0.02 | 0.6 ±0.01 | 1.4 ±0.04 |
| **Strain Amycel 3000** | | | | | | | | |
| control | 1.5 ±0.17 | 2.5 ±0.2 | 3.5 ±0.05 | 2.2 ±0.03 | 1.2 ±0.01 | 0.9 ±0.00 | 0.8 ±0.01 | 1.8 ±0.21 |
| 20% – 2 h | 1.5 ±0.13 | 2.3 ±0.18 | 2.7 ±0.02 | 2.1 ±0.03 | 1.0 ±0.01 | 0.8 ±0.00 | 0.8 ±0.01 | 1.6 ±0.14 |
| 20% – 12 h | 1.5 ±0.12 | 1.9 ±0.12 | 2.5 ±0.04 | 1.8 ±0.01 | 1.0 ±0.02 | 0.7 ±0.00 | 0.6 ±0.01 | 1.4 ±0.11 |
| 20% – 22 h | 1.4 ±0.12 | 1.7 ±0.08 | 2.1 ±0.03 | 1.7 ±0.02 | 0.8 ±0.01 | 0.7 ±0.02 | 0.6 ±0.01 | 1.3 ±0.12 |

Note: Values are means (n = 3) ± standard deviation, $p \leq 0.05$.

The amount of PN changed only after treatment during 22 h (3.1 and 3.2% against 3.3% in the control variant).

Analysis of the IR of mushrooms indicated that after harvesting quantity of carbon dioxide released during respiration begins to increase sharply, and then decreases during storage due to low temperatures (Table **7** and 8).

Significantly affected the amount of $CO_2$ released had postharvest treatment by carbon dioxide, especially in the first 3 days.

As can be seen from Table 7 and Table 8, there is a tendency that with increasing duration of treatment by $CO_2$, the process of respiration is suppressed. It is the least intense respiration was as a result of treatment $CO_2$ during 22 h. It was typical for both AB mushrooms and OP.

Thus, for AP of strain IBK-25 after a day of storage, the amount of $CO_2$ released in the experimental variant was 7.5 but in the control – 9.2 $mL.kg^{-1}.h^{-1}$ for $CO_2$; after two days – 8.1 and 11.3 $mL.kg^{-1}.h^{-1}$ for $CO_2$; after three days – 4.0 and 5.3 $mL.kg^{-1}.h^{-1}$ for $CO_2$, respectively. In the future, the IR of the control and experimental variants was gradually equalized and at the end of storage did not differ significantly (2.1 – 2.3 $mL.kg^{-1}.h^{-1}$ for $CO_2$ in the experimental variants and 2.5 in the control) (Table 7 and 8).

Based on the data on the intensity of respiration, calculations of the HR of mushrooms were performed (Tables 9 and 10).

Postharvest short-term treatment from 2 till 22 h by carbon dioxide at a concentration of 20% affected the average HR of mushrooms during storage.

Since the intensity of HR directly depends on the IR, so the trends of its change are similar.

Thus, for the OP of the strain Amycel 3000, these indexes were: after two hours of treatment – 1.6 kJ. $kg^{-1}.day^{-1}$; after 12 h – 1.4; after 22 hours – 1.3; control – 1.8 $kJ.kg^{-1}.day^{-1}$.

## CONCLUSION

Postharvest short-term handling mushrooms of AB and PO by carbon dioxide at a concentration of 20% is an effective method that reduces the level of natural losses, increases the yield of marketable products, helps preserve their chemical substances, and inhibits the intensity of physiological processes in the product (IR and HR). According to all the obtained data, the most effective is the application of the regime of mushrooms treatment by $CO_2$ during 12 h. Increasing the time of treatment by carbon dioxide up to 22 h most effectively inhibits the processes of respiration (on the 0.4 – 3.2% for AB and the 0.6 – 5.4% for PO) and HR (on the 0.1 – 0.8% for AB and the 0.1 – 1.4% for PO), compared with the control and depending on the mushrooms' strain and duration of their storage. However, this regime promoted sharply increases the quantity of non-marketable products (on the 2.6% for AB and the 1.8 – 2.6% for PO) compared to the control and depending on the strain and wave of fruiting.

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# INFLUENCE OF ROOTSTOCKS ON THE PRODUCTIVITY AND CHEMICAL COMPOSITION OF *PRUNUS DOMESTICA* L. FRUITS

*Svetlana Motyleva, Galina Upadysheva, Tatyana Tumaeva*

## ABSTRACT

The influence of seedling and clonal rootstocks of different spreads on *Prunus domestica* L. plum fruits quality and productivity of Yaichnaya Sinyaya and Utro varieties was studied. The significant change of productivity and the fruit weight of the varieties under study was to determine under the influence of the rootstock. Depending on the scion-stock combination the plum tree's productivity varied from 7.5 kg/tr. (Utro/140-1) to 15.1 kg/tr. (Yaichnaya Sinyaya /Novinka) at the mean value of 11.5 kg/tr. Medium-growing rootstocks Novinka and OPA-15-2 provided the maximum value of the varieties productivity The significant productivity decrease relatively to seedling rootstock was stated for the combinations with low-growing rootstock 140-1. Soluble solids content in the fruits of Yaichnaya Sinyaya variety is higher than in the fruits of Utro variety, moreover, the highest values were determined on Novinka and OPA-15-2 rootstocks: on 4 – 4.5% higher in comparison with the fruits on the seeding rootstock at average. The rootstock causes less influence on titratable acids. The antioxidant activity of Yaichnaya Sinyaya variety fruits is 30% higher than that of Utro variety fruits on average. The maximum values of antioxidant activity in Yaichnaya Sinyaya variety fruits were fixed on OPA-15-2 rootstock (16.37%), the minimal ones – on Skorospelka Krasnaya rootstocks (14.68%). In the fruits of Utro variety the highest values were stated on OP-23-23 rootstock (13.16%), and the lowest ones – on the seedling rootstock (10.93%). The content of phenolic compounds sum is 60% higher in the fruits of Yaichnaya Sinyaya variety than in Utro variety ones on average. The decrease of the content of phenolic compounds sum was stated in the fruits of Utro variety on all the rootstocks in comparison with the combination Utro/seedling rootstock. The decreasing series of ash elements accumulation (K > P > Ca > Mg > Mo > S > Zn > Si) was determined. The strongest variety differences on total mineral element content were overvalued on medium-growing clonal rootstocks (Novinka, OP-23-23 and OPA-15-2). OPA-15-2 and OP-23-23 rootstocks provided the highest fruit quality on the combination of economic and biochemical parameters.

**Keywords:** *Prunus domestica* L; scion-stock combinations; productivity; fruits chemical composition

## INTRODUCTION

The popularity of domestic plum (*Prunus domestica* L.) in different horticultural zones of Russia is connected with ecological plasticity, winter resistance, early maturity, and stable productivity of cultivated varieties **(Upadysheva, 2015; Eremin and Brizhinov, 2011)**. New clonal stocks are used to duplicate new valuable varieties of fruit crops and to create intensive plantations because they favorably influence on adaptivity, early maturity, and productivity of grafted plants **(Upadysheva, 2017; Eremin et al., 2000)**. The spread, the start of fruiting, and the productivity of grafted plants depend on the rootstock **(Blazek and Pistekova, 2012; Hatton et al., 2015)**.

Fruits quality is a genetically associated character for each variety, but in the scientific literature there are data about the significant influence of a rootstock on the weight and quality of fruits **(Usenik et al., 2010; Orazem, Stampar, Hudina, 2011; Bartolini et al., 2014; Reig et al., 2018; Iglesias et al., 2019; Radovića et al., 2020; Karakaya et al., 2021)**.

Seedling rootstocks (seedlings of domestic plum and alucha) and clonal ones of different growing spread (strong-growing – 13-113, medium-growing – OPA-15-2, OP-23-23, SVG-11-19 and Novinka and low-growing – 140-1, VVA-1) are used for plums cultivation in the Central region of Russia **(Eremin, 2000)**. Many questions devoted to plum varieties propagation and scion-stock combination selection are successfully solved in the world; at the same time, the life length of grafted plants and the harvest quality depending on a variety and a rootstock are not studied enough.



**Figure 1** The garden of intensive type during blossoming.



**Figure 2** The plum fruits: Yaichnaya Sinyaya (on the left) and Utro (on the right).

The major part of the researches of the biochemical composition of plum fruits scion-stock combinations was connected with the rootstock influence on the content of soluble solids and titratable acids in the fruits **(Daza et al., 2008; Rato et al., 2008; Reig et al., 2018)**.

The main component of plum fruits' chemical composition is minerals. Among them, K, P, Mg, and Ca are found in big quantities, whereas other minerals such as Fe, B, Cr, Mn, Cu, and Zn are present in much fewer quantities **(Cosmulescu et al., 2017)**. The mineral composition changes and the content of bioactive compounds in fruits *Prunus domestica* L. depending on the rootstock were practically understudied. In scientific literature, the data about the stock influence on the leaves mineral composition are only presented **(Meland, 2010; Milošević and Milošević, 2011; Ionica et al., 2013; Reig et al., 2018)**. The accurate forecast of plum scion-stock combinations germinability and productivity and the stock influence on the nutrients and bioactive substances accumulation in plum fruits is very important for the output yield of high quality.

That is why this work aimed to determine the influence of different growing strength rootstocks on the fruits chemical composition and productivity of the domesticated plum varieties bred by Horticulture. 2 Federal State Budget Scientific Institution «Federal Scientific Selection and Technological Center of Horticulture and Nursery Breeding» (Federal Horticultural Research Center).

**Scientific hypothesis**

The domesticated plum (*Prunus domestica* L.) fruits productivity and quality, their biological composition depending on the used rootstocks are not studied enough. We have checked the influence of the strong-growing seedling and medium and low-growing clonal rootstocks on the fruit productivity, quality, and nutritional value of the plum varieties, i.e. Utro and Yaichnaya Sinyaya grown in Moscow region conditions. We supposed that on the base of the field and laboratory experiments the optimal stock for each variety that will provide the high productivity and valuable bioactive substances accumulation in the plum fruit will be found.

## MATERIAL AND METHODOLOGY

**Conditions of plant growing**

The field researches were held in 2018 – 2019 on the experimental *Prunus domestica* L. plantations, located at laboratory plot of Federal Horticultural Research Center for Breeding, Agrotechnology and Nursery in Moscow region (55° 56′ of North latitude, 37° 64′ East longitude). The plantation overall area was 0.5 ha. The garden of intensive type was set out in 2010 using the scheme 5 x 2.5 m. The soil in the row spacing was black fallow (Figure 1).

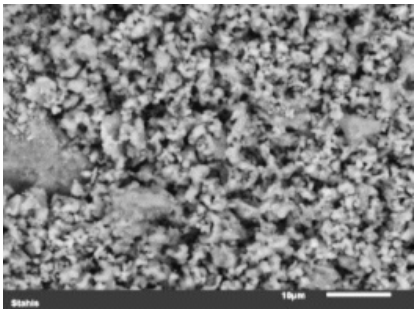
**Biological material**

The fruits of Utro and Yaichnaya Sinyaya varieties on 6 stocks, i.e. strong-growing *P. domestica* L. seedlings, medium-growing clonal rootstocks Skorospelka Krasnaya *(Prunus domestica* L.*),* Novinka (*Prunus bessyi* L.H. Bailey x *Prunus ussuriensis* Kovalev& Kostina), OP-23-23 (*Prunus pumila* L. x *Prunus salicina* Lindl. x *Prunus persica* Stokes), OPA-15-2 (*Prunus pumila* L. x *Prunus salicina* Lindl. x *Prunus cerasifera* Ehrh) and low-growing clonal rootstock 140-1 *(Prunus bessyi* L.H. Bailey x *Aflatunia ilmifolia* L.) were the object of the scientific studies. The samples were taken at the ripeness stage (Figure 2).

The biochemical studies were held in the laboratory of biochemistry and physiology of Federal Horticultural Research Center for Breeding, Agrotechnology, and Nursery.

**Chemicals**

All chemical substances chosen for the analysis were of an analytical sort and were bought from Sigma Aldrich (USA).



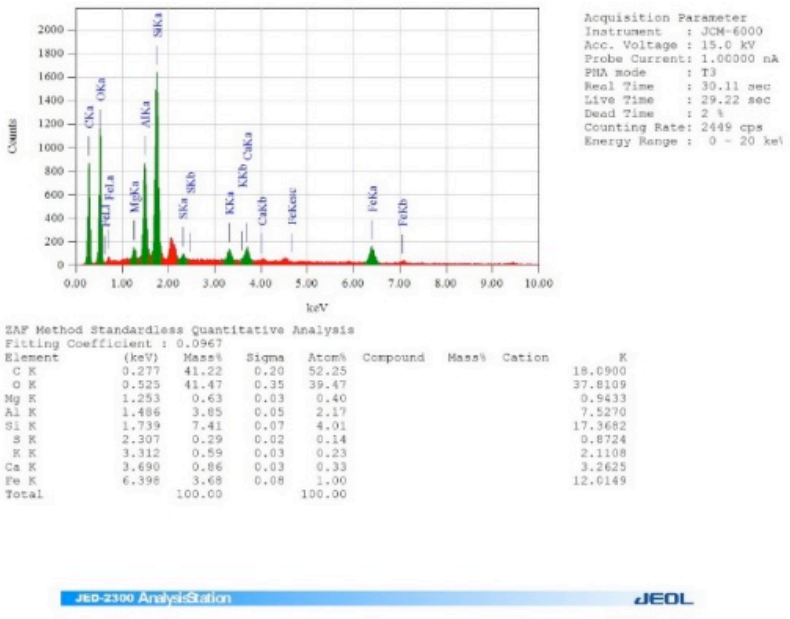



**Figure 3** ESD-analysis protocol.

**Sample preparation**

From representative not less than 500 g fruits probe, 300 g of fruit without stone were prepared. The mass was homogenized using the analytical homogenizer IKAA11 basic (Germany). Then it was extracted by double distilled water (to determine antioxidant activity and phenol compounds sum) and by pure methanol (to study the metabolites composition) and centrifugated at 4000 g (Sigma, Germany) within 10 min. The supernatant was used for measurement purposes. We performed all extractions in triplicate independent samples.

**Basic chemical analyses**

General biochemical parameters, i.e. soluble solids content (SSC) and total titratable acidity (TTA). SSC was determined via refractometric method according to **(GOST ISO 2173, 2013)**, the values were expressed in %. TTA was estimated via the potentiometric method by pH meter HI 2211 HANNA (Germany) via titrating with 10 N. NaOH and expressed in the equivalent of apple acid, % **(GOST ISO 750, 2013)**.

***Total phenolic compounds analysis***

The total phenolics amount was determined with Folin–Ciocalteu reagent according to the method described by **Velioglu et al. (1998)**. A standard curve with gallic acid was used. Different concentrations of gallic acid were prepared in distilled water, and absorbance was recorded at 750 nm. 100 µL of the diluted sample (1:10) was dissolved in 500 µL of Folin–Ciocalteu reagent and 1000 µL of distilled water. The solutions were mixed and incubated at room temperature for 1 min. After 1 min, 1500 µL of 20% sodium carbonate ($Na_2CO_3$) solution was added. The final mixture was shaken and then incubated for 2 h in the dark at room temperature. The absorbance was measured at 750 nm using a Helios Y UV–vis spectrophotometer and the results are expressed in mg of gallic acid (GEA) calculated on the wet weight of plants.

***Total antioxidant capacity***

The scavenging activity on the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically according to the method described by **Brand-Williams et al. (1995)**. The principle of the analysis was based on the colour change of DPPH solution from purple to yellow as the radical was quenched by antioxidants. The homogenized leaves were mixed with distilled water and methanol. The samples were put on the shaker Lab-PU-01 (Russia) for 6 hours, and then they were filtered and the antioxidant activity was measured in 10 minutes after interaction between the extract and the reagent. The absorbance was recorded at 517 nm to determine the concentration of the remaining DPPH. All measurements were performed in triplicate. The radical-scavenging activity was calculated as a percentage as follows:

DPPH radical-scavenging (%) = [(AC - AAt) / AC].100,

Where:
AC – DPPH solution absorption; AAt – absorption at the antioxidant presence.

The lower absorbance of the reaction mixture indicates a higher level of free radical scavenging activity.

***EDS - analysis***

The chemical composition of the basic ash components (Mg, Si, P, S, K, Ca, Zn, Mo) was determined by the method of energy dispersive spectrometry (ESD) on the analytical raster electron microscope JEOL JSM 6090 LA according to the methodology **(Motyleva, 2018).**

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 presented in Figure 3.

***Metabolic analysis by gas chromatography-mass spectrometry***

The metabolites analysis was fulfilled using the method of gas chromatography-mass spectrometry (GC-MS) via GCMS chromatograph JMS-Q1050GC («JEOL Ltd», Japan). Capillary column DB-5HT (Agilent, USA); length 30 m, inner diameter – 0.25 mm, the film thickness – 0.52 um, and gas-carrier — helium) was used. The temperature gradient during the analysis was within 40 – 280 °C, the injector and interface temperature – 250 °C, the ionic source – 200 °C. Gas flow in the column was equal to 2.0 $mL.min^{-1}$, split-flow injection mode, sample injected in volume 1 – 2 mcL of the evaporated extract. The analysis was held for 45 min. The derivation was held using silylation reagent N,O bis (trimethylsilyl) trifluoracetamide (BSTFA) following the method described by **Robbins (2003)**. The substances identification was done according to NIST-5 National Institute of Standards and Technology (USA) retention behavior and mass spectra the scanning range was 33 – 900 m/z. The substance identification credibility was within 75 – 98%.

**Number of repeated analyses:** 3

**Number of experiment replication:** 2

**Statistical analysis**

All the analyses were performed in triplicate. The results were expressed as mean values (n = 3) in standard deviation (*SD*). Statistical analyses were carried out through the Excel package (Microsoft Excel, v. 2016).

**RESULTS AND DISCUSSION**

We studied the plum trees' productivity of different scion-stock combinations in the field conditions, determined the fruit average weight, fulfilled the fruits organoleptic and degustation estimation. As a result of our studies, it was stated that during 2018 – 2019 the plum tree's productivity was 11.5 kg/tree on average, and depending on scion-stock combination it changed from 7.5 kg/tree (Utro/140-1) to 15.1 kg/tree (Yaichnaya Sinyaya/Novinka). This parameter was above average at the understudied varieties grafted on medium-growing clonal rootstocks Novinka and OPA-15-2. The minimum parameters were stated at the combinations with low-growing rootstock 140-1 (Table 1).

The rootstock influence on *P. domestica* L. productivity is discussed in the papers **(Ciobanu et al., 2009; Kaufmane et al., 2007**; **Popova et al., 2020)**.

Studied varieties belong to large-fruited ones with fruits weight more than 30 g. The Utro variety's average fruit weight is 31.2 g, but depending on the stock it varied from

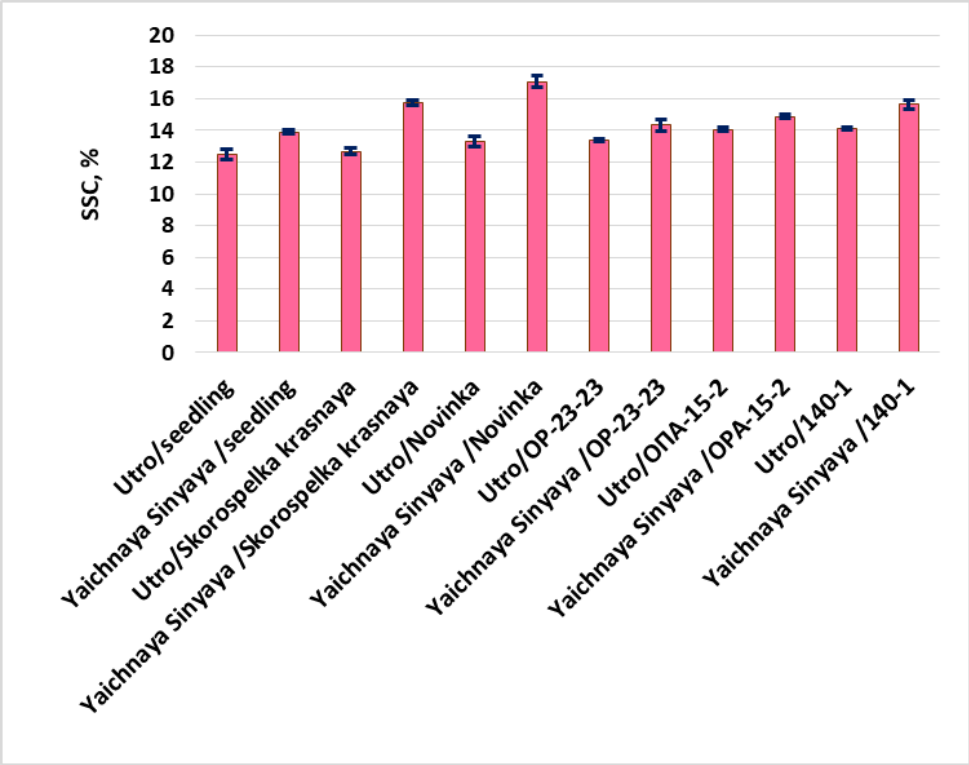



**Figure 4.** Soluble solids content in *Prunus domestica* L. fruits depending on the stock at average in 2018 – 2019; each value represents the mean of three independent experiments (±*SD*).

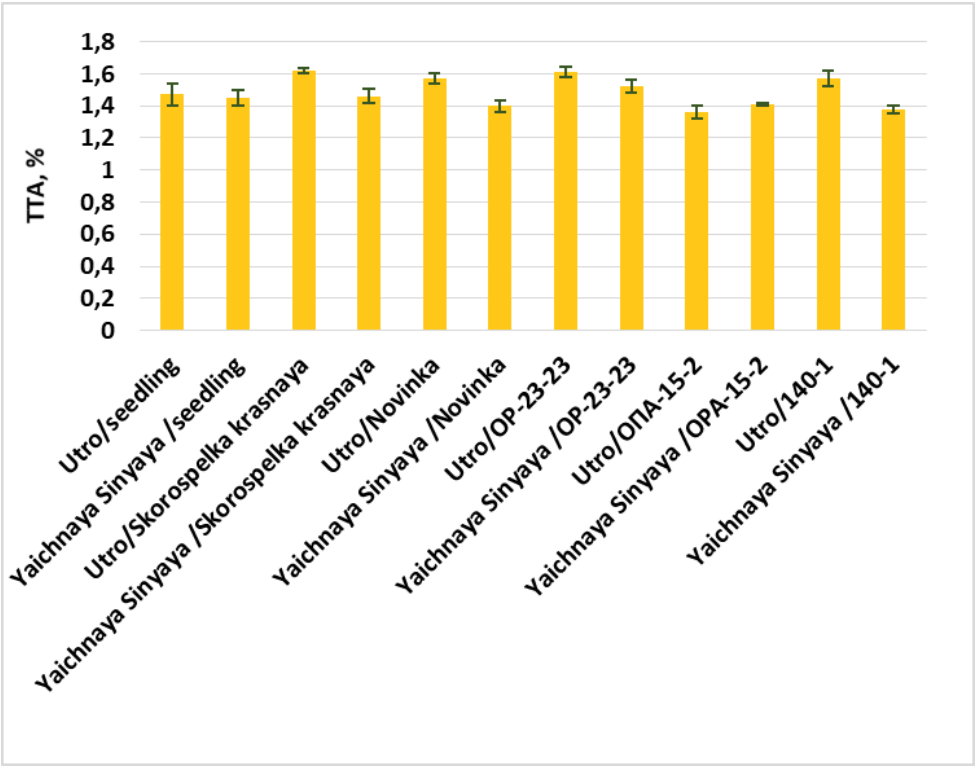



**Figure 5** Titratable acidity content in plum fruits of *Prunus domestica* L. depending on the stock at average in 2018–2019; each value represents the mean of three independent experiments (±*SD*). ).

29.7 g (seedlings *P. domestica* L.) to 33.8 g (OP-23-23). The understudies varieties fruits were significantly larger under OP-23-23 rootstock influence. The Utro variety fruit stone was separated from the flesh well, its portion in the fruit weight was 6% on average with variation from 5.7% (Novinka) to 6.5% (seedlings *P. domestica* L.).

The Yaichnaya Sinyaya variety fruit stone was larger and was near 7% of the fruit weight for all the combinations, it separated from the fruit flesh well. According to the degustation results, the understudied varieties of fruits were characterized with balanced sour-sweet taste and smell (4.2 – 4.8 scores). The degustation estimation had

**Table 1** The productivity, fetal mass and organoleptic estimation of *Prunus domestica* L. fruits depending on the stock at average in 2018 – 2019.

| Scion/rootstock combinations | Productivity (kg/tree) | Average fruit weight(g ±*SD*) | Degustation estimation, point |
|---|---|---|---|
| Utro/140-1 | 7.5±0.85 | 30.8±0.61 | 4.6 |
| Utro/Novinka | 14.6±2.11 | 29.7±0.44 | 4.6 |
| Utro/OPA-15-2 | 14.9±2.07 | 30.3±0.64 | 4.8 |
| Utro/OP-23-23 | 11.9±1.74 | 33.8±0.73 | 4.7 |
| Utro/ Skorospelka Krasnaya | 11.8±1.17 | 32.7±0.87 | 4.5 |
| Utro/ seedlings *P. domestica* | 11.3±1.65 | 29.7±0.65 | 4.2 |
| Yaichnaya Sinyaya/140-1 | 8.2±0.87 | 32.1±0.68 | 4.7 |
| Yaichnaya Sinyaya/Novinka | 15.1±2.69 | 31.3±0.67 | 4.7 |
| Yaichnaya Sinyaya/OPA-15-2 | 13.1±1.13 | 32.1±0.75 | 4.8 |
| Yaichnaya Sinyaya/OP-23-23 | 8.7±0.74 | 33.4±0.61 | 4.8 |
| Yaichnaya Sinyaya/Skorospelka Krasnaya | 10.7±0.95 | 32.4±0.65 | 4.6 |
| Yaichnaya Sinyaya/seedlings *P. domestica* | 10.1±0.99 | 32.2±0.53 | 4.6 |

**Table 2** The rootstock influence on *Prunus domestica* L. fruits antioxidant activity (AA) and phenolic compounds (PC) sum; the values represent the mean of three replicate ±*SD*.

| Scion/rootstock combinations | AA, % | PC, mg gallic acid (GEA)/1g fruit weight |
|---|---|---|
| Utro/seedlings *P. domestica* | 10.93 ±0.174 | 0.99 ±0.032 |
| V, % | 2.77 | 5.35 |
| Utro/Skorospelka Krasnaya | 13.90 ±0.967 | 0.43 ±0.041 |
| V, % | 7.25 | 8.78 |
| Utro/Novinka | 11.38 ±0.254 | 0.22 ±0.049 |
| V, % | 3.76 | 17.82 |
| Utro/OPA-15-2 | 11.33 ±0.239 | 0.43 ±0.024 |
| V, % | 2.08 | 8.73 |
| Utro/140-1 | 12.90 ±0.181 | 0.46 ±0.018 |
| V, % | 2.48 | 6.27 |
| Utro/seedlings *P. domestica* | 16.25 ±0.055 | 0.70 ±0.006 |
| V, % | 0.58 | 1.41 |
| Yaichnaya Sinyaya/Skorospelka Krasnaya | 14.68 ±0.648 | 0.94 ±0.024 |
| V, % | 7.25 | 4.21 |
| Yaichnaya Sinyaya/Novinka | 15.20 ±0.118 | 0.53 ±0.029 |
| V, % | 1.33 | 9.43 |
| Yaichnaya Sinyaya/OP-23-23 | 15.39 ±0.105 | 0.43 ±0.025 |
| V, % | 1.17 | 0.68 |
| Yaichnaya Sinyaya/140-1 | 16.58 ±0.69 | 0.91 ±0.038 |
| V, % | 0.98 | 6.69 |

the maximum score at Utro/OPA-15-2 and Yaichnaya Sinyaya/OP-23-23 combinations (). The soluble solids content (SSC), total titratable acidity (TTA), antioxidant activity (AA), phenolic compounds (PC) sum, and mineral composition change of the understudied plum varieties fruits on different rootstocks were studied in laboratory conditions.

Depending on the variety and the rootstock SSC in the fruits varied from 12.72% (Utro/Skorospelka Krasnaya) to 17.48% (Yaichnaya Sinyaya/Novinka). In the Utro variety fruits on Novinka, OPA-15-2 and 140-1 rootstocks SSC was on 1 – 1.3% higher in comparison with the fruits on the seedling (Figure 4). In the Yaichnaya Sinyaya variety fruits, SSC was higher than in the Utro variety ones; SSC maximum values were determined on Novinka, Skorospelka Krasnaya, and OPA-15-2 rootstocks, i.e. on 4 – 4.5% higher than in the fruits on the seedling at average.

In the understudied scion-stock combinations samples TTA varies within the range 1.1 – 1.7% (Figure 5).

The Yaichnaya Sinyaya variety TTA depended on a scion-stock combination a little. The maximum TTA content was noted at the fruits of Utro variety on Skorospelka Krasnaya, Novinka, OP-23-2,3 and 140-1 rootstocks (1.6 – 1.7%). **Lopez-Ortega et al. (2016)** informed that between the stocks there was no significant difference in SSC accumulation. However, **Gonçalves et al. (2006)** and **Usenik et al. (2010)** found out that at sweet-cherry trees the rootstock influenced SSC and at dwarfing rootstocks SSC was higher. Besides, in the papers of **Daza et al. (2008)** and **Rato et al. (2008)**, it was stated that at plum trees a rootstock affected such quality features as SSC and TTA. While studying the rootstock influence on *Prunus rossica* Erem. Fruits, it was noticed that a rootstock influenced SSC, but did not affect TTA greatly **(Motyleva et al., 2019)**.

**Table 3** The ash composition of Utro variety fruits on different rootstocks.

| Scion/rootstock combinations | The understudied element, mass% | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mg | Si | P | S | K | Ca | Zn | Mo | ∑ |
| Utro/Skorospelka Krasnaya | 2.09 | 0.12 | 3.46 | 0.34 | 27.77 | 3.29 | н/о | 2.11 | 39.18 |
| Utro/Novinka | 1.95 | 0.04 | 4.34 | 0.15 | 28.30 | 1.53 | 0.45 | 1.49 | 38.25 |
| Utro/OP-23-23 | 1.37 | 0.07 | 2.69 | 0.18 | 26.84 | 1.14 | 0.62 | 1.37 | 34.28 |
| Utro/OPA-15-2 | 1.83 | 0.08 | 3.68 | 0.23 | 30.76 | 1.04 | 0.46 | 1.75 | 39.83 |
| Utro/140-1 | 1.88 | 0.29 | 4.16 | 0.11 | 28.95 | 1.26 | 0.25 | 1.15 | 38.05 |
| Utro/seedlings *P. domestica* | 1.76 | 0.11 | 3.29 | 0.16 | 29.73 | 1.34 | 0.17 | 1.42 | 37.98 |
| Min | 1.37 | 0.04 | 2.69 | 0.11 | 26.84 | 1.04 | 0.17 | 1.15 | |
| Max | 2.09 | 0.49 | 4.34 | 0.34 | 30.76 | 3.29 | 0.62 | 2.11 | |
| V, % | 12.5 | 35.9 | 15.7 | 38.6 | 4.9 | 18.8 | 39.6 | 19.7 | |

**Table 4** The ash composition of Yaichnaya Sinyaya variety fruits on different rootstocks.

| Scion/rootstock combinations | The understudied element, mass% | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mg | Si | P | S | K | Ca | Zn | Mo | ∑ |
| Yaichnaya Sinyaya/ OPA-15-2 | 1.13 | 0.06 | 3.39 | 0.29 | 25.94 | 0.45 | 0.42 | 0.99 | 32.67 |
| Yaichnaya Sinyaya/ OP-23-23 | 1.30 | 0.18 | 4.63 | 0.18 | 27.91 | 0.72 | 0.48 | 1.23 | 36.63 |
| Yaichnaya Sinyaya/ Skorospelka Krasnaya | 1.55 | 0.13 | 3.90 | 0.15 | 30.14 | 1.19 | 0.38 | 1.45 | 38.89 |
| Yaichnaya Sinyaya/ seedlings *P. domestica* | 0.88 | 0.16 | 2.96 | 0.06 | 18.99 | 0.62 | 0.19 | 1.19 | 25.05 |
| Yaichnaya Sinyaya / Novinka | 0.81 | 0.10 | 2.90 | 0.11 | 20.72 | 0.73 | 0.29 | 0.83 | 26.49 |
| Yaichnaya Sinyaya / 140-1 | 1.70 | 0.11 | 4.23 | 0.37 | 26.60 | 0.28 | 0.29 | 2.90 | 36.48 |
| Min | 0.81 | 0.06 | 2.9 | 0.06 | 18.99 | 0.28 | 0.19 | 0.83 | |
| Max | 1.7 | 0.18 | 4.63 | 0.37 | 30.14 | 1.19 | 0.48 | 2.9 | |
| V, % | 26.1 | 35.0 | 19.1 | 30.0 | 7.2 | 16.6 | 30.7 | 12.4 | |

**Table 5** The comparative content of the main compounds, found in Yaichnaya Sinyaya variety on different rootstocks, % scale.

| | Substances | Yaichnaya Sinyaya/rootstocks | | | | | |
|---|---|---|---|---|---|---|---|
| | | Seedling Prunus | Skorospelka Krasnaya | Novinka | OP-23-23 | OPA-15-2 | 140-1 |
| 10:02 | L-Norleucine | 18 | 15 | 9 | 18 | 10 | 1 |
| 10:30 | Succinic acid | 9 | 5 | 4 | 4 | 5 | <1 |
| 10:53 | Fumaric acid | 30 | 30 | 28 | 27 | 17 | 1 |
| 11:19 | Malic acid | 8 | 50 | 40 | 40 | 40 | 11 |
| 11:34 | Malonic acid | 8 | 8 | 43 | 40 | 5 | 14 |
| 16:06 | Levoglucosan | 4 | 22 | 28 | 11 | 8 | 4 |
| 16:35 | D-(-)-Fructofuranose | 70 | 75 | 80 | 80 | 80 | 75 |
| 17:03 | D-Allofuranose | 25 | 10 | 40 | 25 | 20 | 20 |
| 17:07 | Quinic acid | 80 | 85 | 94 | 90 | 95 | 20 |
| 17:27 | b-D-Glucopyranose | 70 | 92 | 90 | 80 | 90 | 90 |
| 17:41 | Acrylic acid | 10 | 25 | 32 | 7 | 5 | 8 |
| 17:51 | D-Sorbitol | 25 | 30 | 30 | 50 | 38 | 40 |
| 18:23 | D-Glucose | 70 | 75 | 70 | 88 | 75 | 40 |
| 17:59 | D-(+)-Talofuranose | 1 | 2 | 10 | 12 | 10 | 40 |
| 19:15 | Myo-inositol | 18 | 17 | 12 | 17 | 20 | 10 |
| 24:18 | Sucrose | 90 | 95 | 92 | 92 | 90 | 90 |

Widely spread substances-antioxidants, contained in plants, belong to different classes of chemical compounds. They play a significant role in human nutrition as protective factors that decrease the risk of various diseases **(Fazzari et al. 2008; Usenik et al., 2008)**. These bioactive compounds concentration varies depending on different factors, i.e. climate, soil, rootstocks **(Spinardi et al. 2005)**, сорта **(Mozetic, Trebse and Hribar, 2002; Usenik et al. 2008)**.

Our study results confirm the rootstock influence on bioactive substance synthesis in *Prunus domestica* L fruits. The antioxidant activity of Yaichnaya Sinyaya variety fruits is 30% higher than Utro variety fruits on average (Table 2). The maximum AA values are determined in Yaichnaya Sinyaya variety fruits on the rootstocks OPA - 15-2 (16.37%) and 140-1 (16.58%), and the minimal ones – on Skorospelka Krasnaya rootstock (4.68%). The highest AA values in Utro variety fruits were registered on Skorospelka Krasnaya (13.9%) and OP-23-23 (13.16%) rootstocks, and the lowest ones – on the seedlings (10.93%).

PC content in Yaichnaya Sinyaya variety fruits was 60% higher than in Utro variety ones on average (Table 2). The maximum PC content was registered in Yaichnaya Sinyaya variety fruits on Skorospelka Krasnaya and 140-1 rootstocks, i.e. 0.94 and 0.91 $mg.g^{-1}$ of the fruits equivalent of gallic acid relatively, and the minimum PC content were stated on OP-23-23 rootstock, i.e. 0.43 $mg.g^{-1}$ of the fruits equivalent of gallic acid.

In Utro variety fruits on all the rootstocks, the decrease of PC sum content was stated in comparison with Utro/seedling combination, and on Skorospelka Krasnaya, OPA-15-2, and 140-1 rootstocks the content was twice less.

The mineral substances are an important component of human nutrition, they are a part of enzymes and have antioxidant activity; they are not synthesized in the human organism, but get inside it only with meals. Eight main elements, i.e. Mg, Si, P, S, K, Ca, Zn, Mo, were determined in the plum fruits. K is the main element in the plum fruits. Its content varied from 26.84 mass % (Utro/OP-23-23) to 30.76 mass % (Utro/OPA-15-2) (Table 3). K content variation coefficient is low (4.88%) that speaks about the insignificant influence of the rootstock on this element accumulation.

P content varied within the range from 2.69 mass % (Utro/ OP-23-23) to 4.34 mass % (Utro/Novinka). P content variation coefficient is 16.7%, which speaks about a stable input of this element in plum fruits.

The portion of Ca in Utro variety fruits is from 1.04 mass % (Utro/OPA-15-2) to 3.29 mass % (Utro/Skorospelka Krasnaya). This element variation coefficient is medium.

The portion of Mn and Mo in plum fruits does not exceed 2.1 mass %, these elements maximum accumulation is registered in Utro/Skorospelka Krasnaya combination, i.e. 2.09 and 2.11 mass % relatively. Mn variation coefficient is low, i.e. 13.49%, and Mo coefficient is medium, i.e. 21.74%.

Si maximum accumulation is registered in Utro/140-1 combination fruits, i.e. 0.29 mass %.

Zn content varied from 0.17 mass % (Utro/seedlings) to 0.62 mass % (Utro/OP-23-23). The highest values of all the elements sum are registered in Utro/Skorospelka Krasnaya (39.18 mass %) and Utro/OPA-15-2 (39.83 mass %) scion-stock combinations.

In Yaichnaya Sinyaya variety fruits K is accumulated on 4% less than K and twice less than Ca than in Utro variety fruits (Table 4).

The content of Mg, P, S, K, and Zn on all the rootstocks is higher than on seedlings. Among all the scion-stock combinations we can distinguish the fruits of the following combinations on all the elements sum content: Yaichnaya Sinyaya/OP-23-23 (36.63 mass %), Yaichnaya Sinyaya/140-1 (36.48 mass %), and Yaichnaya Sinyaya/ Skorospelka Krasnaya (38.89 mass %).

Significant differences in mineral elements sum content were determined in the fruits between the varieties and the scion-stock combinations of varieties and rootstocks. The greatest differences in the mineral substances sum content were registered on the medium-growing clonal rootstocks Novinka, OP-232-23, and OPA-15-2.

Higher accumulation of Ca, Mg, and K were determined in the fruits of both varieties on Skorospelka Krasnaya rootstock in comparison with other rootstocks. The decreasing series of the accumulation of the elements in plum fruits is as follows: K > P > Ca > Mg > Mo > S > Zn > Si.

The data given in the scientific literature about the mineral element content in plum fruits are limited, especially regarding the influence of the rootstock. The results on the content of K, Ca, P, and Mg, Mn, Cu, and Zn are presented in the papers of **Rop et al. (2009), Cosmulescu et al. (2017), Milosevic and Milosevic (2012)** and **Motyleva, Upadysheva and Tumaeva (2021)**.

K, Ca, and P values received by us as a result of this study correlate to the data given in the scientific literature in a proportional relation. The differences for some elements content can be connected with the environmental conditions, such as the soil type, the precipitation depth, and the fertilizers application **(Kabata-Pendias, Mukherjee and Trace, 2007)**.

The component composition of plum fruits methanol extracts is illustrated by Yaichnaya Sinyaya variety (Table 5). The main components of plum fruits metabolomic profiles are 16 individual chemical compounds, i.e. L-Norleucine, Succinic acid, Fumaric acid, Malic acid, Malonic acid, Levoglucosan, D-(-)-Fructofuranose, D-Allofuranose, Quinic acid, b-D-Glucopyranose, Acrylic acid, D-Sorbitol, D-Glucose, D-(+)-Talofuranose, Myo-inositol, Sucrose, 9 of which are carbohydrates and their derivatives. The main carbohydrates are saccharose, glucose and fructose. Rootstocks influence the ratio of the metabolite (Table 5). On the medium-growing rootstocks, we registered the increase of monocarbohydrates on 5 – 7%, Quinic acid on 3 – 5%, and Myo-inositol on 2.5 – 3% in comparison with strong- and low-growing rootstocks. The high biological role of Quinic acid and Myo-inositol as proved by many types of research **(Akesson et al., 2005; Font i Forcada et al., 2019; Scăeţeanu et al., 2019)**. The maximum content of Quinic acid and Myo-inositol was marked in Yaichnaya Sinyaya variety fruits on OPA-15-2 rootstock. The same results were received during Utro variety fruits analysis.

## CONCLUSION

The present study stated the rootstock influence on the plants' productivity and bioactive substances accumulation in *Prunus domestica* L. fruits. Under the influence of medium-growing rootstocks, i.e. OPA-15-2, Novinka, and OP-23-23, the increase of not only main economic parameters (output yield, the fruit size, and weight, sensor evaluation, SSC and TTA) but biologically important compounds (antioxidants, metabolites, macro, and microelements) was registered as well. The optimal rootstocks for Yaichnaya Sinyaya and Utro varieties are medium-growing rootstocks OPA-15-2, Novinka, and OP-23-23.

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**Conflict of Interest:**

The authors declare no conflict of interest.

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# THE EFFECT OF RAW PEARL MILLET FLOUR INCLUSION ON THE QUALITY AND FORMULATION COST OF BEEF SAUSAGES

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## ABSTRACT

This study investigated the sensory, nutritional, and physicochemical characteristics as well as formulation cost of beef sausages formulated with raw pearl millet flour (RaPMF) as an extender. Four treatments were formulated with 0%, 5%, 10% and 15% RaPMF. Ash, carbohydrate, fat, and protein contents of the beef sausages were not influenced ($p$ <0.05) by the RaPMF. The calcium, magnesium, potassium, and pH contents were highest ($p$ <0.05) in the 15% RaPMF beef sausages. Cooking loss was at least ($p$ <0.05) for the 15% RaPMF beef sausages. Formulation of beef sausages with RaPMF did not affect ($p$ >0.05) the sensory properties (week 1), water holding capacity, peroxide value, lightness (week 1), and yellowness (week 1). The formulation cost was least for 15% RaPMF beef sausages and highest for 0% RaPMF beef sausages. As a general conclusion, RaPMF improved the mineral composition of the beef sausages and reduced production costs without compromising its physicochemical and sensory properties.

**Keywords:** beef sausages; nutrient composition; physicochemical properties; sensory analysis; pearl millet flour

## INTRODUCTION

The quest and desire for meat products including beef sausages have increased due to their good taste, nutrient composition, and demand for ready-to-eat meals **(Ossom et al., 2020a; Yadav and Yadava, 2008)**. The consumption of meat products is hampered by its associated health issues and high price **(Battaglia et al.., 2015)**. These have limited their purchase and consumption to mostly middle and high-income earners **(Ossom et al., 2020a; Stubbs et al., 2002)**. The development and formulation of meat products that are cheaper with better nutrient composition is the way forward. This can be achieved by the use of extenders.

Extenders are food ingredients that are not of meat origin but have an appreciable amount of proteins used in the formulation of meat products to partially replace muscle meat **(Heinz and Hautzinger, 2007; Ossom et al., 2020b)**. They may play one or more of the following roles in meat products, thus, increase product volume, improve texture, produce healthy products, reduce the cost of production among others **(Food and Agriculture Organization, 2013; Heinz and Hautzinger, 2007)**. For instance, **Yadav and Yadava (2008)** reported that the use of cornflour in quail meat rolls increased yield, and did not affect sensory properties at a 6% inclusion level. Also, sorghum flour as an extender in chicken patties promoted lower TBARS, and sensory properties were not significantly affected **(Kumar et al., 2007)**. **Pintado and Delgado-Pando (2020)** indicated that the use of extenders provides opportunities for the formulation of healthier and more sustainable meat products.

Millets are cereal-grain crops widely grown and consumed in most parts of the world **(Lang, 2020; Muthamilarasan et al., 2016)**. They are considered an essential cereal crop at the global level and have a significant economic impact especially in most developing countries **(Lang, 2020; Nithiyanantham et al., 2019)**. **Muthamilarasan et al. (2016)** revealed that millets have gained popularity in the West because of their gluten-free, high protein, high fiber, and antioxidant contents. Millets are drought resistant, pest resistant, and can withstand poor soil conditions **(Lang, 2020; Nithiyanantham et al., 2019)**. They are also a good energy crop for reducing malnutrition, potential prebiotic and probiotic, customary medicine, and a portion of vital food to preserve health **(Lang, 2020; Muthamilarasan et al., 2016; Nithiyanantham et al., 2019)**. The cultivation of millet promotes sustainable production due to the aforementioned advantages. Therefore, using millet in the formulation of meat products such as sausages provides opportunities for diversification.

Beef sausages are meat products made from minced beef mixed with salt, spices, flavoring agents, and other ingredients such as bread crumbs or grains, and stuffed into casings **(El-Magoli and Abd-Allah, 2014; Food and Agriculture Organization, 2020)**. When bread crumbs or grains are included, they act as extenders.



**Figure** 1 Raw pearl millet seeds.



**Figure 2** Pearl millet flour.



**Figure 3** Beef sample used for this experiement

Beef sausages are an important source of protein and other nutrients for humans and are normally consumed as a delicacy by many **(Abu et al., 2019; Adua et al., 2020; Food and Agriculture Organization, 2020; Ossom et al., 2020a)**. Literature on the use of millet in formulating meat products is scarce, despite its importance and the role meat products play in the diet of humans. Therefore, this study investigated the influence of raw pearl millet flour on the sensory, nutritional, physicochemical, and formulation cost of beef sausages. The feasibility of utilizing raw pearl millet flour in formulating nutrient-rich and low-cost beef sausages will be helpful to expand its further application in the meat industry.

**Scientific hypothesis**

The hypothesis is based on the assumption that millet can be used to produce beef sausages that will have a positive effect on chemical and sensory acceptability. It also assumes that sausages prepared from millet could influence production cost. The results showed that pearl millet improved the mineral composition of the beef sausages and reduced cooking loss at the highest inclusion level. Millet reduced the production cost of the sausages.

## MATERIAL AND METHODOLOGY

**Samples**

The samples used in this study were raw pearl millets, millet flour, and beef. The raw pearl millets (RaPM) were bought from the Aboabo market in the Tamale metropolis of Ghana, while the beef was bought from the University for Development Studies Meats Unit. Figures 1 and Figure 2 shows raw millet seeds and millet flour, respectively. Figure 3 shows a sample of the meat used for this study.

**Chemicals**

The chemical used were perchloric acid (70% Purity, Chem-Lab, Belgium), Sodium Thiosulphate (99% Purity, Lobachemie, India), hydrogen peroxide (35% Purity, Chem-Lab, Belgium), acetic acid (>99.7% Purity, Daejung, Korea), chloroform (>99.5% Purity, Daejung, Korea), potassium iodide (99.7% Purity, VWR Prolabo, Belgium), Sulphuric acid (98% Purity, Merck, Germany).

**Animals and Biological Material**

Animals or biological materials were not used in this study.

**Instruments**

The instruments used in this study were mincer (Model star, Telleres Rammon, Spain), bowl chopper (Model 30, Telleres Rammon, Spain), hydraulic stuffer (Model 30, Telleres Rammon, Spain), smoker (Model HCR700L, ABAD, aquinaria (LAINT), Spain), colorimeter (CR 400 Konica Minolta Inc, Japan), pH meter (Crison Basic 20, Alella, Spain), atomic absorption spectrometer (AAS model novAA 400 P, Analytik Jena AG, Germany), centrifuge (Hettich ROTOFIX 32A Centrifuge, Germany), electric oven (Turbofan Blue Seal, UK), Kjeldahl apparatus (VELP Scientifica, 482365, Italy), Soxhlet apparatus (PRO-NITRO II, 0453462, J. P Selecta, Spain), Furnace (Select- Horn, 0453077, J.P Selecta, Spain) and oven dryer (0453598, J. P Selecta, Spain).

**Laboratory Methods**

The RaPM was hand-picked to remove visible debris and washed thoroughly with tap water. Washed RaPM were allowed to sun dry for 18 h and milled using a commercial mill into flour. The flour was stored in an airtight container at room temperature (25 ±1 °C) for later use.

**Preparation of beef sausages using RaPMF**

Table 1 shows the ingredients and composition of the various treatments. RaPMF substituted ground beef at 0, 5, 10, and 15% on kg basis. Meat (beef) samples were ground using a 5 mm mincer (Model star, Telleres Rammon, Spain) and comminuted using a bowl chopper (Model 30, Telleres Rammon, Spain), during which spices and other ingredients as shown in Table 1 were added. The final product was transferred into a hydraulic stuffer (Model 30, Telleres Rammon, Spain) and stuffed into casings. The sausages were manually linked into approximately 100 mm lengths and smoked at 105ºC for 30 min in a smoker (Model HCR700L, ABAD, aquinaria (LAINT), Spain).

After smoking, they were scalded at 55 °C for 20 min and allowed to cool, packaged, and stored at -18 ±1ºC for further analysis. Figure 4 shows pearl millet beef sausages hanging on a rack.

**pH and water activity ($a_w$) of RaPMF beef sausages**

Ten (10) grams of each sausage was ground in mortar and pestle, and homogenized with 10 mL of distilled water. Following this, the probe of a digital calibrated pH meter (Crison Basic 20, Alella, Spain) was dipped into the solution to obtain the pH values. Analyses were carried out in triplicates.

Water activity determination was carried out on 2.5 g of beef sausages using a water activity meter (Model Labswift-aw, Novasina AG, Switzerland) at room temperature (25 ±1 ºC). All measurements were done in triplicates.

**Color measurements of RaPMF beef sausages**

The surface color of RaPMF beef sausages was measured using a colorimeter (CR 400 Konica Minolta Inc, Japan) equipped with a DP-400 data processor. The equipment was calibrated with a standard white plate at D65 illumination (Y = 80.1, x = 0.3219, y = 0.3394). The Commission Internationale de l'Eclairage (CIE) lightness ($L^*$), redness ($a^*$), and yellowness ($b^*$) values were measured on the surface of the sausage samples. Colour measurement was done according to **CIE (1986)**. Measurements were done in triplicates and on days 0, 7, 14 and 21.

**Proximate composition of RaPMF beef sausages**

Proximate composition was done according to the **Association of Official Analytical Chemists (AOAC) (2000)**. Kjeldahl apparatus (method 954.01), Soxhlet apparatus (method 920.39), furnace (method 942.09), and oven drying (method 930.15) were used for protein, fat, ash, and moisture contents determination, respectively. Total carbohydrates were calculated using the formula 100 – (% protein + % fat + % ash% + moisture). Analyses were carried out in triplicates.

**Mineral analysis of RaPMF beef sausages**

Mineral analysis of the beef sausages was done using a slight according to the method used by **Adua et al. (2020)**. Briefly, 2 mL perchloric acid and 10 mL concentrated $H_2SO_4$ was added to 1 g of beef sausage in a digestion tube.

**Table 1** Ingredients and composition of raw pearl millet flour beef sausages (RaPMFS)

| Ingredients | Treatments | | | |
|---|---|---|---|---|
| | 0% RaPMF | 5% RaPMF | 10% RaPMF | 15% RaPMF |
| Beef (kg) | 2.00 | 1.90 | 1.80 | 1.70 |
| RaPMF (g) | 0.00 | 0.10 | 0.20 | 0.30 |
| Curing salt (g) | 0.03 | 0.03 | 0.03 | 0.03 |
| Adobo (g) | 0.002 | 0.002 | 0.002 | 0.002 |
| White pepper (g) | 0.004 | 0.004 | 0.004 | 0.004 |
| Black pepper (g) | 0.004 | 0.004 | 0.004 | 0.004 |
| Chilli pepper (g) | 0.001 | 0.001 | 0.001 | 0.001 |
| Phosphate (g) | 0.01 | 0.01 | 0.01 | 0.01 |
| Ice cubes (g) | 0.14 | 0.14 | 0.14 | 0.14 |
| Soy oil (g) | 0.26 | 0.26 | 0.26 | 0.26 |



**Figure 4** Pearl millet beef sausages.

They were heated until the solution turned clear at a temperature of 350 ºC. It was cooled, 2 mL of hydrogen peroxide was added, and top up with distilled water to the 50 mL mark. The solution was measured using an Atomic Absorption Spectrometer (AAS model novAA 400 P, Analytik Jena AG, Germany) to determine the various nutrients. Analyses were carried out in triplicates.

**Peroxide value determination of RaPMF beef sausages**

Peroxide value was determined using a slightly modified method of **Salih et al. (1989), Abu et al. (2019),** and **Adua et al. (2020)**. Briefly, 10 g of the sausage was added to 30 mL hexane and agitated for 60 min at 250 rpm. It was then transferred into a 50 mL falcon tube and centrifuge for 5 min at 3000 rpm. The residues were then evaporated in an evaporator with 5 mL acetic acid-chloroform solution and later with 10 ml acetic acid-chloroform solution. One (1) mL of saturated potassium iodide solution was added to the extracted samples in a 100 mL Erlenmeyer flask. And then, 5 mL of 1% starch soluble solution was added and the resulting mixture was titrated against 0.01 N sodium thiosulfate solution ($Na_2S_2O_3$) until an endpoint was obtained, that is, color change from orange to a transparent color. A blank test was used for calibration. The peroxide value was calculated as:

$$Peroxide\ value\ (mEq/kg) = (V_1 - V_0) \times N \times 1000/S \qquad (1)$$

Where:

$V_1$ = titre value of sample; $V_0$ = titre value of blank; S = weight of sample; N = normality of sodium thiosulfate. Measurements were taken on week 1, week 2, and week 3, and were carried out in triplicates.

**Water holding capacity of RaPMF beef sausages**

The water holding capacity was done using a slightly modified procedure of **Heywood et al. (2002)**. In brief, 2.5 g of each sample was placed in a pre-weighed 50 mL centrifuge tube and 10 mL distilled water was added. They were thoroughly mixed, allowed to stand for 30 min at room temperature, and centrifuge at 3700 rpm for 30 min using Hettich ROTOFIX 32A Centrifuge, Germany. After centrifugation, the supernatant was decanted and the sample weight was taken. Percentage water holding capacity was calculated according to the following equation:

$$WHC(\%) = \frac{Dw - Sw}{Sw} \times 100 \qquad (2)$$

Where:

Dw – is the decant weight of the sample after centrifugation; Sw – is the sample weight. Analyses were carried out in triplicates.

**Cooking loss of RaPMF beef sausages**

Cooking loss of the beef sausages was done according to **Lee et al. (2008)**. Sausages were weighed (Wr) and cooked to obtain a core temperature of 70 °C at a temperature of 150°C. Cooked sausages were allowed to cool to room temperature and reweighed (Wc). The cooking loss percentage was determined using the equation:

$$Cooking\ loss\ (\%) = \frac{Wr-Wc}{Wr} \times 100 \qquad (3)$$

Where:
Wr = weight of raw sausage; Wc = weight of cooked sausage. Analyses were carried out in triplicates.

**Sensory analysis of RaPMF beef sausages**

Before this, the sausages were grilled in an electric oven (Turbofan Blue Seal, UK) at a temperature of 105 °C for 45 min. Grilled beef sausages were cut into 2 cm sizes and wrapped in coded aluminum foil for sensory evaluation using 15 trained panelists **(British Standard Institute Guidelines, 1993)**. Beef sausages were assessed using a 9-point hedonic scale, that is, 1: extremely liked to 9 disliked for color, flavor liking, flavor intensity, juiciness, taste, tenderness, texture, and overall liking. Sensory evaluations were done on weeks 1, 2, and 3.

**Number of samples analyzed**

Thirty-six (36) raw pearl millet flour (RaPMF) beef sausages were analyzed. The samples comprised of 9 each of 0, 5, 10, and 15% RAMPF beef sausages.

**Number of repeated analyses**

The experimental analysis was triplicated.

**Number of experiment replication**

The entire experiment was triplicated.

**The production cost of RaPMF beef sausages**

The cost of producing a kilogram of beef sausages with and without raw pearl millet was determined according to **Yaro et al. (2021)**. The cost of each inclusion level (0, 5, 10, and 15%) was determined as a proportion of the respective kilogram cost, thus the cost of beef, spices, curing salt, and ice cubes for processing a kilogram of beef sausage.

## Statistical Analysis

Data analysis was performed using one-way analysis of variance in Genstat Discovery 4th Edition. Significant differences were determined at a 5% significant level using Tukey studentized range test.

## RESULTS AND DISCUSSION

The cooking loss, water holding capacity, pH, water activity, and peroxide value of the beef sausages can be found in Table 2. The cooking loss, pH, and water activity of the beef sausages differed significantly ($p$ <0.05) from each other. However, the water holding capacity and peroxide value of the beef sausages did not differ ($p$ >0.05) from each other. The values ranged from 18.76±0.36 to 26.74 ±1.18%, 1.18 ±0.07 to 1.24 ±0.14%, 5.94 ±0.02 to 6.03 ±0.01, 0.805 ±0.002 to 0.815 ±0.002, 1.917 to 3.542 meq.kg$^{-1}$ for cooking loss, water holding capacity, pH, water activity, and peroxide value, respectively. The instrumental color (lightness, redness, and yellowness) measurements of the beef sausages are presented in Table 3. The lightness of the beef sausages differed significantly ($p$ <0.05) on day 14, redness on days 0, 7, and 14, and yellowness on days 7, 14, and 21. The instrumental color measurements of the beef sausages ranged from 50.22 to 55.64 (lightness), 7.14 to 8.73 (redness), and 18.17 to 22.69 (yellowness). Meat products formulated with food resources to improve their nutritional compositions and to promote good health upon consumption are essential. This is because the consumption of meat products in some cases has been associated with cardiovascular and other related diseases **(Alisson-Silva et al., 2016; Battaglia et al., 2015)**. Millets have been reported to be a gluten-free cereal grain with sufficient protein, fiber, and antioxidants contents **(Lang, 2020; Taylor and Emmambux, 2008)** which when incorporated into sausages, will promote better nutrient composition and consequently health benefit. Meat products formulated with other food resources also bring diversity in meat consumption, ensure responsible consumption and cater to the increasing population that has less time for food preparation **(Pintado and Delgado-Pando, 2020; Yaro et al., 2021)**.

In this study, the water holding capacity and peroxide value were not influenced by the use of raw pearl millet flour in the beef sausages. Water holding capacity contributes to and influences the juiciness and tenderness of meat products **(Cheng and Sun, 2008)**. **Serdaroglu et al. (2018)** found higher water holding capacity for beef patties formulated with dried pumpkin pulp and seed. The non-significant differences in the peroxide values mean that beef sausages with or without raw pearl millet flour are equally stable to rancidity, which is a major cause of unpleasant smell, taste, and flavor in meat products **(Pereira and Abreu, 2018)**. The peroxide values recorded in this work were far below or not above 10 meq/kg, the peroxide value at which rancidity is eminent **(Feiner, 2006; Pereira and Abreu, 2018)**. The cooking loss, pH, water activity, and some instrumental color parameters were influenced by the use of raw pearl millet flour. Generally, raw pearl millet flour increased the pH of the beef sausages and decreased the water activity, but within the recommended pH and water activity range for meat products **(Feiner, 2006; Warris, 2010)**. The pH and water activity as found in this study will limit the growth of microorganisms and improve on the shelf life of the beef sausages. The cooking loss of the beef sausages decreased with the increasing amount of raw pearl millet, therefore, raw pearl millet flour improved the yield of the sausages during cooking. Colour is an important factor when it comes to consumers' acceptance of meat and meat products.

The lightness of the beef sausages was influenced on day 14, redness on days 0, 7, and 14, and the yellowness on days 7, 14, and 21. Myoglobin is responsible for the redness of meat and meat products. Consumers prefer red meat color for certain meat products and attribute it to freshness **(Warris, 2010)**. Raw pearl millet at a high inclusion level (15%) reduced the redness of the beef sausages except on day 21 where significant differences did not occur. The natural color of the pearl millet used which was grey is responsible for the reduction in the red color. The raw pearl millet diluted the red color of meat and reduced myoglobin and red coloration.

**Table 2** Physicochemical properties of raw pearl millet flour beef sausages (RaPMFS).

| Treatment (%) | Parameters | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cooking loss (%) | Water holding capacity (%) | pH | Water activity | Peroxide value (meq/kg) | | |
| | | | | | Week 1 | Week 2 | Week 3 |
| 0%RaPMFS | 25.00±0.41[a] | 1.24±0.14 | 5.94±0.02[c] | 0.812±0.002[ab] | 3.542 | 3.305 | 3.175 |
| 5%RaPMFS | 26.74±1.18[a] | 1.22±0.06 | 6.00±0.01[b] | 0.815±0.002[a] | 3.155 | 3.405 | 3.271 |
| 10%RaPMFS | 21.48±1.28[b] | 1.18±0.07 | 5.98±0.00[b] | 0.805±0.002[b] | 3.289 | 3.369 | 3.202 |
| 15%RaPMFS | 18.76±0.36[c] | 1.22±0.07 | 6.03±0.01[a] | 0.807±0.004[b] | 3.855 | 2.188 | 1.917 |
| *p* – value | < 0.001 | 0.916 | < 0.001 | 0.005 | 0.911 | 0.397 | 0.346 |

Note: Values are means ± standard deviation, values with different superscripts under the same column are significantly different (p <0.05).

**Table 3** Instrumental colour measurements of raw pearl millet flour beef sausages (RaPMFS).

| Parameter | Treatment | Storage time (days)[1] | | | |
|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 21 |
| Lightness (L*) | 0% RaPMFS | 53.69±1.97 | 53.48±0.38 | 52.83±0.91[ab] | 53.11±1.47 |
| | 5% RaPMFS | 52.47±1.66 | 54.87±1.71 | 50.22±1.53[b] | 55.98±1.21 |
| | 10% RaPMFS | 53.80±0.76 | 55.01±0.53 | 55.64±0.47[a] | 55.47±1.17 |
| | 15% RaPMFS | 50.31±1.96 | 55.45±0.09 | 54.35±1.71[a] | 55.72±1.35 |
| | *p* value | 0.320 | 0.130 | 0.050 | 0.400 |
| Redness (a*) | 0% RaPMFS | 7.99±0.12[ab] | 8.50±0.48[ab] | 8.34±0.06[a] | 8.09±0.60 |
| | 5% RaPMFS | 8.48±0.21[a] | 8.73±0.40[a] | 8.33±0.16[a] | 8.20±0.46 |
| | 10% RaPMFS | 7.97±0.28[ab] | 8.40±0.17[ab] | 8.07±0.42[a] | 8.44±0.37 |
| | 15% RaPMFS | 7.85±0.25[b] | 7.30±0.70[b] | 7.25±0.30[b] | 7.14±0.64 |
| | *p* value | 0.040 | 0.030 | 0.000 | 0.070 |
| Yellowness (b*) | 0% RaPMFS | 20.53±0.51 | 18.91±0.35[c] | 18.31±0.18[c] | 18.17±0.50[c] |
| | 5% RaPMFS | 20.90±0.46 | 19.94±0.49[b] | 18.46±1.04[bc] | 19.09±1.13[bc] |
| | 10% RaPMFS | 21.96±0.74 | 21.25±0.36[a] | 20.14±0.83[ab] | 20.71±0.60[ab] |
| | 15% RaPMFS | 21.51±1.34 | 21.56±0.22[a] | 21.44±0.33[a] | 22.69±1.36[a] |
| | *p* value | 0.240 | 0.000 | 0.000 | 0.000 |

Note: Values are means ± standard deviation, values with different superscripts under the same column are significantly different (*p* <0.05); [1] storage at -18 °C.

**Table 4** Proximate compositions of raw pearl millet flour beef sausages (RaPMFS).

| Treatments | Parameters | | | | |
|---|---|---|---|---|---|
| | Moisture (%) | Ash (%) | Fat (%) | Protein (%) | Carbohydrate (%) |
| 0% RaPMFS | 68.42 ±0.43[a] | 4.47 ±0.57 | 5.53 ±3.22 | 18.81 ±1.50 | 2.77 ±1.70 |
| 5% RaPMFS | 65.91 ±0.75[b] | 3.85 ±1.31 | 8.19 ±2.11 | 19.25 ±1.22 | 2.80 ±1.58 |
| 10% RaPMFS | 65.03 ±1.13[b] | 5.00 ±0.28 | 6.63 ±2.68 | 16.90 ±1.35 | 6.44 ±3.64 |
| 15% RaPMFS | 62.14 ±0.60[c] | 5.24 ±1.13 | 9.86 ±0.56 | 17.83 ±1.39 | 4.93 ±0.26 |
| *p* – value | <0.001 | 0.326 | 0.206 | 0.232 | 0.187 |

Note: Values are means ± standard deviation, values with different superscripts under the same column are significantly different
(*p* <0.05).

**Table 5** Mineral compositions of raw pearl millet flour beef sausages (RaPMFS).

| Treatments | Parameters | | | |
|---|---|---|---|---|
| | Magnesium (mg.kg$^{-1}$) | Calcium (mg.kg$^{-1}$) | Potassium (mg.kg$^{-1}$) | Zinc (mg.kg$^{-1}$) |
| 0% RaPMFS | 594.99±0.94[d] | 64.26±0.12[d] | 1137.46±1.24[b] | 33.96±0.07[a] |
| 5% RaPMFS | 712.66±1.13[b] | 80.78±0.53[c] | 1072.52±0.91[d] | 33.45±0.13[a] |
| 10% RaPMFS | 670.54±0.60[c] | 86.54±0.54[b] | 1116.63±1.62[c] | 33.39±0.41[a] |
| 15% RaPMFS | 795.73±2.19[a] | 99.11±0.93[a] | 1156.90±1.26[a] | 30.61±0.04[b] |
| *p* – value | <0.001 | <0.001 | <0.001 | <0.001 |

Note: Values are means ± standard deviation, values with different superscripts under the same column are significantly different
(*p* <0.05).

Similarly, **Argel et al. (2020)** found that green pea and chickpea flour emulsions used in pork patties lowered redness in the products. Also, **Serdaroglu et al. (2018)** reported that dried pumpkin pulp and seed lowered the redness of beef patties. The natural color of raw pearl millet also influenced the yellowness of the beef sausages especially during storage in that the beef sausages with raw millet flour were more yellow than the 0% RaPMF beef sausage. Green pea and chickpea flour emulsions also increased the yellowness of pork patties **(Argel et al., 2020)**. However, this natural-color did not affect the lightness of the beef sausages except on day 14. The lightness, redness, and yellowness recorded in this study were higher than values recorded for uncooked meatballs during storage at 4 °C for 6 days **(Ergezer et al., 2014)**.

The proximate composition results for the beef sausages are presented in Table 4. There were no significant differences ($p$ >0.05) in the ash, carbohydrate, fat, and protein contents of the beef sausages. However, the moisture content of the 0% RaPMF beef sausages was significantly higher ($p$ <0.05) than the 0, 5, and 15% RaPMF beef sausages. The results ranged from 62.14 ±0.60 to 68.42 ±0.43% (moisture), 3.85 ±1.31 to 5.24 ±1.13% (ash), 5.53 ±3.22 to 9.86 ±0.56% (fat), 16.90 ±1.35 to 19.25 ±1.22% (protein) and 2.77 ±1.70 to 6.44 ±3.64% (carbohydrate).

Incorporating raw pearl millet flour into beef sausages did not negatively affect the ash, fat, protein, and carbohydrate contents, but reduced the moisture content. This means raw pearl millet flour up to 15% can be used for beef sausage preparation without adverse effects on the proximate composition. A similar observation was made by **Yaro et al. (2021)**. **Yaro et al. (2021)** reported differences in the moisture and protein content of roasted pearl millet flour beef sausages, but not the ash, fat, and carbohydrate contents. **Amadi (2020)** found significant differences in the moisture, protein, ash, carbohydrates, and fat content of full-fat soy flour beef sausages. Also, the inclusion of soy and finger millet flours in beef sausages increased the moisture, crude fat, crude protein, crude ash, and crude fiber contents **(Behailu and Abebe, 2020)**.

The mineral composition of the beef sausages is shown in Table 5. There were significant differences ($p$ <0.05) in all the minerals measured. The iron, magnesium, and calcium contents were significantly higher ($p$ >0.05) in the 5, 10, and 15% RaPMF beef sausages than the 0% RaPMF beef sausages. The 15% RaPMF beef sausages had the highest ($p$ <0.05) potassium and least ($p$ <0.05) zinc contents. The 5% RaPMF beef sausages had the lowest ($p$ <0.05) potassium content. The minerals in the beef sausages also ranged from 594.99 ±0.94 to 795.73 ±2.19 $mg.kg^{-1}$ (magnesium), 64.26 ±0.12 to 99.11 ±0.93 $mg.kg^{-1}$ (calcium), 1072.52 ±0.91 to 1156.90 ±1.26 $mg.kg^{-1}$ (potassium) and 30.61 ±0.04 to 33.96 ±0.07 $mg.kg^{-1}$ (zinc). Developing beef sausages with raw pearl millet flour increased the iron, magnesium, calcium, and potassium contents. Thus, raw pearl millet flour for sausage development positively influenced the mineral composition of beef sausages. The zinc contents were similar except for beef sausages developed with 15% raw pearl millet flour. The calcium, magnesium, and zinc contents of the beef sausages recorded in this study were higher than those reported by **Amadi (2020)** in full-fat soy flour beef sausages. The magnesium, calcium, potassium, and zinc contents were similar to those reported by **Yaro et al. (2021)** in roasted pearl millet flour beef sausages.

The sensory properties of the beef sausages are shown in Table 6. There were no significant differences ($p$ >0.05) in the color, flavor intensity, flavor liking (except week 2), texture, tenderness, juiciness, and overall liking of the beef sausages. Sensory scores for color, flavor intensity, flavor liking, texture, tenderness, juiciness, and overall liking ranged from 4.53 ±2.13 to 6.47 ±0.92, 4.73 ±1.75 to 6.80 ±1.27, 5.53 ±1.96 to 7.20 ±1.61, 4.87 ±1.77 to 6.00 ±1.51, 4.93 ±2.05 to 6.53 ±1.19, 5.20 ±1.37 to 6.33 ±1.40 and 5.93 ±1.16 to 7.53 ±1.36, respectively. The color, flavor intensity, flavor liking, texture, tenderness, juiciness, and overall liking of the beef sausages were not influenced by raw pearl millet flour, except flavor liking and overall liking in week 2. Even though the flavor liking and overall liking were lower for the 15% RaPMF beef sausages in week 2, this did not occur in week 3. This means that beef sausages with or without raw pearl millet were equally acceptable. Also, the panelist described the color, flavor intensity, flavor liking, texture, tenderness, juiciness, and overall liking of the beef sausages as intermediate to slightly pale, intermediate to moderately strong, like slightly to like moderately, intermediate to slightly smooth, intermediate to moderately tender, intermediate to slightly juicy, and like slightly to like very much, respectively. It was interesting to find that, overall liking was scored better than the rest of the sensory attributes. **Behailu and Abebe (2020)** found that beef sausages formulated with 20% soy and finger millet flours were liked very much. The results of the present study also collaborate with that of **Yaro et al. (2021)** and **Serdaroglu et al. (2018)**. **Yaro et al. (2021)** reported that roasted pearl millet flour did not cause changes in the sensory attributes of beef sausages. **Serdaroglu et al. (2018)** observed that sensory properties were not affected when dried pumpkin pulp and seed were used for beef patties.

The cost of producing the RaPMF beef sausages is presented in Table 7. The production cost in US dollars were $5.41, $5.30, $5.20 and $5.08 for 0% RaPMF, 5% RaPMF, 10% RaPMF and 15% RaPMF beef sausages, respectively. Price is important to factor consumers consider in purchasing meat and meat products. The costs of producing 5, 10, and 15% RaPMF beef sausages were lower than that of the 0% RaPMF beef sausages. This can be attributed to the lower cost of obtaining a kilogram of raw pearl millet as compared to a kilogram of lean beef. It also follows that RaPMF beef sausages will be more affordable compared with 0% RaPMF beef sausages.

**Adua et al. (2020)** formulated beef sausages with raw *Citrullus vulgaris* chaff and found that the incorporation of the raw *Citrullus vulgaris* chaff reduced the cost of producing the beef sausages. Similarly, **Behailu and Abebe (2020)** reported a reduction in the cost of producing beef sausages when soy and finger millet flours were used to prepare beef sausages. It has been reported that the expensive nature of meat products limits their patronage by average income earners **(Malav et al., 2013)**. Therefore, the formulated RaPMF beef sausages will increase affordability and promote the consumption of beef sausages required to complement protein intake.

**Table 6** Sensory properties of raw pearl millet flour beef sausages (RaPMFS).

| Storage period[1] | Treatments (%) | Colour | Flavour intensity | Flavour liking | Texture | Tenderness | Juiciness | Overall liking |
|---|---|---|---|---|---|---|---|---|
| | 0% RaPMFS | 5.67 ±2.13 | 6.00 ±1.69 | 6.47 ±1.81 | 4.87 ±1.77 | 4.93 ±2.05 | 5.67 ±2.02 | 6.20 ±1.90 |
| Week 1 | 5% RaPMFS | 5.73 ±1.79 | 6.13 ±1.69 | 5.53 ±1.96 | 5.27 ±1.83 | 6.13 ±1.35 | 6.33 ±1.40 | 6.33 ±1.72 |
| | 10% RaPMFS | 5.40 ±1.55 | 5.47 ±1.89 | 6.33 ±1.11 | 5.27 ±1.22 | 6.27 ±1.34 | 5.47 ±1.55 | 6.67 ±1.45 |
| | 15% RaPMFS | 4.53 ±2.13 | 4.73 ±1.75 | 5.67 ±1.40 | 5.67 ±1.59 | 5.80 ±1.47 | 5.20 ±1.37 | 5.93 ±1.16 |
| *p* – Value | | 0.286 | 0.121 | 0.164 | 0.538 | 0.266 | 0.125 | 0.352 |
| | 0% RaPMFS | 5.20 ±1.52 | 6.80 ±1.27 | 7.20 ±1.61$^{a}$ | 5.47 ±1.73 | 5.40 ±1.81 | 5.73 ±1.58 | 7.53 ±1.36$^{a}$ |
| Week 2 | 5% RaPMFS | 5.20 ±1.52 | 5.60 ±1.45 | 6.47 ±1.51$^{ab}$ | 5.80 ±1.15 | 6.27 ±1.34 | 5.80 ±1.27 | 6.60 ±1.50$^{ab}$ |
| | 10% RaPMFS | 4.73 ±1.39 | 5.47 ±1.51 | 6.67 ±1.40$^{ab}$ | 5.47 ±1.36 | 6.53 ±1.19 | 5.93 ±1.16 | 6.53 ±1.85$^{ab}$ |
| | 15% RaPMFS | 5.13 ±1.77 | 5.40 ±1.92 | 6.13 ±0.92$^{b}$ | 6.00 ±1.51 | 5.67 ±1.59 | 5.73 ±1.44 | 6.40 ±1.40$^{b}$ |
| *p* – Value | | 0.792 | 0.059 | 0.038 | 0.489 | 0.222 | 0.906 | 0.032 |
| | 0% RaPMFS | 6.47 ±0.92 | 5.87 ±1.41 | 6.47 ±1.73 | 5.33 ±1.45 | 6.20 ±1.42 | 6.13 ±1.64 | 6.73 ±1.98 |
| Week 3 | 5% RaPMFS | 5.80 ±1.57 | 5.67 ±0.90 | 6.60 ±0.91 | 5.67 ±1.29 | 6.07 ±1.44 | 5.67 ±1.63 | 6.40 ±1.30 |
| | 10% RaPMFS | 6.13 ±1.19 | 5.67 ±0.90 | 5.93 ±1.22 | 5.87 ±1.36 | 6.53 ±0.74 | 6.33 ±1.40 | 6.67 ±1.54 |
| | 15% RaPMFS | 5.87 ±1.13 | 5.60 ±1.12 | 5.73 ±1.44 | 5.80 ±1.86 | 6.07 ±1.67 | 5.53 ±1.51 | 6.33 ±1.29 |
| *p* – Value | | 0.546 | 0.908 | 0.257 | 0.668 | 0.606 | 0.411 | 0.480 |

Note: Values are means ± standard deviation, values with different superscripts under the same column are significantly different ($p < 0.05$).

**Table 7** Formulation cost of raw pearl millet flour beef sausages (RaPMFS).

| Ingredient | Amount (US $/kg) | 0% RaPMFS ($) | 5% RaPMFS ($) | 10% RaPMFS ($) | 15% RaPMFS ($) |
|---|---|---|---|---|---|
| Minced beef | 4.46 | 4.46 | 4.24 | 4.02 | 3.79 |
| Pearl millet | 9.61 | - | 0.04 | 0.09 | 0.13 |
| Milling | 0.34 | - | 0.02 | 0.03 | 0.05 |
| Water for processing millet | 0.17 | - | 0.01 | 0.02 | 0.03 |
| Spice mix | 0.09 | 0.12 | 0.12 | 0.12 | 0.12 |
| Curing salt | 0.09 | 0.09 | 0.09 | 0.09 | 0.09 |
| Soy oil | 0.14 | 0.14 | 0.14 | 0.14 | 0.14 |
| Casing | 0.51 | 0.51 | 0.51 | 0.51 | 0.51 |
| Ice cube | 0.09 | 0.09 | 0.09 | 0.09 | 0.09 |
| Transportation | 0.8 | - | 0.04 | 0.09 | 0.13 |
| Total cost (US$) | | 5.41 | 5.30 | 5.20 | 5.08 |

## CONCLUSION

Raw pearl millet flour can be used to replace up to 15% of lean meat in beef sausages without generally affecting the nutritional composition, physicochemical properties, and sensory evaluation. Raw pearl millet flour positively influenced the mineral composition of beef sausages. It also reduced the formulation cost of producing beef sausages. The cooking loss was at least for the 15% RaPMF beef sausages. Millet showed great potential for sausage formulation and will make an appreciable contribution to a healthy diet. Phytochemical and microbial analyses are recommended for further research.

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# THE EFFECT OF CHAYOTE LEAVES (*SECHIUM EDULE*)'S FLAVONOID FRACTION ON THE REDUCTION OF THE SERUM URIC ACID LEVELS THROUGH THE INHIBITION OF XANTHINE OXIDASE ACTIVITY

***Diah Pitaloka Putri, Aulia Putri Wahyuningtyas, Ahmad Ni'matullah Al-Baarri, Nani Maharani***

## ABSTRACT

Uric acid is the final product of purine metabolism and is categorized as hyperuricemia when it reaches >6.0 mg.dL$^{-1}$ for women and >7.0 mg.dL$^{-1}$ for men. The chayote leaves (*Sechium edule*) contain a high amount of flavonoid and might be used as an alternative to reduce hyperuricemia. The purpose of this study is to analyze the effect of chayote leaves (*Sechium edule*)'s flavonoid fraction on the level of uric acid and the activity of xanthine oxidase (XO) in *Sprague Dawley* Rats. The flavonoid fraction (FF) was obtained by extracting the chayote leaves, fractionating with n-hexane, hydrolyzing with HCl, and finally re-fractionating with ethyl acetate. Thirty male *Sprague Dawley* rats were induced for hyperuricemia by potassium oxonate and broth block for 21 days, and the interventions were given orally for 14 days. The rats were divided randomly into five groups: normal control (K-), hyperuricemia control (K+), hyperuricemia with FF dose 50 mg.200g$^{-1}$ body weight (P1), hyperuricemia with FF dose 100 mg.200g$^{-1}$ body weight (P2) and hyperuricemia with allopurinol 1.8 mg.200g$^{-1}$ body weight. Xanthine oxidase activity was measured by *CheKine$^{TM}$ Xanthine Oxidase Assay Kit*, with simple colorimetry methods. The statistical analysis for XO activity was done using *Kruskal-Wallis* followed by *Mann Whitney*. The results showed that chayote leaves (*Sechium edule*)'s flavonoid fraction contains apigenin, apigenin o-glucoside, and luteolin. It also has antioxidant activity with 98.45% inhibition. There was a significant reduction in xanthine oxidase activity in groups treated with FF ($p$ <0.005). The best dose of FF affecting XO activity was 100 mg.200g$^{-1}$ body weight. The combination of FF and allopurinol can be more effective in decreasing uric acid levels by inhibiting XO activity.

**Keywords:** flavonoids; *Sechium edule*; xanthine oxidase; uric acid

## INTRODUCTION

Uric acid is the final product of purine metabolism, which is produced endogenously, and mostly comes from purines in foods consumed excessively. The uric acid level is high if it is more than 6.0 mg.dL$^{-1}$ in women and more than 7.0 mg.dL$^{-1}$ in men. An increase in uric acid levels is called hyperuricemia **(Dalbeth et al., 2016; Kushiyama et al., 2016)**. The prevalence of hyperuricemia has increased rapidly in recent years, reaching 10% of the world's population. The increase in hyperuricemia does not merely occur in developed countries but also in developing countries **(Kuo et al., 2015)**. Lifestyle factors include excessive purine diet (the habit of consuming seafood, internal meats such as liver, kidney, intestines), alcohol consumption, obesity, gender, and age, which are risk factors for increased hyperuricemia **(Raja et al., 2019)**. Meanwhile, several other epidemiological studies have shown that hyperuricemia has an impact on the development of diabetes mellitus, dyslipidemia, kidney disease, obesity, hypertension, cardiovascular disease, and arthritis **(Ma et al., 2019; Nurshad et al., 2018)**. Chayote is a type of vegetable that is widely found and consumed, especially in Mexico, Costa Rica, Guatemala, and Indonesia. The part of chayote that can be consumed apart from the fruit is its leaves. Chayote leaves have the potential as a source of nutrition and health benefits, such as hyperuricemia. However, chayote leaves are not as popular as the other part of the plant regardless of its content of bioactive compounds higher than the fruit. The bioactive compounds in chayote leaves include phenolics, carotenoids, and flavonoids **(Managa et al., 2021; Siciliano et al., 2004)**. The flavonoids found in chayote leaves are apigenin and luteolin. The apigenin and luteolin content in chayote leaves is, respectively, 0.081 g.100g$^{-1}$ and 0.141 g.100g$^{-1}$ **(Loizzo et al., 2016; Vieira et al., 2019)**.

Flavonoids found in plants are often in demand due to their various bioactivity **(Xiao, 2017)**. Currently, plants containing flavonoids are widely used as alternatives to help treat several diseases because the side effects are lower, and the price is relatively cheaper than drugs **(Sahnoun and Saibi, 2018)**. Flavonoids have biological and pharmacological properties such as anti-oxidative, anti-bacterial, anti-viral, and anti-mutagenic. Flavonoids also play a role in reducing the activity of several enzymes, one

of which is the xanthine oxidase enzyme **(Lin et al., 2002)**. Xanthine oxidase is an enzyme responsible for catalyzing hypoxanthine and xanthine into uric acid, to control purine catabolism **(Huang et al., 2011)**. Apigenin and luteolin have the effect of reducing uric acid levels by inhibiting the activity of the xanthine oxidase enzyme **(Huang et al., 2011; Lin et al., 2018)**.

Another researcher indicates that administration of the flavonoid apigenin at a dose of 50 mg.200g$^{-1}$ body weight and 100 mg.200g$^{-1}$ body weight for seven days affects xanthine oxidase activity in both normal and hyperuricemia rats **(Huang et al., 2011)**. Another report describes that luteolin is also a bioactive compound that plays a role in inhibiting the activity of the xanthine oxidase enzyme so that it affects the decrease in uric acid levels **(Pauff and Hille, 2009)**.

## Scientific hypothesis

Chayote leaves (*Sechium edule*)'s flavonoid fraction reduces serum uric acid levels through the inhibition of xanthine oxidase activity in *Sprague Dawley* rats hyperuricemia.

## MATERIAL AND METHODOLOGY

This study was a true experimental research design using a randomized pre-post-test control group design. The study was conducted at the Animal Laboratory of PAU, Universitas Gadjah Mada, Yogyakarta for animal care, and Integrated Laboratory of Universitas Diponegoro, Semarang for the production of chayote leaves (*Sechium edule*)'s flavonoid fraction. This study had been approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Diponegoro Semarang, with the number 05/EC/H/FK-UNDIP/I/2020.



**Figure 1** Chayote (*Sechium edule*) leaves.

### Samples

The sample in this study used *Rattus norvegicus*, strain *Sprague Dawley* rats, weighing 150 – 300 g and aged 2 – 3 months.

### Chemicals

Chemicals used in the fractionation of chayote leaves consisted of HCl (Merck 1.00317.2500, Germany), methanol (Brataco, Indonesia), ethyl-acetate, and n-hexane (Merck, Germany, packed by PT. Brataco, Indonesia). Chemicals of antioxidant activity test are methanol (Brataco, Indonesia), DPPH liquid (Sigma Aldrich, Germany). Chemicals of XO activity test (assay buffer, sample diluent, WST-8, enhancer, and xanthine oxidase standard produced by Abbkine Scientific co., Ltd, Wuhan, China. Chemicals for uric acid levels test are uric acid reagent FS TBHBA (Diagnostic Systems GmbH, Germany).

### Animals and Biological Material

*Sprague dawley* rats were obtained from Animal Laboratory of Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats (except K-) induced with Maggi brand block broth (PT Nestle, Indonesia) at an amount of 140 mg.200g$^{-1}$ rat's body weight and potassium oxonate (PO; Sigma Aldrich, USA) at a dose of 50 mg.200g$^{-1}$ body weight for 21 days. Thirty rats were divided into five groups, namely the normal control group (K-) which received no induction and no treatment, the hyperuricemia control group (K+), the hyperuricemia group with the administration of FF from chayote leaves at a dose of 50 mg.200g$^{-1}$ body weight (P1), the hyperuricemia group which was administered FF from chayote leaves at a dose of 100 mg.200g$^{-1}$ body weight (P2), and the hyperuricemia group which was given allopurinol at the dose of 1.8 mg.200g$^{-1}$ body weight (P3). The rats were given oral intervention (FF) for 14 days. The FF was obtained by the fractionation method from chayote leaves (*Sechium edule*). The criteria of chayotes were fresh leaves with no hollow or mold, and leaves were >28 days old. The chayote leaves were obtained from chayote farm in Ngablak, Magelang, Central Java, Indonesia.

### Instruments

iMark™ microplate absorbance reader, Bio-Rad.Microprocessor Controlled Spectrophotometer, (*OPTIMA SP-300,* Japan) for reader absorbance of uric acid levels and XO activity; Shimadzu UV-1800 UV-Vis Spectrophotometer (Japan) for reader absorbance of antioxidant activity by DPPH method.

### Laboratory Methods

Before and after the intervention (2 times), the blood tests were carried out to measure the serum uric acid level and XO activity. The uric acid level was measured using the enzymatic method using the uric acid reagent FS TBHBA (2,4,6-tribromo 3 hydroxybenzoic acid), while the XO activity was measured using the CheKine$^{TM}$ Xanthine Oxidase Assay Kit with a colorimetric test method. The antioxidant activity was measured by the DPPH method. DPPH method's principle is the sample's ability to reduce the oxidation process of DPPH free radicals (1,1-diphenyl-2-picrylhydrazyl) in methanol solution.

### Description of the Experiment

**Sample preparation:** After 14 days of intervention, blood was collected from the *sinus orbital* (±2mL) **a**nd centrifuged at 1,000 rpm for 15 minutes at 2 – 8 °C, plasma was collected and stored at -80 °C for later analysis. The analytical procedures were done according to the instruction of the kits. Thirty samples were analyzed, and each sample was measured twice (duplicate). Each treatment group consisted of six rats.

## Statistical Analysis

The results are presented as median (min-max) data. The difference in XO activity was tested using Kruskal Wallis (nonparametric) test followed by Mann-Whitney post-hoc test. The differences were considered significant at a

*p*-value <0.005. The SPSS software version 21 (SPSS Inc, Chicago, IL, USA) was used to perform the tests.

## RESULTS AND DISCUSSION

In this study, the antioxidant activity of chayote's flavonoid fraction was measured prior to the start of the experiment. Antioxidant activity from the flavonoid fraction of chayote (*Sechium edule*) leaves has a stronger ability to ward off free radicals than allopurinol (Table 1). High or low ability to reduce free radicals can be seen from the amount of percent inhibition. The higher percent of inhibition shows more materials to reduce free radicals. In contrast, a lower percent of inhibition shows less material to reduce free radicals. The inhibition process is thought to occur when the DPPH radical reacts with antioxidant compounds through the uptake of hydrogen ions. Flavonoids have a hydroxyl group (OH) so that the flavonoid can ward off free radicals by donating hydrogen and change free radicals from unstable to more stable **(Sangeetha et al., 2016)**.

Rats were weighed every week, starting at the time of acclimatization, during the intervention, and after the completion of the intervention. Weighing the rats aimed to monitor weight gain and determine the initial dose of flavonoid fractions and allopurinol. The results of body weight measurements showed an increase during the study (data not shown). In hyperuricemia groups, the increase in body weight is higher than in the control group. Different metabolic responses to different treatments might cause the difference in rats' weight gains.

Serum uric acid level measurement showed a decrease after treatment in P1 and P2 (flavonoid fraction) and P3 (allopurinol) groups, as shown in Table 2. In contrast, the K- and K+ groups (the control group) experienced an increase in uric acid, as shown in Table 1. The most significant decrease in serum uric acid level was found in the allopurinol group. Thus, allopurinol administration was proven to be more effective in reducing uric acid levels than flavonoid fractions at either dose. Among the flavonoid-treated group, the one gave 100 mg.200g$^{-1}$ BW flavonoid showed a better reduction than the lower one, indicating that the flavonoid fraction's effect is dose-dependent.

Based on the results of this study, it is observed that the chayote leaves (*Sechium edule*)'s flavonoid fraction affects the reduction of uric acid levels in rats for 14 days. The research found out that the administration of a fraction of the breadfruit leaves affects decreasing blood uric acid levels in rats **(Sinata, 2019)**. Research on human samples proved that administering *Sechium edule* extract three times a day for six weeks can reduce uric acid levels in subjects with metabolic syndrome disease **(Rosado-Pérez et al., 2019)**. The result of previous studies reported that intervention using allopurinol was more effective in decreasing uric acid levels compared to flavonoids from plants, but the side effects were more dangerous **(Jiang et al., 2020; Harwoko and Warsinah, 2020; Abdulhafiz et al., 2020)**. Chayote leaves (*Sechium edule*)'s flavonoid fraction and allopurinol have the same effect on reducing blood uric acid levels. Still, allopurinol has more side effects, including fever, skin rashes, allergies, hepatitis, kidney disease, and the risk of cardiovascular diseases **(Shah and Shah, 2015; Presa et al., 2019; Stamp et al., 2011; Bove et al., 2017)**.

The XO activity measurements were undertaken twice, before and after the intervention. The results showed that the treatment groups experienced a decrease, and the control group showed an increase in XO activity. The reduction in XO activity was shown by the groups given treatment, the P1 (flavonoid fraction 50 mg.200g$^{-1}$ BW), P2 (flavonoid fraction 100 mg.200g$^{-1}$ BW), and P3 (allopurinol 1.8 mg.200g$^{-1}$ BW). This reduction in XO activity reflected the inhibition of uric acid formation.

**Table 1** Antioxidant Activity (percent inhibition).

| Methods | Materials | % inhibition |
|---|---|---|
| DPPH | Chayote leaves (*Sechium edule*)'s flavonoid fraction | 91.45 |
| | Allopurinol | 78.00 |

**Table 2** Statistical Analysis of Xanthine Oxidase Activity.

| Xanthine Oxidase Activity (%) | Groups | | | | |
|---|---|---|---|---|---|
| | K- | K+ | P1 | P2 | P3 |
| Pre | 29.53 (27.54-31.79) | 69.04 (67.86-71.43) | 69.60 (68.09-70.41) | 68.81 (67.11- 9.58) | 68.43 (65.87-69.07) |
| Post | 30.70 (29.32-32.69) | 70.12 (68.95-71.69) | 51.85 (46.99-55.32) | 37.10 (34.14-40.13) | 32.74 (32.00-35.14) |
| $p^1$ | 0.028 | 0.028 | 0.028 | 0.028 | 0.028 |
| △ | 1.36 (0.64-1.78) | 0.93 (0.28-2.04) | 17.37 (-23.42- -14.49) | -32.10 (-34.68- -26.98) | -35.79 (-36.37- -30.73) |
| $p^2$ | | | 0.000 | | |

Note : Five groups of rats consisted of K-: normal rats; K+: hyperuricemia rats; P1:flavonoid fraction from chayote leaves (*Sechium edule*) treatment at dose 50 mg.200g$^{-1}$ bodyweight; P2: flavonoid fraction from chayote leaves (*Sechium edule*) treatment at dose 100 mg.200g$^{-1}$ bodyweight; P3: allopurinol treatment at dose 1.8 mg.200g$^{-1}$ bodyweight; $p^1$ : *p* values were obtained by *Wilcoxon* test between pre and post intervention; $p^2$ : *p* values were obtained by *Kruskal Wallis* test (all groups); △ : changes between pre and post treatment. The results are presented as median (min-max) for not normally distributed data.

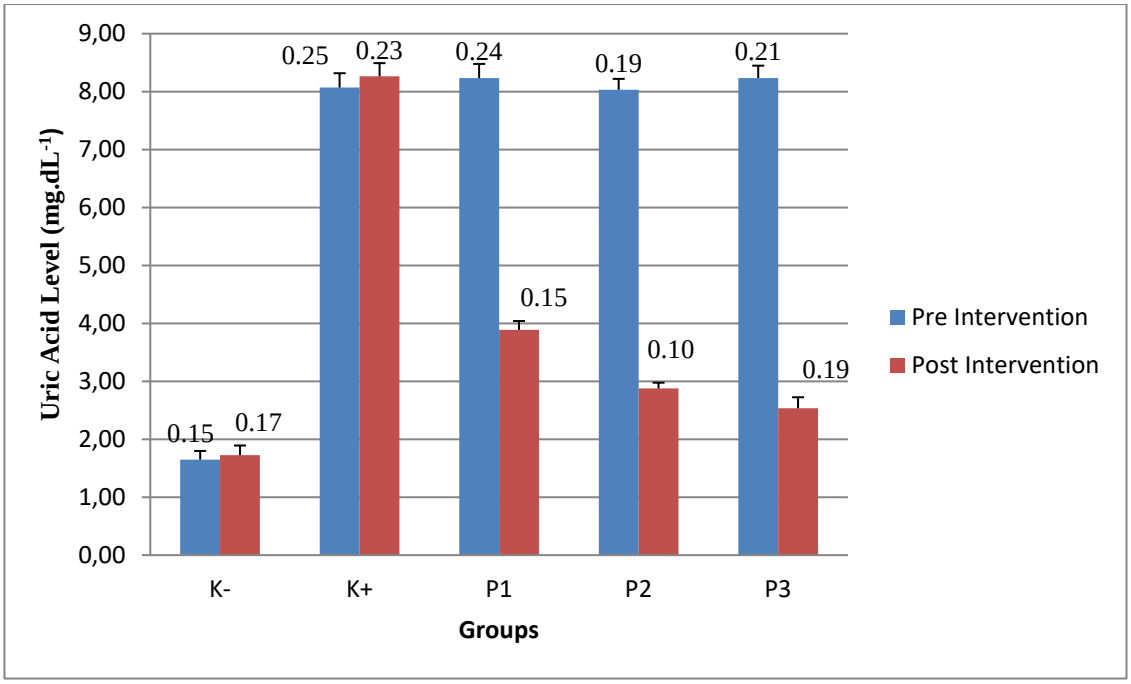


**Figure 2** Changes of uric acid level (pre and post intervention chayote leaves (*Sechium edule*)'s flavonoid fraction.

The average ability of the flavonoid fractions from chayote leaves to reduce XO activity was 18.19% and 31.46% for P1 and P2, respectively, while the P3 (allopurinol group) showed a 40.95% reduction.

In the groups without any treatment, XO activity increased, with the largest increase was shown by the K- group (1.25%). An increase in xanthine oxidase activity indicated the formation of serum uric acid.

The Wilcoxon test results showed that the XO activity before and after the intervention was significantly different ($p$ = 0.028) (Table 2). XO activity tended to increase in the control group, while in the treatment group, XO activity decreased. The results showed that flavonoid fraction intervention for 14 days affected the decrease of XO activity in hyperuricemic rats. The results of the Kruskal-Wallis test in this study showed that there were differences in XO activity after intervention in all groups ($p$ = 0.000) (Table 2). In the control group, XO activity tended to increase, while in the treatment group, XO activity decreased. This result showed that the intervention of flavonoid fraction for 14 days affected the decrease of xanthine oxidase activity in hyperuricemic rats.

In this study, the highest decrease in XO activity in the treatment group occurred from chayote leaves at the dose of 100 mg.200g$^{-1}$ BW. However, allopurinol administration was proven to be more effective in reducing the highest XO activity than the chayote leaves (*Sechium edule*)'s flavonoid fraction at a dose of 100/200 mg.g$^{-1}$ BW and 50/200 mg.g$^{-1}$ BW. The decrease in uric acid levels in the treatment groups, P1 and P2, was probably due to the content of bioactive compounds in the flavonoid fraction, which can inhibit the activity of the XO enzyme so that uric acid was not formed. The results of previous studies in vitro and in vivo reported that flavonoids contribute to antihyperuricemic activity by inhibiting XO activity **(Mohos et al., 2020; Yunita, Santoso and Subandi, 2021)**.

Xanthine oxidase is an enzyme that contributes to the production of uric acid by catalyzing hypoxanthine to xanthine and xanthine to uric acid in purine metabolism. These higher uric acid concentrations are in response to higher levels of XO activity and oxidative stress. Previous research reported that *Cudrania tricuspidata* leaf extract with high flavonoids administered to rats could inhibit XO activity **(Song et al., 2018)**. The flavonoid content of plants has antioxidant properties, which could suppress the XO enzyme so that more hypoxanthine and xanthine are excreted in the urine. The formation of uric acid can be inhibited, shown by the lower level in the blood **(Casagrande et al., 2013; Serrano et al., 2020)**. Other research shows that flavonoids extract from *Marantodes Pumilum var pumila* leaves can decrease XO activity in hyperuricemia mice **(Aladdin et al., 2020)**.

The decrease in uric acid levels is due to flavonoids content in this plant, which has a very good effect on XO activity, inhibits purine biosynthesis in the body **(Zeng et al., 2017)**, and increases the concentration of uric acid in the urine **(Kuo et al., 2015; Ling and Bochu, 2014; Shi and Williamson, 2016)**. Another mechanism by which the flavonoids reduce serum uric acid level is inhibition of uric acid reabsorption in the kidney **(Hamada et al., 2008)**. The bioactive compounds in plants reduce the expression of mRNA and protein transporter URAT1 and GLUT9 in hyperuricemic rats, associated with uricosuric effects with inhibition of uric acid reabsorption **(Hu et al., 2012; Yang et al., 2015; Dolati et al., 2018)**.

Based on the results of this study, the flavonoid fraction (apigenin and luteolin) can affect XO activity. In addition, the results of the FTIR test showed that the flavonoid fraction from chayote leaves contains the structure of C2, which has an effect of reducing XO activity. Previous research reported that the basic chemical structure of the flavanol group with C2 and C3 double bonds in the flavonoid compound was found to be very influential in the inhibition of high XO activity **(Van Hoorn et al., 2002; Spanou et al., 2012)**.

## CONCLUSION

Chayote leaves (*Sechium edule*) contain flavonoids (apigenin and luteolin), which affected serum uric acid levels in the hyperuricemic rat model after 14 days of treatment; however, its effect was lower than those of allopurinol. This reduction was due to the inhibition of XO activity, and the best result of this flavonoid fraction was observed at 100 mg.200g$^{-1}$ BW. Combining FF or flavonoids from plants and allopurinol can be more effective to decrease uric acid levels through the inhibition of XO activity. This finding might be useful to be implemented in humans to improve hyperuricemia conditions.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

This research was approved by the Ethics Committee of the Health Research Diponegoro University, Indonesia following the legislation of the number 05/EC/H/FK-UNDIP/1/2020 on the June 18th, 2020.

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# THE EFFECT OF OREGANO ESSENTIAL OIL ON CHICKEN MEAT LIPID OXIDATION AND PEROXIDATION

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## ABSTRACT

The study aimed to investigate and evaluate the oxidative stability of chicken thighs with skin stored in freezing conditions due to the effect of oregano essential oil for various times. The results were compared with a control group without the use of oregano essential oil. Samples of chicken thighs with skin were obtained from an experiment performed on a poultry farm in a deep litter breeding system. The results obtained from the application of oregano essential oil to chicken thighs with skin did not show a statistically significant difference ($p$ >0.05) in the dry matter content, fat content and acid value compared to the control group, where coccidiostats were used in starter and growth feed mixtures. A statistically significant difference was found in the peroxide value by applying oregano essential oil to chicken thighs with skin compared to a control group containing coccidiostats in starter and growth feed mixtures when stored for 1 day at room temperature ($p$ ≤0.01) and 12 months in freezing conditions at -18 °C (p ≤0.05) and a statistically non-significant difference ($p$ >0.05) when thighs with skin were stored for 6 and 9 months in -18 °C freezing conditions. In the conclusion, it was stated that maintaining the oxidative stability of chicken meat means knowing the factors that affect it and prepare the conditions for its maintenance. Chicken meat is generally susceptible to oxidative damage because it is characterized by a high concentration of polyunsaturated fatty acids. With a sufficient amount of effective antioxidants, chicken meat could be a homoeostatic system, but it remains limited or free of oxidized compounds and reactive components. These questions are the subject of further research in the field of oxidative stability of chicken meat.

**Keywords:** oregano essential oil; chicken thigh with skin; freezing; storage; oxidative

## INTRODUCTION

In addition to microbial degradation, lipid oxidation is a major cause of quality degradation and one of the main factors limiting the quality and acceptability of meat and meat products. Products that result from the peroxidation of polyunsaturated fatty acids affect meat quality parameters and reduce shelf life. in addition, many of these peroxidation products, including malondialdehyde, are toxic, genotoxic, cause intracellular oxidative stress, and could increase the frequency of tumor and atherosclerosis. Consumption of foods containing malondialdehyde, therefore, poses a risk to human health. The use of antioxidants is one of the main strategies to prevent the oxidation of lipids in meat and meat products. Although most synthetic antioxidants show high efficacy at low concentrations and are classified as a grass, i.e. generally accepted as safe, the acceptable dosage of these substances is low and the potential health risk is limited by their widespread use. In addition, in recent years, consumer concerns about the relation between food composition and human health and the increasing demand for natural-based foods have resulted in the food industry's interest in alternative methods to prevent and slow down lipid oxidation. One of these alternatives is the use of plant extracts, especially essential oils **(Moghrovyan et al., 2019)**.

Oxidation significantly reduces the shelf life of prepared meat products. The addition of antioxidants to foods can be an effective solution to reduce oxidation and maintain its properties such as softness, juiciness, palatability, and shelf life. Both natural and synthetic antioxidants are available. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can effectively inhibit oxidation-induced adverse changes in meat products, but these antioxidants can have potential genotoxic effects. Therefore, the consumer prefers natural antioxidants **(Feng et al., 2017)**.

Oregano essential oil, as well as thyme essential oil, is among the 10 most popular essential oils used as preservatives in food. Both are categorized as GRAS by the Food and Drug Administration. Due to the high content of phenolic compounds, these essential oils are effective

antioxidants. Standardization of natural preservatives is needed **(Boskovic et al., 2019)**.

Poultry meat is particularly susceptible to oxidative damage due to its high concentration of polyunsaturated fatty acids. The rate and extent of lipid oxidation in muscle tissue have been found to depend on the degree of muscle tissue damage during handling before the slaughter of broiler chickens, such as stress and physical injury, as well as after slaughter, such as early postmortem conditions, pH and carcass temperature.

Under practical conditions, synthetic antioxidants such as butylated hydroxytoluene or butylated hydroxyanisole are often used as antioxidants. However, there is a trend to look for compounds that allow the transition from synthetic to natural antioxidants. The activity of *Origanum vulgare* L. is mainly attributed to its main components carvacrol and thymol, substances that modify the permeability of the bacterial cell membrane and react with lipid and hydroxyl radicals, which convert them into stable products **(Luna et al., 2010)**.

**Scientific hypothesis**: Oregano essential oil supports the oxidative stability of chicken thigh muscle fat.

## MATERIAL AND METHODOLOGY

### Technique carried out of the experiment

The feeding experiment was carried out under practical conditions on a broiler chicken farm. A hybrid combination of Cobb 500 was used as the final fattening type of broiler chickens. A feeding experiment was performed to obtain samples for chemical analyzes. Each box had dimensions of 2 m wide and 3.8 m long, which is following the principle of unrestricted movement of broiler chickens, i.e. 33.0 kg live weight per $m^2$ when removed from storage. 100 one-day-old chickens were placed in each group. The experiment in practical conditions lasted 40 days. Broiler chickens were fed from plate feeders and sucked water from hat feeders until the age of 14 days, and later until the end of the experiment, they received a feed from tube feeders and drank water from bucket feeders. Feed mixtures starter, growth, and final were used for feeding, which were the same in the basic raw materials in both the control and experimental groups. The difference in feed mixtures between the control and experimental groups was in the active substance added by the feed supplement in the control group and the additive applied to the thighs after the slaughter of the broiler chickens in the experimental group. The control group used a commercially produced feed mixture starter in chickens aged 1 to 14 days with the feed supplement coccidiostat Maxiban G160, a growth feed mixture in chickens aged 15 to 33 days with a feed supplement coccidiostat Sacox and a final feed mixture aged 34 to 40 days. In the experimental group, the feed mixtures were the same as in the control group, but without coccidiostats. Feed mixtures were manufactured by Biofeed, a.s., Kolárovo, where they were manually mixed from feed materials 4 times in a row.

### Sample preparation for laboratory measurements

Broiler chickens were randomly selected at the end of the feeding experiment in the number of 24 pcs from the control group (which means obtaining 48 pcs of thighs) and 24 pcs from the experimental group (i.e. 48 pcs of thighs). Broiler chickens were killed humanely and technologically processed. The thighs were separated from the carcass in a total of 48 pcs in each group. Each thigh was packed in a microtene bag and labeled with K (control group) and P (experimental group). All thigh samples with the skin from the control and experimental groups were boned. The thigh samples with the skin of the control group were packed in a microtene bag and marked K (control group) and further marked 1, 6, 9, and 12, which means 12 thighs for analysis depending on the different storage time (1 means 1 day after slaughter the broiler chickens and kept at room temperature, 6, 9 and 12 means 6, 9 and 12 months after the slaughter of broiler chickens and kept under freezing storage conditions at -18 °C.

### Application of oregano essential oil to the chicken thighs in the experimental group and their storage

Oregano essential oil was applied to each thigh sample with the skin of the experimental group. A thigh sample was placed in a porcelain mortar and 4.0 mL of oregano essential oil was applied to the surface of the entire thigh. Oregano essential oil was applied with a syringe and hand-thoroughly spread over the entire thigh surface using surgical gloves.

Thigh samples with the skin of the experimental group were packed in a microtene bag and marked P (experimental group) and another designation 1, 6, 9, and 12, which means 12 thighs for analysis depending on different storage time (1 means 1 day after slaughter broiler chickens and application of oregano essential oil and stored at room temperature, 6, 9 and 12 means 6, 9 and 12 months after the slaughter of broiler chickens and application of oregano essential oil and stored in freezing conditions -18 °C.

Each thigh with skin wrapped in a microtene bag and labeled was stored under the specified conditions according to the scheme in Table 1.

Oregano essential oil was obtained as a commercial additive from Calendula, a. s., Nová Ľubovňa. Oregano essential oil was accompanied by an A-test. The main components of oregano essential oil according to **Özkan et al. (2017)** are carvacrol (63.97%), p-cymene (12.63%), linalool (3.67%), α-terpineol (2.54%), and (-)- terpinen-4-ol (2.24%).

### Examined indicators

A feeding experiment was performed to obtain samples for chemical analysis. Chemical analysis of thigh samples was aimed at determining:

(a) the dry matter of thigh muscle with skin depending on the storage period,

(b) the fat of thigh muscle with skin depending on the storage period,

(c) the acid value of thigh muscle with skin depending on the storage period,

(d) the peroxide value of the thigh muscle with the skin depending on the storage period.

### The procedure of investigation established indicators

***Weighing of samples***

All samples and laboratory equipment requiring weighing for chemical analyzes of the thigh were performed on automatic scales of the Kern 440-49N type with an accuracy of d = 0.01 g.

**Table 1** Scheme of sample preparation for storage.

| Group | n (broilers) | Thigh samples prepared for storage | n (thighs) | Weight of grinded thigh samples prepared for chemical analysis, g |
|---|---|---|---|---|
| Control | 24 | 1 day | 12 | 70 |
| | | 6 months | 12 | 70 |
| | | 9 months | 12 | 70 |
| | | 12 months | 12 | 70 |
| Experimental | 24 | 1 day | 12 | 70 |
| | | 6 months | 12 | 70 |
| | | 9 months | 12 | 70 |
| | | 12 months | 12 | 70 |

Note: thigh sample: boned thigh with skin and in the experimental group with oregano essential oil application,
1 day after slaughter of broiler chickens and in the experimental group after application of oregano essential oil, stored samples at room temperature,
6 months after the slaughter of broiler chickens and in the experimental group after application of oregano essential oil, stored samples at under freezing conditions -18 °C,
9 months after the slaughter of broiler chickens and in the experimental group after application of oregano essential oil, stored samples at under freezing conditions -18 °C,
12 months after the slaughter of broiler chickens and in the experimental group after application of oregano essential oil, stored samples at under freezing conditions -18 °C,
weight of sample for chemical analysis – weighed 70 g from each mixed thigh with skin.

***Grinding and homogenization of samples***

The AOAC Official Method 983.18 which is Codex-Adopted – AOAC Method especially for Meat and Meat Products was used for the samples preparation.
Before each chemical analysis, each sample was individually ground and homogenized in a Grindomix 200 laboratory mixer and quantitatively transferred to a glass laboratory beaker. 70 g of the homogenized fresh mass of each thigh sample was weighed.

***The procedure for analyzed sample drying***

Sea sand was poured to the bottom of the petri dish. A petri dish with sea sand was weighed. The homogenized sample was transferred from the mixer to sea sand of the petri dish and the sample thus prepared was weighed. The prepared samples with Petri dish and sea sand for chemical analysis were dried in an oven of type HS 62A at a temperature of 103 ±2 °C for 12 hours to constant weight. The dried samples together with the petri dish and sand were transferred to a desiccator using laboratory tongs. The samples in the desiccator were allowed to cool. Cooled samples with a petri dish and sand were weighed. The difference between the weight of the dried sample with the petri dish together with the sea sand and the weight of the petri dish together with the sea sand represented the sample for chemical analysis. The weight of each dried thigh sample with skin ranged on average 19.3 g.

***Dry matter per fresh mass***

The standard reference method (AOAC Method 950.46) for measurements of moisture in meat was used.
Analyzed samples of thigh muscle of 5.0 g were placed in the pre-weighed aluminum dishes ≥50 mm diameter and ≤40 mm deep then placed in the drying oven (type: HS 62A) at temperature 103 ±2 °C for 4 hours to a constant weight. Samples in aluminum dishes were partially dried covered. Samples with covered aluminum bowls after drying were transferred to the desiccator to cool. Then the dishes and their dried samples were reweighed.
Calculation of dry matter per fresh mass of thigh muscle with skin and dry matter of analyzed sample:

$$\text{Dry mater} = \frac{a}{n} \times 100 \quad (1)$$

where:
*dry matter per fresh mass and dry matter of the analyzed sample (same calculation procedure), %,*
*a = weight of the dried sample, g,*
*n = sample weight, g.*

***The procedure for analyzed sample extraction***

The fat content of the samples was determined 1 day after the slaughter of the broiler chickens, 3, 6, 9, and 12 months after the slaughter of the broiler chickens. The samples were stored for 1 day at room temperature, 6, 9, and 12 months in freezing conditions at -18 °C. The analyzed sample (dried) was extracted with boiling petroleum ether, the fat is determined by evaporation of the extraction system by mass.
The AOAC Official Method 991.36 for Fat (Crude) in Meat and Meat Products was used. In this experiment were used petroleum ether as a solvent and Det-gras N apparatus, Model 4002842 – capable of simultaneous extraction of 6 test portions.

**Procedure for determining the fat of the analyzed sample**

Empty aluminum cartridges were weighed, and 6 pcs were needed for one analysis. Each assay sample was first mixed in a Grindomix 200 laboratory mixer from which 12 g was weighed. Each weighed sample was transferred to an extraction cellulose cartridge, which was sealed with

cotton wool. The filled sample cartridge was placed in an aluminum cartridge and connected to a Detras N-type extractor. The operating temperature on the apparatus was 120 °C and the fat extraction itself took 60 minutes. The fat extraction from the analysis sample was into the aluminum cartridge of the instrument. After the fat extraction was complete, the remaining petroleum ether was evaporated from the fat sample and each aluminum fat cartridge was weighed. The difference between the weight of the aluminum grease cartridge and the weight of the empty aluminum cartridge was considered to be the fat of the analysis sample. The weight of each fat analysis sample ranged from 8.55 to 9.97 g.

Calculation of the fat for an analysed sample of the thigh muscle with skin:

$$Fat = \frac{a}{b} \times 100 \quad (2)$$

where:

*fat of the analysed sample, %,*

*a* = weight of fat extracted*, g,*

*b* = weight of the analysed sample*, g.*

***Acid value***

The acid value was determined in fat samples performed 1 day after the slaughter of the broiler chickens, 6, 9, and 12 months after the slaughter of the broiler chickens. The samples were stored for 1 day at room temperature, 6, 9, and 12 months in freezing conditions at -18 °C. The acid value expresses the quantity of potassium hydroxide needed to neutralize the free fatty acids present in 1.0 g of fat (mg $KOH.g^{-1}$). The acid number of the fat expresses the amount of potassium hydroxide (KOH) in mg needed to neutralize the fatty acids in 1 g of the sample.

The acid value is the quantity of potassium hydroxide (KOH) in mg necessary to neutralize the fatty acids in 1 g of sample.

$$RCOOH + KOH \rightarrow RCOOK + H_2O \quad (3)$$

Often, the acid value is converted to a free fatty acid (FFA) content by multiplying the acid value with a factor that equals the molecular weight of the fatty acid concerned (usually oleic acid, MW = 282.4) divided by ten times the molecular weight of the potassium hydroxide (56.1). This factor ten stems from the fact that the acid value is expressed as $mg.g^{-1}$, whereas the free fatty acid content is expressed as a percentage. When the free fatty acid content is expressed as 'wt% oleic acid,' this factor, therefore, equals 0.50. When examining the risk from food fat, the acid value expresses the degree of hydrolysis of fats. It is an indicator for evaluating the condition of processed food raw material and its state in the storage conditions.

***Principle of determining the acid value***

The principle of determining the acid value is to dissolve the fat in the extract with an ethanol-diethyl ether mixture using alkalimetric titration in the presence of phenolphthalein.

***Procedure for determining the acid number of fat***

First, 25.0 mL burettes and extraction flasks were prepared. 2.5 g of fat was weighed into each extraction flask, and a 1:1 mixture of ethanol-diethyl ether in a volume of 25 mL was added. Before titration, 2 drops of phenolphthalein indicator were added to the mixture in the extraction beaker. Each extraction beaker was slightly heated with a laboratory gas burner. The burette was made up to 25.0 mL with potassium hydroxide solution before each measurement of the sample. The titration was completed when the sample in the extraction flask acquired a pale pink color, which had a shelf life of 30 seconds.

Calculation:

$$AV = \frac{V}{n} \times T \quad (4)$$

where:

*AV = acid value, mg KOH.g$^{-1}$,*

*V = quantity of standard KOH solution during titration, mL,*

*n = weight of extracted fat, g, i.e. 2.5 g,*

*T = concentration of KOH in the measuring solution, $mg.mL^{-1}$ (at the concentration of the solution exactly c (KOH) = 0.05 $mol.L^{-1}$ is T = 2.8055 $mg.mL^{-1}$).*

The weight of extracted fat was determined based on the results of the assumed acid value in the thighs of broiler chickens according to **Tkačová and Angelovičová (2013), Klimentová (2019)**.

***Peroxide value***

Fat peroxide value was determined in fat samples performed 1 day after slaughter of broiler chickens, 6, 9, and 12 months after the slaughter of broiler chickens. The samples were stored for 1 day at room temperature, 6, 9, and 12 months in freezing conditions at -18 °C. The peroxide number is an indicator to examine the oxidation of fats. It expresses the amount of active oxygen in 1.0 g of fat (μmol $O_2.g^{-1}$). The peroxide value is defined as the amount of peroxide oxygen per 1 kg of fat or oil. Traditionally this was expressed in units of milliequivalents, although if we are using SI units then the appropriate option would be in mmol per kg (N.B. 1 milliequivalents = 0.5 mmol; because 1 mEq of $O_2$ = 1 mmol/2 = 0.5 mmol of $O_2$, where 2 is valence). The unit of milliequivalent has been commonly abbreviated as mequiv or even as meq.

The peroxide value is determined by measuring the amount of iodine that is formed by the reaction of peroxides (formed in fat or oil) with iodide ions.

$$2\,I^- + H_2O + HOOH \rightarrow HOH + 2OH^- + I_2 \quad (5)$$

The base produced in this reaction is taken up by the excess of acetic acid present. The iodine liberated is titrated with sodium thiosulphate.

$$2S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2\,I^- \quad (6)$$

The acidic conditions (excess acetic acid) prevent the formation of hypoiodite (analogous to hypochlorite), which would interfere with the reaction.

The indicator used in this reaction is a starch solution where amylose forms a blue to the black solution with iodine and is colorless where iodine is titrated.

A precaution that should be observed is to add the starch indicator solution only near the endpoint (the endpoint is near when fading of the yellowish iodine color occurs) because at high iodine concentration starch is decomposed to products whose indicator properties are not entirely reversible.

***Principle of determination of peroxide value***

The principle of determining the peroxide value is the determination of iodine by titration during its release from iodide by hydroperoxide-unsaturated lipids in an acidic medium.

***Procedure for the determination of the peroxide value***

Erlenmeyer flasks with ground glass joint and glass stoppers and a starch indicator were prepared. 5.0 g of starch was weighed, to which 30.0 mL of water was added. The mixture was mixed thoroughly. 2.0 g of fat was weighed and transferred to each Erlenmeyer flask. Chloroform (10.0 mL) was added to the fat. The flask was then closed and the contents were shaken well by hand until the fat was completely dissolved.

Saturated 1.0 mL aqueous potassium iodide solution and 15.0 mL concentrated acetic acid were added to the dissolved sample. The flask was immediately closed and the contents were manually shaken again for 1 minute. Following this procedure, the sample was stored in a closed flask in a dark place at room temperature with the windows covered with blinds for exactly 5 minutes. Then 75.0 mL of water was added to the sample in the flask and again the contents were shaken thoroughly by hand in a closed flask. After mixing, a prepared starch indicator of 5.0 mL (starch solution) was added to the contents of the flask. The sample thus prepared was titrated with a solution of sodium thiosulfate 0.01 mol.$L^{-1}$ c ($Na_2S_2O_3$ x 5 $H_2O$). Its concentration was determined by discoloration. In parallel with the determination of the peroxide value of the fat sample, a blank experiment of the fat-free sample was performed.

Calculation:

$$PV = \frac{[(V_1 - V_0) \times T \times 1000]}{n} \quad (7)$$

where:

*PV = peroxide value, µmol $O_2.g^{-1}$,*

*$V_0$ = sodium thiosulphate standard solution used in the blank, mL,*

*$V_1$ = sodium thiosulphate standard solution used for sample titration, mL,*

*T = concentration of sodium thiosulphate standard solution used, mol.$L^{-1}$,*

*n = weighed sample of fat, g.*

The weight of extracted fat was determined based on the results of the presumed peroxide value in the thighs of broiler chickens according to **Tkačová and Angelovičová (2013), Klimentová (2019)**.

## Statistical analysis of results

The data obtained by the measurements were statistically evaluated according to statistical characteristics such as mean (*M*), average value, and standard deviation (*SD*). The difference in the indicator between the groups with each other, i.e. two dependent selections of control and experimental groups were evaluated by Student's t-test. The value of the t-test was commented as statistically significant $p \leq 0.05$ and $p \leq 0.01$, and statistically non-significant $p > 0.05$. The statistical evaluation of the results was by the methods of the SAS program package version 8.2.

## RESULTS AND DISCUSSION

### Dry matter in thigh muscle with skin

The average dry matter content in the thigh muscle with skin 1 day, 6, 9, and 12 months after the slaughter of broiler chickens is given in Table 2.

**Table 2** Average dry matter content in the chicken thigh muscle with skin, g.100 $g^{-1}$.

| Storage time thigh muscle | Group | n | *M* ±*SD* | t-test |
|---|---|---|---|---|
| 1 day at room temperature | control | 12 | 26.91 ±2.03 | 0.341– |
| | experimental | 12 | 27.21 ±1.85 | |
| 6 months at -18 °C | control | 12 | 27.17 ±1.44 | 0,701– |
| | experimental | 12 | 28.31 ±1.53 | |
| 9 months at -18 °C | control | 12 | 27.89 ±1.41 | 0,401– |
| | experimental | 12 | 28.26 ±2.01 | |
| 12 months at -18 °C | control | 12 | 28.41 ±1.52 | 0, 302– |
| | experimental | 12 | 28.72 ±1.52 | |

Note: *n* = multiciplity, *M* = mean, *SD* = standard deviation,
numerical values in the column at t-test marked -: statistically non-significant difference between the control and experimental group ($p > 0.05$).

In recent years, it has become common use prophylactic drugs and antibiotic growth stimulants in the feeding of broiler chickens and other farm animals. However, the continued use of these compounds has led to results such as the development of resistant bacteria and antibiotic residues in meat and other animal products that pose a risk to public health and the environment. This concern has led many countries, including European Union countries, to limit the use of antibiotics in feed. Therefore,
it is necessary to identify safe alternatives to feed antibiotics **(Jazi et al., 2020)**.

In our study, we focused on the verification of oregano essential oil by application to chicken thighs in relation to oxidative stability depending on the storage time under freezing conditions. Positive results in the application of essential oils whether in feed or on the product itself, are achieved by the effects of bioactive substances contained in the essential oils. These bioactive compounds include hydrocarbons, phenols, esters, alcohols, acids, and steroids, which have a positive effect on the health of broiler chickens and their performance or meat quality **(Shirani et al., 2019)**.

**Sharma et al. (2020)** state that essential oils can influence the performance and physiological processes of broiler chickens, both quantitatively and qualitatively.

The aim of the study by **Sabikun et al. (2019)** was to investigate the *post-mortem* effects on physico-chemical properties and oxidative stability of chicken thigh and breast muscles depending on the storage time in freezing conditions. Chicken breast and thigh muscles were obtained from 24 broiler chickens and measured from slaughter for 30 minutes or 1.5 hours. They were immediately frozen at -75 °C and then stored at -20 °C for 90 days to measure meat quality characteristics. The results showed that longer freezing led to the deterioration of meat quality and greater deterioration of post-rigid frozen muscles. Frozen muscles before stiffening had a higher pH, water holding capacity (about 90%) and sarcomere length with lower melting point and boiling loss than muscles after solidification. The thigh muscles had better physico-chemical properties than the breast muscles, except for loss during cooking. Therefore, immediate freezing could be an effective way to minimize the deterioration of the quality of frozen chicken.

The condition of *rigor-mortis* or *post-mortem* causes various physical and biochemical changes in the muscles through the activity of the proteolytic system after the slaughter of broiler chickens. This proteolytic system is associated with qualitative features such as sensitivity, juiciness, water retention, taste, protein, and structural muscle degradation **(Carvalho et al., 2017)**.

When frozen chicken meat is thawed, the muscles lose weight and lose a lot of water, leading to a reduction in the juiciness of the meat. Their research aimed to evaluate *pre-* and *post-rigor* frozen muscles concerning the quality of chicken meat. The research focused on changes in muscle pH, color, water retention capacity, heating losses, cooking losses, shear strength, sarcomere length, and oxidative stability before and after solidification of frozen thigh and breast muscles during different storage times for 90 days in freezing conditions (-20 °C). Based on the results, a conclusion was formulated. Prolonged *post-mortem* aging before freezing could have adverse effects on the quality characteristics of frozen chicken muscles. The results showed positive effects of freezing before the onset of *rigor-mortis* (within 30 minutes after the slaughter of broiler chickens) on the quality of chicken muscles. Frozen muscles before solidification had a higher pH, lower dripping, shorter thawing, and less cooking losses than frozen muscles after solidification **(Sabikun et al. (2019)**.

We recorded the average dry matter content of the thigh muscle with skin 1 day after the slaughter of broiler chickens and kept at room temperature in the experimental group of 27.21 g.100 $g^{-1}$ compared with the average dry matter content of the thigh muscle with skin in the control group of 26.91 g.100 $g^{-1}$. The difference in dry matter content with skin 1 day after the slaughter of broiler chickens and kept at room temperature was not statistically significant ($p$ >0.05) between the control and experimental groups. Based on the statistical evaluation of the results expressed by the standard deviation, we can state that the measured values of dry matter content in thigh muscle with skin kept at room temperature 1 day after the slaughter of broiler chickens fluctuated more in the control group compared to the experimental group ($SD$ = 2.03 versus $SD$ = 1.85).

Freezing is the preferred way to preserve food on the world meat export market at more than $ 13 billion a year. Despite their ability to maintain the quality and safety of meat, problems with freezing and thawing cycles remain a major problem for processors and consumers. Temperature fluctuations or abuse caused by the freeze-thaw cycle stimulate lipid oxidation and accelerated discoloration of the meat surface. Temperature fluctuations, which are a major problem in the cold-chain meat industry, especially in developing countries, are related to physiological and biochemical changes in animal muscles. The storability of meat is usually determined by its appearance, structure, color, aroma, microbial activity, and nutritional value and is affected by frozen storage and subsequent thawing. The main damage to frozen meat during storage is caused by the breakdown of fats and proteins. The meat industry has a strong interest in verifying a meat product that is actually fresh or that has been previously frozen, due to the large price differences between fresh, frozen, and thawed meat. It is difficult for consumers to detect changes in the quality of meat that occur in some products when they have been frozen. Most research on freezing and thawing red meat focuses on reducing moisture loss. Water loss is directly proportional to water retention capacity by muscle proteins, and reduced water content alters key quality parameters such as color and texture. The freeze-thaw cycle is a major contributor to reduced water retention capacity and unacceptably low water retention capacity causes great deterioration of products. However, limited progress has been made in understanding the real mechanisms for changing meat quality in freeze-thaw cycles. The reduction in water retention capacity is directly related to the denaturation of proteins in the muscle fiber structure. Protein oxidation due to freeze-thaw cycles has been largely ignored, especially in the commercial broiler chicken production chain. Therefore, studies are needed to understand the effect of freeze-thaw cycles on protein stability and its relation to lipid and protein oxidation. The result of increasing freezing and thawing cycles is a higher degree of oxidation of lipids and proteins, as evidenced by a higher content of malondialdehyde and carbonyl compounds and a lower content of sulfhydryl groups. Repeated freeze-thaw cycles increase lipid and protein oxidation and reduce water retention and color fastness of chicken meat **(Ali et al., 2015)**.

We recorded an average dry matter content in the thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens in the experimental group of 28.31 g.100 $g^{-1}$. This value is slightly higher than compared to the control group of 27.17 g.100 $g^{-1}$. The difference in dry matter content of the thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p$ >0.05) between the control and experimental groups. By statistical evaluation of the results expressed by the standard deviation, we found that the measured values of the dry matter content of thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the experimental group compared to control group ($SD$ = 1.53 vs. $SD$ = 1.44).

The average value of dry matter content in the thigh muscle with skin was measured in the experimental group of 28.26 g.100 $g^{-1}$ for 9 months of our storage in freezing conditions at -18 °C after the slaughter of broiler chickens and in the control sample 27.89 g.100 $g^{-1}$. The value of the experimental group was slightly higher. The difference in dry matter content of the thigh muscle with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p$ >0.05) between the control and experimental groups. According to the statistical evaluation expressed by the standard deviation, we found that the measured values of dry matter content in the thigh muscle with skin, which were stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the experimental group than in the control group ($SD$ = 2.01 vs. $SD$ = 1.41).

After 12 months from the slaughter of broiler chickens kept in freezing conditions at -18 °C, the average dry matter content in the thigh muscle with the skin of the experimental group was measured 28.72 g.100 $g^{-1}$ and in the control sample 28.41 g.100 $g^{-1}$. The difference in the dry matter content of the thigh muscle with skin stored for 12 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p$ >0.05) between the control and experimental groups.

Statistical evaluation of the results, which are expressed by the standard deviation, we found that the measured values of the dry matter content of the thigh muscle with skin stored for 12 months in freezing conditions at -18 °C after the slaughter of broiler chickens was lower in the experimental group compared to control group ($SD$ = 1.36 vs. $SD$ = 1.52).

To compare the dry matter content in the thigh muscle of our results, we selected the results of **Haščík et al. (2012)** for a hybrid combination of Ross 308 broiler chickens. Their results are higher compared to ours. They recorded dry matter content 31.51 g.100 $g^{-1}$ in the control group in which the broiler chickens were fed a feed mixture with coccidiostats and 30.21 g.100 $g^{-1}$ or 29.88 g.100 $g^{-1}$ in the experimental group with pollen extract with different doses.

In an experiment with broiler chickens, **Semjon et al. (2020)** investigated by chemical analysis the dry matter content in the thigh muscle of the Cobb 500 hybrid combination, which measured slightly lower values compared to our results. These authors report 26.21 g.100 $g^{-1}$ and 26.04 g.100 $g^{-1}$ of dry matter content in the thigh muscle. Their object of research with broiler chickens was different doses of humic substances in feed mixtures.

**Fat in thigh muscle with skin**

The average fat content in the thigh muscle with skin 1 day, 6, 9, and 12 months after the slaughter of broiler chickens is given in Table 3.

The fat content of chicken meat is closely related to the nutrition and feeding of broiler chickens. The lipid profile in these tissues can be assumed to reflect the lipid profile of the feed (type and dose) **(Sirri et al., 2003)**.

The interactions that take place between the nutrients that make up the feed and the synthesis and activity of lipogenic enzymes are responsible for a wide range of lipid storage options in adipose tissue. In addition, the biological activity of some fatty acids stimulates or inhibits specific lipogenic genes encoding enzymes **(Jump, 2002)**.

**Sierżant et al. (2018)** state in their study that meat obtained from broiler chickens is a source of the high biological value of animal proteins and is therefore valued for the consumer. On the other hand, the consumer prefers chicken meat due to its low-fat content and low energy value compared to meat from large livestock.

Fats are vital substances for proper human nutrition. In addition to providing energy for the body's biological processes, fats contain large amounts of substances such as essential fatty acids or fat-soluble vitamins that only a diet can provide. On the other hand, when obtaining fats from food, it is necessary to know the conditions for their qualitative attributes **(Purriños et al., 2011)**, in our case chicken thigh muscle, such as the focus of this study in the methodology.

Fats are involved in processes that affect the taste of meat and contribute to improving its tenderness and juiciness **(Amaral et al., 2018)**.

Therefore, the fat content and its composition are crucial for consumers due to their importance for meat quality and nutritional value **(Wood et al., 2004)**.

However, fats are prone to degradation. Fat oxidation is a major non-microbial cause of deterioration in the quality of meat and meat products **(Lorenzo et al., 2012)**.

Degradation of the chicken begins with the killing of the broiler chicken and continues gradually until the final product is consumed **(Chaijan et al., 2017)**.

**Table 3** Average fat content in the chicken thigh muscle with skin, g.100 $g^{-1}$.

| Storage time thigh muscle | Group | n | *M* ±*SD* | t-test |
|---|---|---|---|---|
| 1 day at room temperature | control | 12 | 2,56 ±0.16 | 0.332– |
| | experimental | 12 | 2.77 ±0.09 | |
| 6 months at -18 °C | control | 12 | 2.68 ±0.15 | 0.208– |
| | experimental | 12 | 2.57 ±0.09 | |
| 9 months at -18 °C | control | 12 | 2.72 ±0.18 | 0.621– |
| | experimental | 12 | 2.66 ±0.23 | |
| 12 months at -18 °C | control | 12 | 2.76 ±0.13 | 0.371– |
| | experimental | 12 | 2.52 ±0.19 | |

Note: $n$ = multiciplity, $M$ = mean, $SD$ = standard deviation; numerical values in the column at t-test marked -: statistically non-significant difference between the control and experimental group ($p$ >0.05).

Therefore, all stages of the processes of handling broiler chickens and chicken meat, processing and storage of chicken meat must be carefully controlled to avoid possible spoilage **(Richards, 2006)** and to minimize economic losses in the meat industry **(Králová, 2015)**.

We recorded the average fat content of the thigh muscle with skin 1 day kept at room temperature in the experimental group of 2.77 g.100 $g^{-1}$ after the slaughter of broiler chickens compared to the average fat content in the thigh muscle with the skin of the control group of 2.56 g.100 $g^{-1}$. The difference in the fat content of the thigh muscle with the skin was not statistically significant ($p$ >0.05) between the control and experimental groups. Based on the statistical evaluation of the results expressed by the standard deviation, we can state that the measured values of fat content in the thigh muscle with skin kept at room temperature 1 day after the slaughter of broiler chickens fluctuated more in the control group compared to the experimental group ($SD$ = 0.16 vs. $SD$ = 0.09).

In thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens, the average fat content in the experimental group was recorded at 2.57 g.100 $g^{-1}$. This value is lower compared to a control sample of 2.68 g.100 $g^{-1.}$

The difference in fat content in the thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p$ >0.05) between the control and experimental groups. Statistical evaluation of the results expressed by the standard deviation, we found that the measured values of thigh muscle fat content with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the control group compared to the experimental group ($SD$ = 0.15 and $SD$ = 0.09). We found the average value of fat content with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens in the experimental group 2.66 g.100 $g^{-1}$ and the control group slightly higher 2.72 g. 100 $g^{-1}$. Statistical evaluation of the results expressed by the standard deviation, we found that the measured values of thigh muscle fat content with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the experimental group compared to the control group ($SD$ = 0.23 against $SD$ = 0.18).

After 12 months from the slaughter of the broiler chickens when the thigh muscles were stored in freezing conditions at -18 °C, the average fat content in the thigh muscle with the skin of the experimental group was found 2.52 g.100 $g^{-1}$ versus control group 2.76 g.100 $g^{-1}$. The difference in the fat content of the thigh muscle with skin stored for 12 months under freezing conditions at -18 °C was not statistically significant ($p$ >0.05) between the control and experimental groups. By statistical evaluation of the measured results, which are expressed by the standard deviation, we found that the values of fat content in the thigh muscle with the skin of the experimental group stored for 12 months in freezing conditions at -18 °C fluctuated more compared to control samples ($SD$ = 0.19 vs. $SD$ = 0.13).

Freeze storage cannot prevent oxidative degradation and microbial or enzymatic degradation **(Jay et al., 2005)**.

It has been known in the past, as is the case today, that chemical preservation methods for meat are quite beneficial in combination with refrigeration to optimize stability, product quality while maintaining freshness and nutritional value **(Cassens, 1994)**.

As reported by **Ali et al. (2015)**, the main degradation of frozen meat during storage is caused by the processes of fat and protein degradation. It is through these changes that it is possible to find out whether a given product is fresh or has been frozen before. Especially in the meat industry, this is important from a price point of view, as fresh products have a higher price than frozen or thawed products.

All foods that contain lipids, even in very small amounts (<1%), can undergo oxidation, leading to yellowing **(Wąsowicz et al., 2004)**.

Common livestock species, such as beef and lamb (ruminants), contain higher levels of saturated fatty acids in muscle mass and adipose tissue compared to chicken meat, which contains higher levels of polyunsaturated fatty acids **(Wood et al., 2008)**.

**Acid value in the thigh muscle with skin**

The average acid value in the thigh muscle with skin 1 day after the slaughter of broiler chickens, 6, 9, and 12 months from the slaughter of broiler chickens is given in Table 4.

**Table 4** Average acid value in the chicken thigh muscle with skin, g.100 $g^{-1}$.

| Storage time thigh muscle | Group | n | $M \pm SD$ | t-test |
|---|---|---|---|---|
| 1 day at room temperature | control | 12 | 4.68 ±1.16 | 0.563– |
| | experimental | 12 | 4.31 ±1.13 | |
| 6 months at -18 °C | control | 12 | 6.46 ±0.88 | 0.196– |
| | experimental | 12 | 6.08 ±1.19 | |
| 9 months at -18 °C | control | 12 | 7.68 ±1.07 | 0.273– |
| | experimental | 12 | 7.21 ±1.18 | |
| 12 months at -18 °C | control | 12 | 9.78 ±1.23 | 0.481– |
| | experimental | 12 | 9.26 ±1.16 | |

Note: n = multiciplity, $M$ = mean, $SD$ = standard deviation; numerical values in the column at t-test marked -: statistically non-significant difference between the control and experimental group ($p$ >0.05).

**Leonel et al. (2007)** analyzed breast and thigh muscle. They considered that higher levels of fat found in the thigh muscle compared to the breast muscle could indicate a higher probability of oxidative processes in the thigh muscle. The different fat content in the thigh muscle and the breast muscle is not related to the oxidative processes of fat.

These conclusions were reported in the study by **Gardini (2000)**.

However, there is a more recent study which states that muscles with a higher fat content show a greater tendency to oxidize through a continuous chain reaction of free radicals **(Ruban, 2009)**.

The main goal of the meat industry and food safety researchers is to understand the mechanisms of fat oxidation and to identify the most effective methods of managing this process **(Domínguez et al., 2018)**.

Lipid oxidation leads to a deterioration of some quality characteristics of the meat, such as taste, texture, and color, and also reduces the shelf life along with the formation of some toxic compounds **(Mohamed et al., 2008)**.

A similar view is reported by **Ali et al. (2015)**.

We recorded the average acid value in the thigh muscle with skin for 1 day stored at room temperature after the slaughter of broiler chickens in the experimental group of 4.31 mg $KOH.g^{-1}$ compared to a control sample of 4.68 mg $KOH.g^{-1}$. The difference in the average value of the acid value of thigh muscle fat with skin for 1 day kept at room temperature after the slaughter of broiler chickens was not statistically significant ($p$ >0.05) between the control and experimental group. Based on the statistical evaluation of the results expressed by the standard deviation, we can state that the measured acid value of thigh muscle fat with skin kept at room temperature 1 day after slaughter of broiler chickens fluctuated slightly more in the control group compared to the experimental group ($SD$ = 1.16 vs. $SD$ = 1.13).

In thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens, we recorded the average acid value in the experimental group of 6.08 mg $KOH.g^{-1}$. This value is lower compared to the control group of 6.46 mg $KOH.g^{-1}$. The difference in the acid value in thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p$ >0.05) between the control and experimental group. Statistical evaluation of the results expressed by the standard deviation, we found that the measured values of the acid value of thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the experimental group compared to the control group ($SD$ = 1.19 vs. $SD$ = 0.88).

In the thigh muscle stored under freezing conditions at -18 °C 9 months after the slaughter of broiler chickens, the average acid value was measured in the experimental group of 7.21 mg $KOH.g^{-1}$ and the control sample 7.68 mg $KOH.g^{-1}$. No statistically significant difference ($p$ >0.05) was found between the control and experimental group in the in acid value of thigh muscle with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens. By statistical evaluation of the results expressed by the standard deviation, we found that the measured acid values of thigh muscle fat with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the experimental group compared to the control group ($SD$ = 1.18 vs. $SD$ = 1.07).

In thigh muscles stored for 12 months in freezing conditions at -18 °C after the slaughter of broiler chickens, we recorded an average acid value in the experimental thigh muscle group with the skin of 9.26 mg $KOH.g^{-1}$ and the control group 9.78 mg $KOH.g^{-1}$. The difference in measured acid values of thigh muscle with skin stored for 12 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not found statistically significant ($p$ >0.05) between the control and experimental group. Statistical evaluation of the results, which are expressed by the standard deviation, we found that the measured acid values of thigh muscle with skin stored for 12 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the control group compared to the experimental group ($SD$ = 1.23 and vs. $SD$ = 1.16).

It is well known that unsaturated fatty acids and oxygen are components that react during the fat oxidation process. In addition, other components may promote or prevent oxidative reactions. Fats can be oxidized in three main ways, which involve complex reactions: autoxidation, enzymatically catalyzed oxidation, and photooxidation. Of the three mechanisms, the most important process of lipid oxidation in meat is autoxidation, which is a continuous chain reaction of free radicals **(Cheng, 2016)**.

Enzymatic and photooxidation mechanisms differ from autoxidation only by the formation of hydroperoxides during the initiation phase **(Chaijan et al., 2017)**.

The mechanism of free radicals, despite the explanation of many of the changes observed in meat, does not provide a detailed and complete description of the changes induced in the reactants and derived products during the oxidation process. The main challenge, therefore, is to complete a scheme that can fully explain all the agents, intermediates, and reactions in fat oxidation **(Ghinimi et al., 2017)**.

**Peroxide value in thigh muscle with skin**

The average peroxide value in the thigh muscle with skin 1 day, 6, 9, and 12 months after the slaughter of broiler chickens is given in Table 5.

We found the average peroxide value in the thigh muscle with the skin after 1 day of storage at room temperature after the slaughter of the broiler chickens in the experimental group of 0.99 μmol $O_2.g^{-1}$ compared with the control group of 1.11 μmol $O_2.g^{-1}$. The difference in peroxide value in the thigh muscle with skin for 1 day kept at room temperature after the slaughter of broiler chickens was statistically significant ($p \leq 0.01$) between the control and experimental groups. Based on the statistical evaluation of the results expressed by the standard deviation, we can state that the measured values of peroxide value in the thigh muscle with skin kept at room temperature 1 day after the slaughter of broiler chickens fluctuated more in the control group compared to the experimental group ($SD$ = 0.52 vs. $SD$ = 0.43).

An increase in hydroperoxides is observed in the initial stages of fat oxidation because the level of formation is higher than during decomposition. These compounds are unstable. The process of decomposition of hydroperoxides is larger than the process of formation in more advanced stages of oxidation, therefore a decrease in the content of hydroperoxides (peroxide value) is observed. This means that low peroxide levels can result in early as well as advanced fat oxidation (**Estévez et al., 2009**).

The peroxide value is a used indicator to know the degree of oxidation. Previous research as well as review and arguments rather point to its effectiveness in the initial stages of oxidation processes (**Shahidi and Wanasundara, 2002**).

From this aspect, it can be concluded that in the advanced stages of fat oxidation, the peroxide value indicator as an oxidation indicator could lead to an underestimation of the degree of oxidation (**Ross and Smith, 2006**).

**Table 5** Average peroxide value in the chicken thigh muscle with skin, g.100 $g^{-1}$.

| Storage time thigh muscle | Group | n | *M* ±*SD* | t-test |
|---|---|---|---|---|
| 1 day at room temperature | control | 12 | 1.11 ±0.52 | $0.004^{++}$ |
| | experimental | 12 | 0.99 ±0.43 | |
| 6 months at -18 °C | control | 12 | 1.37 ±0.68 | $0.076^{-}$ |
| | experimental | 12 | 1.02 ±0.56 | |
| 9 months at -18 °C | control | 12 | 2.47 ±0.93 | $0.083^{-}$ |
| | experimental | 12 | 2.01 ±0.98 | |
| 12 months at -18 °C | control | 12 | 3.48 ±1.23 | $0.031^{+}$ |
| | experimental | 12 | 2.96 ±1.09 | |

Note: *n* = multiciplity, *M* = mean, *SD* = standard deviation; numerical values in the column at t-test marked ++, +: statistically significant difference between the control and experimental group ($p \leq 0.01$, $p \leq 0.05$); numerical values in the column at t-test marked -: statistically non-significant difference between the control and experimental group ($p > 0.05$).

In thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens, we recorded the average peroxide value in the experimental group of 1.02 μmol $O_2.g^{-1}$. This value is lower than the compared control sample 1.37 μmol $O_2.g^{-1}$. The difference in peroxide value in thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p > 0.05$) between the control and experimental group. By statistical evaluation of the results expressed by the standard deviation, we found that the measured peroxide values of the thigh muscle with skin stored for 6 months in freezing conditions at -18 °C after the slaughter broiler chickens fluctuated more in the control group compared to the experimental group (*SD* = 0.68 vs. *SD* = 0.56).

In thigh muscle with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens, we recorded an average peroxide value in the experimental group of 2.01 μmol $O_2.g^{-1}$ and a control sample of 2.47 μmol $O_2.g^{-1}$. The average value of the experimental group is lower. The difference in peroxide value of thigh muscle with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens was not statistically significant ($p > 0.05$) between the control and experimental groups. According to the statistical evaluation expressed by the standard deviation, we found that peroxide values in the thigh muscle with skin stored for 9 months in freezing conditions at -18 °C after the slaughter of broiler chickens fluctuated more in the experimental group compared to the control sample (*SD* = 0.98 vs. *SD* = 0.93).

In thigh muscle with skin stored for 12 months in freezing conditions at -18 °C after the slaughter of broiler chickens, we recorded an average peroxide value in the experimental group of 2.96 μmol $O_2.g^{-1}$ and the control group of 3.48 μmol $O_2.g^{-1}$. The difference in peroxide value in thigh muscle with skin stored for 12 months under freezing conditions at -18 °C after the slaughter of broiler chickens was statistically significant ($p \leq 0.05$) between the control and experimental groups. By statistical evaluation of the results, which are expressed by the standard deviation, we found that the measured values of peroxide value in thigh muscle with skin stored for 12 months in freezing conditions at -18 °C after the slaughter broiler chickens fluctuated more in the control group compared to the experimental group (*SD* = 1.23 vs. *SD* = 1.09).

Based on the comparison of our results of the peroxide value of thigh muscles with the results of the experiment **Klimentová and Angelovičová (2019**) it can be stated, that the values differ slightly. The authors did not find a statistically significant difference in peroxide value between the experimental group using oregano essential oil and the control group. In our experiment, a statistically significant difference ($p \leq 0.01$) was confirmed for 1 day of sample storage at room temperature between the control and experimental group. The second difference compared to the above study was observed, when the thigh muscle was stored in freezing conditions at -18 °C for 12 months, while our results showed ($p \leq 0.05$) a statistically significant difference between the control and experimental groups. The study of these authors includes the evaluation of the peroxide number of frozen chicken thighs by the application of oregano essential oil as a feed supplement for broiler chickens. The authors recommended the use of oregano essential oil in feed mixtures in a proportion of 0.05%.

Research results of **Marcinčák et al. (2008)** showed that oregano essential oil was effective in slowing down the oxidation of fats compared to the control group. Another fact found by these authors was that the thigh muscle was more prone to fat oxidation compared to the breast muscle ($p \leq 0.05$), which is a different opinion than reported **Gardini (2000), Leonel et al. (2007)**. According to these

authors, the different fat content in the thigh muscle and the breast muscle is not related to the oxidative processes of fat. Their conclusion is identical with the opinion of **Ruban (2009)**, which states that muscles with a higher fat content show a greater tendency to oxidize through a continuous chain reaction of free radicals.

## CONCLUSION

The evaluated results of the application of oregano essential oil to chicken thighs resulted in the following conclusion:

(a) no statistically significant difference was found in dry matter content, fat content of thigh muscle with skin when stored for 1 day at room temperature, 6, 9 and 12 months under freezing conditions at -18 °C compared to a control group whose starter and growth feed mixtures contained coccidiostats,

(b) no statistically significant difference was found in the acid value of thigh muscle with skin when stored for 1 day at room temperature, 6, 9 and 12 months under freezing conditions at -18 °C compared to a control group whose starter and growth feed mixtures contained coccidiostats,

(c) statistically significant difference was found in the peroxide value of thigh muscle with skin when stored for 1 day at room temperature and 12 months under freezing conditions at -18 °C, and no statistically significant difference when stored for 6, 9 months under freezing conditions at -18 °C compared to a control group whose starter and growth feed mixtures contained coccidiostats.

In conclusion, we can state that maintaining the oxidative stability of chicken meat means knowing the factors that affect it and prepare the conditions for its maintenance. The rate and extent of lipid oxidation in muscle tissue depends on many factors, including broiler chicken feed, handling of meat after slaughter. Chicken meat is generally prone to oxidative degradation because it is characterized by a high concentration of polyunsaturated fatty acids. With a sufficient number of effective antioxidants using chicken meat can be a homoeostatic system that remains valid or without of oxidized compounds and reactive components.

These issues are the subject of further research in the field of oxidative stability of chicken meat.

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The authors declare no conflict of interest.

**Ethical Statement:**

The use of animals in this research was approved by the Ethics Committee of the Slovak University of Agriculture in Nitra following the legislation of the Slovak Republic.

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# THE EFFECT OF ESSENTIAL OILS ON CHOLESTEROL CONTENT IN CHICKEN MEAT

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## ABSTRACT

The study aimed to investigate cholesterol content in chicken breast and thigh muscles by the influence of feed supplements of various content of essential oils. The experiment was carried out under practical conditions in a poultry farm with broiler chickens of the Cobb 500 hybrid combination according to the feed supplement used thyme essential oil, cinnamon essential oil, commercial citrus fruit essential oil, and their combination. The control group was without the use of experimental feed supplements and commercial coccidiostats were used in their feed mixtures. The experiment lasted 40 days in welfare conditions. Broiler chickens were used for sample preparation of breast and thigh muscles with the skin and their analysis for dry matter, fat and cholesterol contents. Samples were analyzed using a Nicolet 6700 FT-IR Fourier transform infrared spectrometer. The results were processed by the SAS system program, version 8.2. The results, which were evaluated, indicated a tendency to reduce the cholesterol content in chicken breast muscle due to cinnamon essential oil and the combination of cinnamon essential oil with citrus fruit essential oil as well as thyme essential oil with citrus fruit essential. In the achieved results of dry matter, fat, and cholesterol content in breast and thigh muscles, the difference between the effects of the used feed supplements based on essential oils and concerning the control group were not statistically significant $p > 0.05$. The correlation was a statistically significant strong linear relation only between dry matter content and fat content due to thyme and cinnamon essential oils. In conclusion, it was stated that the investigation of the feed supplement effect based on essential oils is an open question concerning the production of safe food of animal origin.

**Keywords:** essential oil; feed mixture; chicken thigh muscle; chicken breast muscle; cholesterol

## INTRODUCTION

In recent years, epidemiological work and laboratory experiments have focused on the causes and modified factors associated with cardiovascular disease. It is generally accepted that the incidence of cardiovascular disease is closely related to the intake of cholesterol and saturated fatty acids in the diet. Therefore, great attention has been paid to the production of animal products and the modulation of animal products enriched with bioactive compounds, to improve their quality and protect consumer health against diseases **(Simitzis et al., 2011)**.

Achieving a better balance of fatty acids in the diet by reducing cholesterol and saturated fat intake is considered an effective and important strategy to reduce the incidence of cardiovascular diseases. Therefore, knowledge about the cholesterol content of foods is important. About a healthy diet, knowledge of the chemical composition of chicken and fish meat is important, because the consumption of these types of meat is increasing, even on the recommendations of nutritional experts **(Milićević et al., 2014)**.

An interesting focus of the research was realized by **Attia et al. (2017)** with broiler chickens. The results of their research showed that cholesterol metabolism in the brain and muscles are associated with the aggressive behavior of broiler chickens and with a reduction in meat quality. Liver cholesterol is an indicator of the chicken fatty liver syndrome. The fatty liver syndrome is a common metabolic disease that seriously affects the health and efficiency of broiler chicken production and causes major economic losses to the poultry industry.

Differences in cholesterol content between different muscles of the same chicken species and between the same muscles in different species are generally explained by changes in cholesterol absorption and biosynthesis, lipoprotein metabolism, distribution of muscle fiber types, genetic variations, subcutaneous and intramuscular fat **(Padre et al., 2006)**.

Meat and broiler chicken products are important worldwide in human nutrition as they address global food shortages due to their availability. In general, the energy value for chicken breast muscle is 435 kJ and for thigh muscle 481 kJ. It is similar to cholesterol. Its content is 62

**Table 1** Design of a group feeding experiment on a poultry farm in the hall.

| Grop | n | Pen area | Feed mixtures | Feed supplement | Content/proportion of active substance in feed and water |
|---|---|---|---|---|---|
| Control | 20 | 1.5 $m^2$ | Commercial: Starter | Coccidiostats: Maxiban G160 | Narasin 80 mg per kg feed and Nicarbazin 80 mg per kg feed |
| | | | Growth | Coccidiostats: Sacox® | Salinomycin sodium 70 mg per kg feed |
| | | | Final | - | - |
| Thyme essential oil | 20 | 1.5 $m^2$ | Experimental: Starter | Thyme essential oil | 0.05% in feed |
| | | | Growth | Thyme essential oil | 0.05% in feed |
| | | | Final | Thyme essential oil | 0.05% in feed |
| Cinnamon essential oil | 20 | 1.5 $m^2$ | Experimental: Starter | Cinnamon essential oil | 0.05% in feed |
| | | | Growth | Cinnamon essential oil | 0.05% in feed |
| | | | Final | Cinnamon essential oil | 0.05% in feed |
| Citrus fruit essential oil | 20 | 1.5 $m^2$ | Experimental: Starter | Commercial: Citrus fruit essential oil | 1.0 ml per L of water |
| | | | Growth | Citrus fruit essential oil | 1.0 ml per L of water |
| | | | Final | Citrus fruit essential oil | 1.0 ml per L of water |
| Thyme essential oil + Citrus fruit essential oil | 20 | 1.5 $m^2$ | Experimental: Starter | Thyme essential oil + Citrus fruit essential oil | 0.05% in feed + 1.0 ml per L of water |
| | | | Growth | Thyme essential oil + Citrus fruit essential oil | 0.05% in feed + 1.0 ml per L of water |
| | | | Final | Thyme essential oil + Citrus fruit essential oil | 0.05% in feed + 1.0 ml per L of water |
| Cinnamon essential oil + Citrus fruit essential oil | 20 | 1.5 $m^2$ | Experimental: Starter | Cinnamon essential oil + Citrus fruit essential oil | 0.05% in feed + 1.0 ml per L of water |
| | | | Growth | Cinnamon essential oil + Citrus fruit essential oil | 0.05% in feed + 1.0 ml per L of water |
| | | | Final | Cinnamon essential oil + Citrus fruit essential oil | 0.05% in feed + 1.0 ml per L of water |

Note: n = number of broiler chickens in a group feeding experiment.

mg in breast muscle and 80 mg in thigh muscle **(Haščík et al., 2020)**.

**Dehghani et al. (2018)** reported that essential oils and some of their components can lower plasma cholesterol and triglyceride levels. This may be the result of the inhibition of the enzymes involved in the synthesis of these lipids by monoterpenes.

Essential oil extracts are a possible alternative to antibiotic growth stimulators because they are natural, readily available, non-toxic, and residue-free. Due to these properties, they are acceptable as natural feed additives for broiler chickens. Essential oils made from aromatic plants have become more interesting due to their potential effects as hypocholesterolemic, antioxidants, antimicrobials, antifungals, and digestive enzyme stimulants **(Abd El-Hack et al., 2020)**.

**Jamroz et al. (2005)** also demonstrated increased pancreatic and intestinal lipase activity in broilers fed diets with added plant extract.

The effect of the feed supplements with essential oils on the cholesterol content in chicken breast and tight muscles was evaluated.

**Scientific hypothesis:** feeding feed mixtures supplemented with thyme, cinnamon, and citrus fruit essential oils for broiler chickens lowers the cholesterol content in their meat.

## MATERIAL AND METHODOLOGY

### Technical implementation of the feeding experiment

The experiment will take place on a poultry farm. At the entrance gate to the hall, space was set aside for 6 groups of broiler chickens, 20 for each. Change pens small dimensions: 1.5 m wide and 1.0 m long for unrestricted movement of broiler chickens. Definitive Cobb 500 broiler chickens for fattening are used as experimental animals. The groups were marked according to the schemes (Table 1) depending on the active substance test. Commercial feed mixtures were used in control groups, commonly used in poultry farms based on wheat, corn, and soybean extracted meal. Coccidiostats, which are commonly used and form part of feed mixtures, have been used in starter and growth compound feeds. Control feed mixtures did not contain any essential oils. The same feed mixtures were used in the experimental groups as in the control group but without coccidiostats.

In the thyme group, the active substance thyme essential oil was used with a proportion of 0.05% in the feed. In the cinnamon group, the active ingredient cinnamon essential oil was used with a proportion of 0.05% in the feed. In the

citrus fruit essential oil group, a commercial feed supplement made from citrus raw materials was used and applied to the water. In the experimental group thyme essential oil + citrus fruit essential oil, thyme essential oil with a proportion of 0.05% in the feed was used and citrus fruit essential oil was applied to the water of 1.0 $ml.l^{-1}$. In the experimental group cinnamon essential oil + citrus fruit essential oil, cinnamon silica with a proportion of 0.05% in the feed was used and citrus fruit essential oil was applied to water 1.0 $ml.l^{-1}$.

Essential oils were first mixed with vegetable oil, which was the feed material of the feed mixture to achieve the desired energy value of the feed mixture. Subsequently, the mixture of vegetable oil and essential oil was mixed with corn meal and then manually homogenized with other feed materials. The drinkers were cleaned daily and topped 2 to 3 times with fresh water enriched with citrus fruit essential oil. Feed feeds were made (mixed) in the feed company Biofeed, a.s. Kolárovo.

The hall, which included our experiment, was equipped with deep bedding of straw, automatic ventilation equipment in the ceiling, heat sources – radiators, and a light regime as recommended by the producer of fattening type broiler chickens. One-day-old broiler chickens were placed in the experiment. Feeding was provided from plate feeders and hat feeders until the age of 14 days. After 14 days, the experimental feeding and watering equipment was replaced by a tube feeder in each group of 1 piece and a bucket feeder in each group of 1 piece. The trial period was divided into 3 phases: starter: days 1 to 18 with the feeding of starter feed mixture, growth: days 15 to 33 with the feeding of the growth feed mixture, final: days 34 to 40 with the feeding of the final feed mixture. The feed mixtures used in the control and experimental groups differed from each other according to the use of the active substance (see scheme, Table 1). Essential oils were obtained from Calendula, a.s. Nová Ľubovňa, which supplied us with samples with an a-test, according to which it declared the safety and effectiveness of thyme and cinnamon essential oils. The citrus essential oil was obtained from a feed company.

***Composition of the thyme essential oil, cinnamon essential oil, and a commercial feed supplement citrus fruit essential oil***

Compound percentages of the essential oil of *Thymus vulgaris*: *α*-thujene 0.71%, *α*-pinène 0.49%, *cis*-sabinene hydrate 0.46%, camphene 0.14%, *β*-myrcene 0.71%, *α*-terpinene 0.58%, *β*-phelladrene 0.37%, *γ*-terpinene 1.92%, *ρ*-cymene 9.89%, carvacryl acetate 1.19%, *β*-linalool 1.51% **(Ben Jabeur et al., 2017)**.

Compound percentages of the essential oil of bark of *Cinnamomum zeylanicum:* styrene benzene, ethenyle-(CAS) 5.5%, *α*-pinene 0.50%, benzaldehyde 1.10%, cyclohexane, 1-methyl-3-(1-methylet) 0.52%, benzenepropanal (CAS) B-phenylpr 1.21%, L-borneol 0.46%, *trans*-cinnamaldehyde 1.07%, cinnamic aldehyde 52.30%, *α*-longipinene tricyclo (5.4) 0.70%, *α*-copaene 11.42%, sativene 0.48%, *trans*-caryophyllene bicyclo (7.2) 0.80%, ar-curcumene 0.55%, naphtalene, 1,2,3,4,4a,5,6,8a-octa 2.28%, *α*-muurolene naphtalene, 1 0.70% *δ*-cadinene 6.25%, *cis*-calamenene 3.61%, naphtalene, 1,2,3,4,4a,7-hexahydro 0.83%, *α*-calacorene 0.68%, naphtalene, 1,2,3,4,4a,7-hexahydro 0.78%, *t*-muurolol 1-naphtslenol 1,2,3 1.95% **(Kazemi and Mokhtariniya, 2016)**.

Compound percentages of the essential oil of *Citrus paradisi, Citrus reticulata Blanco*, *Citrus sinensis and Citrus aurantium* L. *subsp. bergamia:α*-pinene 0.27%, sabinene 0.08%, myrcene 3.03%, Z,Z,Z-1,4,6,9-nonadecatetraene 0.20%, limonene 50.42%, Z-β-ocimene 0.12%, *γ*-terpinene 0.05%, n-octanol 0.11%, linalool 0.45%, n-nonanal 0.17%, *trans*-para-mentha-2,8-dien-1-ol 0.77%, P-mentha-E-2,8(9)-dien-1-ol 0.73%, 3-(methoxy)-3-methyl-6-prop-1-en-2-cylcyclohexene 0.03%, *cis*-β-terpineol 0.04%, *α*-2,4-cyclohexadiene-1-methanol 0.08%, n-octanol 0.08%, (-)-terpinen-4-ol 0.45%, *trans*-isocarveol 0.13%, *α*-terpineol 1.19%, [1,1'-bicyclopentyl]-2-one 1.16%, decanal 1.28%, octyl-acetate 0.09%, *trans*-carveol 3.09%, carvomenthol 0.15%, 5-isoprorpenyl-2-methyl-2-cyclohexen-1-ol 0.81%, 2-methyl-5-(1-methylethene) 2-cyclohexen-1-one 0.50%, 3-methyl-6-(1-methylethene) 2-cyclohexen-1-one 0.07%, perillaldehyde 0.22%, undecanol 0.18%, limonen-10-ol 0.15%, perilla alcohol 0.04%, n-undecanal 0.12%, 4-vinyl- guaiacol 0.92%, *α*-a-cubebene 0.24%, 3,7-dimethyl 6-octen-1-ol 0.19%, *α*-copaene 1.49%, neryl acetate 0.06%, *β*-copaene 0.54%, *α*-, *trans*-bergamotene 0.06%, dodecanal 0.84%, (E)-caryophyllene 0.34%, *β*-copaene 0.06%, *α*-guaiene 0.07%, 6,10-dimethylundeca-5,9-dien-2-one 0.07%, *α*-humulene 1.23%, cadina-1,4-diene 0.07%, *γ*-muurolene 0.07%, *β*-copaene 1.30%, bicyclogermacrene 0.22%, *α*-muurolene 0.24%, (E,E)-, *α*-farnesene 1.67%, *γ*-cadinene 0.03%, δ-cadinene 2.53%, sesquisabinene 0.20%, *trans*-cadina-1,4-diene 0.07%, *α*-elemol 0.79%, (-)-spathulenol 0.54%, n-dodecanoic acid 1.28%, ethyl iso-allocholate 0.08%, epicubenol 0.22%, *γ*-eudesmol 0.31%, *α*-cadinol 0.08%, cadin-4-en-10-ol 0.99%, 3,7-dimethyl 6-octenal 0.15%, humulene 0.22%, *β*-sinensal 1.65%, 2,6,10-trimethyl 2,6,9,11-dodecatetraenal 0.05%, *α*-sinensal 3.14%, 2-pentyl-2-nonenal 0.19%, tetradecanoic acid 0.94%, nootkatone 0.28%, cryptomeridiol 0.05%, farnesyl acetone 0.05%, methyl-hexadecanoate 0.26%, 2-dodecen-1-yl(-) succinic anhydride 0.12%, n-hexadecanoic acid 5.65%, methyl ester 9,12-octadecadienoic acid 0.32%, linoleic acid 0.94%, methyl linoleate 0.19%, dodecenyl succinic anhydride 0.31% **(Goyal and Kaushal, 2018)**.

The results of the chemical composition of Citrus peel essential oil can slightly various in literature because it depending upon the age of the plant, harvesting time, geographical and ecological conditions **(Wu et al., 2013)**.

At the end of the feeding experiment, 6 broiler chickens were randomly selected from each group for chemical analysis. Selected broiler chickens were transported to the technological laboratory of the Department of Technology and Quality of Animal Products, where they were slaughtered humanely and technologically processed. The breast and thigh muscles with skin were separated from the carcass. These samples of breast and thigh muscles were transported in a portable refrigerator to the chemical laboratory of the Department of Food Hygiene and Safety, where the preparation of samples for chemical analysis was performed.

**Preparation of breast and thigh muscle samples with skin for chemical analysis**

The thighs were boned and labeled with the TEO, STO, B, TEO + B, and SEO + B experimental groups, and in each group with a sample number from 1 to 6. Breast muscle samples were similarly labeled. Samples of the control group were marked K and with sample numbers from 1 to 6. Each sample was mixed in a Grindomix 200 laboratory grinder and weighed 50.0 g. Samples of breast and thigh muscles with skin were transferred to laboratory flasks with ground glass. Weighing of breast and thigh muscle samples was performed on an automatic scale of the Kern 440-49N type with an accuracy of d = 0.01 g.

**Chemical analysis of breast and thigh muscle samples**

Chemical analysis of breast and thigh muscle samples with skin was performed using a Nicolet 6700 FT-IR Fourier Transform Infrared Spectrometer. The samples were measured by the attenuated total reflectance method, which uses a diamond crystal attachment. The device works fully automatically, using OMNIC computer software. Infrared spectroscopy is a method whose principle is the ability of substances to absorb radiation from the infrared region, i.e. 800 – 106 nm of the electromagnetic spectrum. The sample was placed on the surface and in contact with the diamond crystal. Subsequently, a total reflection was formed at the interface of the crystal, which represents an optically denser environment, with the sample, which represents an optically denser environment. In this type of reflection, part of the radiation penetrates slightly into the sample and spectral information is obtained. The analytical output is the infrared spectrum. This spectrum graphically shows the functional dependence of the energy expressed as a percentage of the transmittance, i.e. transmittance (T) or absorbance units (A) at the wavelength of the incident radiation. Chemical analyzes were performed at the Department of Special Zootechnics. Samples of breast and thigh muscles with skin were measured for moisture (calculated on the dry matter), fat, and cholesterol contents using a Nicolet 6700 FT-IR Fourier transform infrared spectrometer.

It is more advantageous to use Fourier transform spectrometers today, which have dispersive elements replaced by an interferometer, which prevents the decomposition of radiation and allows folding and recombination (**Challa Kumar, 2011; Čech Barabaszová, 2012**).

## Statistical Analysis

The statistically evaluated results are reported in the study as arithmetic mean (*M*), standard deviation (*SD*), which are commonly used in descriptive statistics. The F test was used to test the difference in the observed indicator between the groups, followed by the Scheffe test, which was set to test at a significance level of $p = 0.05$.

The relation between the two variables in each group was tested using Pearson's correlation coefficient ($r$). The value of the coefficient is between –1 and +1. If its value is –1, it means a high negative linear relationship between two variables, +1 means a high positive linear relation between two variables. If the correlation coefficient reaches 0, it means that there is no linear relation between the two variables. The procedure according to Cohen (1988) was chosen to evaluate the results: below 0.1 trivial relations (simple, easy), 0.1 – 0.3 weak relation, 0.3 – 0.5 medium relation, above 0.5 strong relations (0.7 – 0.9 is often reported as very strong and 0.9 – 1.0 as almost perfect). The results of the correlation coefficient were based on a statistically significant at the significance level $\alpha = 0.05$, $\alpha = 0.01$, $\alpha = 0.001$. The SAS system program, version 8.2 was used for statistical analysis of the results.

## RESULTS AND DISCUSSION

**Dry matter of the chicken breast and thigh muscles with skin**

***Dry matter of the chicken breast muscle with skin***

The average dry matter content of the chicken breast muscle with skin and the statistical evaluation of the results are given in Table 2.

The average dry matter content of breast muscle with skin in the experimental groups ranged from 26.73 g.100 $g^{-1}$ with citrus fruit essential oil, followed by an average dry matter content of 26.98 g.100 $g^{-1}$ with thyme essential oil, 27.07 g.100 $g^{-1}$ with thyme essential oil + citrus fruit essential oil, 27.12 g.100 $g^{-1}$ with cinnamon essential oil nd 27.89 g.100 $g^{-1}$ with cinnamon essential oil + citrus fruit essential oil. These values of the average dry matter content in the breast muscle with the skin are lower compared to the average dry matter content in the breast muscle with the skin of the control group. The difference in the dry matter content of the pectoral muscle with the skin was not statistically significant among the groups ($p$ >0.05). Statistical evaluation of the measured breast muscle dry matter content data expressed as the standard deviation revealed that the smallest variation was in the thyme essential oil experimental group and the highest in the thyme essential oil + citrus fruit essential oil experimental group, i.e. $SD = 0.98$ versus $SD = 1.56$.

**Mosca et al. (2018)** in their study report breast muscle dry matter content in broiler chicken hens and cocks with an average value of 28.21 g.100 $g^{-1}$, which is higher compared to our results. **Evaris et al**. **(2021)** report a dry matter content of breast muscle of only 23.47 g.100 $g^{-1}$ and point out that the addition of essential oil to feed reduces the dry matter content of breast muscle broiler chickens.

***Dry matter of the chicken thigh muscle with skin***

The average dry matter content of the chicken thigh muscle with skin and the statistical evaluation of the results are given in Table 3. The average dry matter content of the thigh muscle with skin in the experimental groups ranged from 27.33 g.100 $g^{-1}$ with cinnamon essential oil, followed by an average dry matter content of 27.42 g.100 $g^{-1}$ with thyme essential oil, 28.07 g .100 $g^{-1}$ with cinnamon essential oil + citrus fruit essential oil, 28.09 g.100 $g^{-1}$ with citrus fruit essential oil and 28.14 g.100 $g^{-1}$ with thyme essential oil + citrus fruit essential oil. These values of the average dry matter content of the skin thigh muscle are lower compared to the average dry matter content of the skin of the control group.

The difference in the dry matter content of the thigh muscle with the skin was not statistically significant among the groups ($p$ >0.05).

**Table 2** The average dry matter content of the chicken breast muscle with skin, g.100 $g^{-1}$, and the statistical evaluation of the results.

| Group | n | *M* | *SD* | Thyme EO | Cinnamon EO | Citrus fruit EO | Thyme EO + Citrus fruit EO | Cinnamon EO + Citrus fruit EO |
|---|---|---|---|---|---|---|---|---|
| | | | | F test: 1.326$^{-}$ | | | | |
| Control | 6 | 28.61 | 1.49 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Thyme EO | 6 | 26.98 | 0.98 | | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Cinnamon EO | 6 | 27.12 | 1.32 | | | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Citrus fruit EO | 6 | 26.73 | 1.24 | | | | *p* >0.05 | *p* >0.05 |
| Thyme EO + Citrus fruit EO | 6 | 27.09 | 1.56 | | | | | *p* >0.05 |
| Cinnamon EO + Citrus fruit EO | 6 | 27.89 | 1.23 | | | | | |

Note: EO = essential oil, n = number of samples obtained from 6 broiler chickens, *M* = mean, *SD* = standard deviation, *p* >0.05: no statistically significant difference among groups.

**Table 3** The average dry matter content of the chicken thigh muscle with skin, g.100 $g^{-1}$, and the statistical evaluation of the results.

| Group | n | *M* | *SD* | Thyme EO | Cinnamon EO | Citrus fruit EO | Thyme EO + Citrus fruit EO | Cinnamon EO + Citrus fruit EO |
|---|---|---|---|---|---|---|---|---|
| | | | | F test: 1.326$^{-}$ | | | | |
| Control | 6 | 28.66 | 1.22 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Thyme EO | 6 | 27.42 | 0.96 | | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Cinnamon EO | 6 | 27.33 | 1.44 | | | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Citrus fruit EO | 6 | 28.09 | 1.19 | | | | *p* >0.05 | *p* >0.05 |
| Thyme EO + Citrus fruit EO | 6 | 28.14 | 1.28 | | | | | *p* >0.05 |
| Cinnamon EO + Citrus fruit EO | 6 | 28.07 | 1.36 | | | | | |

Note: EO = essential oil, n = number of samples obtained from 6 broiler chickens, *M* = mean, *SD* = standard deviation, *p* >0.05: no statistically significant difference among groups.

**Table 4** The average fat content of the chicken breast muscle with skin, g.100 $g^{-1}$, and the statistical evaluation of the results.

| Group | n | *M* | *SD* | Thyme EO | Cinnamon EO | Citrus fruit EO | Thyme EO + Citrus fruit EO | Cinnamon EO + Citrus fruit EO |
|---|---|---|---|---|---|---|---|---|
| | | | | F test: 1.952$^{-}$ | | | | |
| Control | 6 | 1.32 | 0.14 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Thyme EO | 6 | 1.78 | 0.11 | | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Cinnamon EO | 6 | 1.81 | 0.13 | | | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Citrus fruit EO | 6 | 1.56 | 0.16 | | | | *p* >0.05 | *p* >0.05 |
| Thyme EO + Citrus fruit EO | 6 | 1.68 | 0.17 | | | | | *p* >0.05 |
| Cinnamon EO + Citrus fruit EO | 6 | 1.49 | 0.16 | | | | | |

Note: EO = essential oil, n = number of samples obtained from 6 broiler chickens, *M* = mean, *SD* = standard deviation, *p* >0.05: no statistically significant difference among groups.

Statistical evaluation of the measured data of the dry matter content of the thigh
muscle with skin expressed by the standard deviation revealed that the smallest fluctuation of values was in the experimental group with thyme essential oil and the highest in the experimental group with cinnamon essential oil, i.e. *SD* = 0.96 vs. *SD* = 1.44.

**Haščík et al. (2012)** report higher values of dry matter content in the thigh muscle as are our data. They achieved results of 31.51 g.100 $g^{-1}$ in the control group with coccidiostats, and 30.21 g.100 $g^{-1}$ or 29.88 g.100 $g^{-1}$ in the experimental groups with pollen extract. They experimented with a hybrid combination of the Ross 308 broiler chickens. **Mosca et al. (2018)** report the dry matter content in the thigh muscle of broiler chickens without taking into account sex 26.3 g.100 $g^{-1}$, which is a lower value compared to our results. The stated lower dry matter value in the thigh muscle may be due to another hybrid combination of broiler chickens or the feeding of feed mixtures of a different composition than our feed mixtures. **Evaris et al. (2021)** report a dry matter content of the thigh muscle of only 24.14 g.100 $g^{-1}$.

**Fat of the chicken breast and thigh muscles with skin**

***Fat of the chicken breast muscle with skin***

The average fat content of the chicken breast muscle with skin and the statistical evaluation of the results are given in Table 4.

The average fat content of breast muscle with skin in the experimental groups ranged from 1.49 g.100 $g^{-1}$ with cinnamon essential oil + citrus fruit essential oil, followed by an average fat content of 1.56 g.100 $g^{-1}$ with citrus fruit essential oil, 1.68 g.100 $g^{-1}$ with thyme essential oil + citrus fruit essential oil, 1.78 g.100 $g^{-1}$ with thyme essential oil and 1.81 g.100 $g^{-1}$ with cinnamon essential oil. These values of the average fat content of the breast muscle with the skin are higher compared to the average fat content of the breast muscle with the skin of the control group.

The difference in the fat content of the breast muscle with the skin was not statistically significant among the groups ($p$ >0.05). Statistical evaluation of the measured data of fat content in the breast muscle with skin expressed by the standard deviation revealed that the smallest fluctuation of values were in the experimental group with thyme essential oil and the highest in the experimental group with cinnamon essential oil + citrus fruit essential oil, i.e. *SD* = 0.11 vs. *SD* = 0.16.

A study by **Zotte et al.** (**2020**) reports a breast fat content of 1.21 g.100 $g^{-1}$, which is lower than our results, which may indicate that the addition of essential oils to feed for broiler chickens has the effect of increasing the fat content as in our results. In another study, **Zampiga et al.** (**2019**) report a fat content in breast muscle of 1.71 g.100 $g^{-1}$, which is lower compared to the results of our study after feeding of feed mixtures with thyme and cinnamon essential oils and higher compared to the group, where cinnamon essential oil + citrus fruit essential oil, thyme essential oil + citrus fruit essential oil and citrus fruit essential oil were used. These results may indicate that feeding of thyme and cinnamon essential oils tends to increase fat storage.

**Kim et al.** (**2020**) recorded a fat content of chicken breast muscle of 1.10 to 1.44%, who examined this indicator in terms of the impact of conventional farming systems and welfare systems with the application of welfare principles. Our results of the fat content of the control group and cinnamon essential oil + citrus fruit essential oil are the closest to their highest value of fat content in breast muscle.

***Fat of the chicken thigh muscle with skin***

The average fat content of the chicken thigh muscle with skin and the statistical evaluation of the results are given in Table 5.

The average fat content of the thigh muscle with skin in the experimental groups ranged from 2.59 g.100 $g^{-1}$ with cinnamon essential oil + citrus fruit essential oil, followed by an average fat content of 2.71 g.100 $g^{-1}$ with thyme essential oil, 2.83 g.100 $g^{-1}$ with cinnamon essential oil, 2.91 g.100 $g^{-1}$ with thyme essential oil + citrus fruit essential oil of 2.93 g.100 $g^{-1}$ with citrus fruit essential oil. These values of the average fat content of the thigh muscle with the skin are higher compared to the average fat content of the thigh muscle with the skin of the control group. The difference in the fat content of the original thigh muscle mass with the skin among the groups was not statistically significant ($p$ >0.05). Statistical evaluation of the measured data of the fat content of the thigh muscle with skin expressed by the standard deviation revealed that the smallest fluctuation of values was in the experimental group with thyme essential oil and the highest in the experimental group with cinnamon essential oil, i.e. *SD* = 0.96 vs. *SD* = 1.44.

**Evaris et al.** (**2021**) report a fat content in the thigh muscle of 9.66 g.100 $g^{-1}$, which is a significant difference compared to our results.

**The cholesterol of the chicken breast and thigh muscles with skin**

***The cholesterol of the chicken breast muscle with skin***

The average cholesterol content of the chicken breast muscle with skin and the statistical evaluation of the results are given in Table 6.

The average cholesterol content of the breast muscle with skin in the experimental groups ranged from 0.028 g.100 $g^{-1}$ with cinnamon essential oil, cinnamon essential oil + citrus fruit essential oil, and thyme essential oil + citrus fruit essential oil, followed by average cholesterol content of 0.034 g .100 $g^{-1}$ with citrus fruit essential oil and 0.042 g.100 $g^{-1}$ with thyme essential oil. These values of the average cholesterol content of the breast muscle with the skin are lower compared to the control group (when using a cinnamon essential oil, cinnamon essential oil + citrus fruit essential oil, thyme essential oil + citrus fruit essential oil and when using citrus fruit essential oil) or higher (when using thyme essential oil). The difference in the cholesterol content of the breast muscle with the skin between the groups was not statistically significant ($p$ >0.05).

**Table 5** The average fat content of the chicken thigh muscle with skin, g.100 $g^{-1}$, and the statistical evaluation of the results.

| Group | n | *M* | *SD* | Thyme EO | Cinnamon EO | Citrus fruit EO | Thyme EO + Citrus fruit EO | Cinnamon EO + Citrus fruit EO |
|---|---|---|---|---|---|---|---|---|
| | | | | F test: 1.952$^{-}$ | | | | |
| Control | 6 | 2.64 | 1.22 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Thyme EO | 6 | 2.71 | 0.96 | | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Cinnamon EO | 6 | 2.83 | 1.44 | | | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Citrus fruit EO | 6 | 2.93 | 1.19 | | | | *p* >0.05 | *p* >0.05 |
| Thyme EO + Citrus fruit EO | 6 | 2.91 | 1.28 | | | | | *p* >0.05 |
| Cinnamon EO + Citrus fruit EO | 6 | 2.59 | 1.36 | | | | | |

Note: EO = essential oil, n = number of samples obtained from 6 broiler chickens, *M* = mean, *SD* = standard deviation, *p* >0.05: no statistically significant difference among groups.

**Table 6** The average cholesterol content of the chicken breast muscle with skin, g.100 $g^{-1}$, and the statistical evaluation of the results.

| Group | n | *M* | *SD* | Thyme EO | Cinnamon EO | Citrus fruit EO | Thyme EO + Citrus fruit EO | Cinnamon EO + Citrus fruit EO |
|---|---|---|---|---|---|---|---|---|
| | | | | F test: 0.146$^{-}$ | | | | |
| Control | 6 | 0.035 | 0.003 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Thyme EO | 6 | 0.042 | 0.005 | | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Cinnamon EO | 6 | 0.028 | 0.003 | | | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Citrus fruit EO | 6 | 0.034 | 0.001 | | | | *p* >0.05 | *p* >0.05 |
| Thyme EO + Citrus fruit EO | 6 | 0.028 | 0.003 | | | | | *p* >0.05 |
| Cinnamon EO + Citrus fruit EO | 6 | 0.028 | 0.002 | | | | | |

Note: EO = essential oil, n = number of samples obtained from 6 broiler chickens, *M* = mean, *SD* = standard deviation, *p* >0.05: no statistically significant difference among groups.

**Table 7** The average cholesterol content of the chicken thigh muscle with skin, g.100 $g^{-1}$, and the statistical evaluation of the results.

| Group | n | *M* | *SD* | Thyme EO | Cinnamon EO | Citrus fruit EO | Thyme EO + Citrus fruit EO | Cinnamon EO + Citrus fruit EO |
|---|---|---|---|---|---|---|---|---|
| | | | | F test: 0.146$^{-}$ | | | | |
| Control | 6 | 0.037 | 0.003 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Thyme EO | 6 | 0.034 | 0.002 | | *p* >0.05 | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Cinnamon EO | 6 | 0.037 | 0.002 | | | *p* >0.05 | *p* >0.05 | *p* >0.05 |
| Citrus fruit EO | 6 | 0.044 | 0.004 | | | | *p* >0.05 | *p* >0.05 |
| Thyme EO + Citrus fruit EO | 6 | 0.045 | 0.005 | | | | | *p* >0.05 |
| Cinnamon EO + Citrus fruit EO | 6 | 0.039 | 0.003 | | | | | |

Note: EO = essential oil, n = number of samples obtained from 6 broiler chickens, *M* = mean, *SD* = standard deviation, *p* >0.05: no statistically significant difference among groups.

Statistical evaluation of the measured cholesterol content data in the breast muscle with the skin expressed by the standard deviation revealed that the smallest fluctuation of values was in the experimental group with citrus fruit essential oil and the highest in the experimental group with thyme essential oil, i.e. $SD = 0.001$ vs. $SD = 0.005$.

In general, raw poultry meat has approximately 27 to 90 mg of cholesterol.100 $g^{-1}$ (**Bragagnolo, 2009**). **Shang et al.** (**2020**) report a cholesterol content in the breast muscle of 0.054 g.100 $g^{-1}$, which is a higher value of the cholesterol content in the breast muscle compared to our results. **Krauze et al.** (**2021**) note that broiler chickens fed cinnamon essential oil in feed showed better growth performance due to the beneficial effect of the product on the small intestinal microbiome, metabolism. Their study states that HDL cholesterol level was increased and levels of total cholesterol and non-esterified fatty acids as well as triacylglycerols were decreased. Their opinion on the total cholesterol also confirmed our results in an experiment with broiler chickens, which was used only cinnamon oil or cinnamon essential oil also combined with citrus fruit essential oil. **Lin et al.** (**2003**) also state cinnamon essential oil has, among other things, strong hypocholesterolemic effects. **Koochaksaraie et al.** (**2011**) believe that cinnamon aldehyde found in cinnamon essential oil is primarily responsible for the hypocholesterolemic effect of stimulating the excretion of cholesterol from the body.

In the context of citrus fruit essential oil, it is known that the active compound in sweet oranges such as tannins, saponins, flavonoids, and essential oil can inhibit the absorption of cholesterol in the intestine. The presence of tannins will stick or lining the intestine membrane thus inhibiting the absorption of cholesterol (**Oluremi et al., 2007**). In our experiment, the cholesterol content was reduced mainly when using citrus fruit essential oil in combination with thymus and cinnamon essential oils. Some studies have shown that bioactive compounds from plant materials, including sterols, polysaccharides, and polyphenols, exhibit cholesterol lowering effects both *in vitro* and *in vivo*. Condensed tannins belong to the family of flavonoids, which occur in plants as mixtures of flavan-3-ol oligomers or polymers (**Desrues et al., 2017**). To determine the potential cholesterol-lowering activities of polyphenols, researchers usually investigate their inhibitory effects on the cholesterol solubility in mixed micelles *in vitro* at first. It is believed that reduction in cholesterol micellar solubility by phenolic compounds may inhibit the cholesterol absorption in the intestinal lumen and is attributed to their interactions with phosphatidylcholine or bile acid (**Ogawa et al., 2015**). In recent years, some research teams put forward that the direct interactions of plant functional components with cholesterol may be another possible mechanism of their hypocholesterolemic effects. For instance, β-sitosterol can be co-crystallized with cholesterol to form insoluble mixed crystals to further reduce the absorption of cholesterol in the small intestine (**Christiansen et al., 2003**).

There is also a study by **Kriaa et al.** (**2019**), who report that bacteria found in the intestinal microbiome use cholesterol from the intestinal contents to form their cell walls and thereby reduce the amount of exogenous cholesterol in the body. In their study, **Koeth et al.** (**2013**) showed that dietary carnitine and direct supplementation with trimethylamine-N-oxide both reduced reverse cholesterol transport ($p < 0.05$) in mice only in case of the intact gut microbiome.

The main issue regards the molecular mechanisms, which link microflora and trimethylamine-N-oxide synthesis to reverse cholesterol transport reduction. In the intestines, trimethylamine-N-oxide is referred to diminish cholesterol uptake by reducing Niemann-Pick C1-like 1, which takes cholesterol from the lumen into the enterocytes (**Altmann et al., 2004**). However, the intestinal metabolism of the cholesterol cannot justify the decrease of reverse cholesterol transport. Of note, in the liver, trimethylamine-N-oxide has been referred to reduce the expression of Cyp7a1, a fundamental bile enzyme in the metabolism, and transport of cholesterol (**Bronzato and Durante, 2017**).

Chicken meat is recognized for several health benefits due to its high nutritional value and, in addition to its high protein content, also its low cholesterol content, low energy value, and low-fat content (**Sujiwo et al., 2018**).

***The cholesterol of the chicken thigh muscle with skin***

The average cholesterol content of the chicken thigh muscle with skin and the statistical evaluation of the results are given in Table 7.

The average cholesterol content of the thigh muscle with skin in the experimental groups ranged from 0.034 g.100 $g^{-1}$ with thyme essential oil, followed by an average cholesterol content of 0.037 g.100 $g^{-1}$ with cinnamon essential oil, 0.039 g.100 $g^{-1}$ with cinnamon essential oil + citrus fruit essential oil, 0.044 g.100 $g^{-1}$ with citrus fruit essential oil up to 0.045 g.100 $g^{-1}$ with thyme essential oil + citrus fruit essential oil.

These values of average cholesterol content in the thigh muscle with skin are either lower (when using thyme essential oil), the same (when using cinnamon essential oil), or higher (when using citrus fruit essential oil, thyme essential oil + citrus fruit essential oil or cinnamon essential oil + citrus fruit essential oil).

The difference in the cholesterol content of the thigh muscle with the skin between the groups was not statistically significant ($p > 0.05$).

Statistical evaluation of the measured data of the cholesterol content of the thigh muscle with skin expressed by the standard deviation revealed that the smallest fluctuation of values was in the experimental group with cinnamon essential oil and the highest in the experimental group with thyme essential oil + citrus fruit essential oil, i.e. $SD = 0.002$ vs. $SD = 0.005$.

**Shang et al.** (**2020**) report a cholesterol content in the thigh muscle of 0.060 g.100 $g^{-1}$, which is a higher value of cholesterol content compared to our results. The cholesterol content of raw and cooked meat and poultry products ranges from 40 to 90 mg.100 $g^{-1}$ (**Honikel, 2009**).

Phytobiotics are products of plant origin extracted from medicinal plants, spices, or even fungi. They have a high impact on the body due to the high content of bioactive compounds provided by secondary metabolism. Numerous studies performed on various livestock, including broiler chickens, have confirmed the multidirectional effects of phytobiotics in animal nutrition on improving health and productivity.

**Table 8** Correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the breast muscle with skin by groups.

| Indicator | Fat | Cholesterol |
|---|---|---|
| Group | Control | |
| Dry matter | 0.71<br>0.14$^{-}$ | 0.38<br>0.31$^{-}$ |
| Fat | | 0.34<br>0.29$^{-}$ |
| Group | Thyme EO | |
| Dry matter | 0.42<br>0.83$^{-}$ | 0.18<br>0.87$^{-}$ |
| Fat | | 0.57<br>0.39$^{-}$ |
| Group | Cinnamon EO | |
| Dry matter | 0.51<br>0.49$^{-}$ | 0.47<br>0.99$^{-}$ |
| Fat | | 0.38<br>0.53$^{-}$ |
| Group | Citrus fruit EO | |
| Dry matter | 0.74<br>0.11$^{-}$ | 0.17<br>0.69$^{-}$ |
| Fat | | 0.09<br>0.98$^{-}$ |
| Group | Thyme EO + Citrus fruit EO | |
| Dry matter | 0.66<br>0.91$^{-}$ | 0.33<br>0.55$^{-}$ |
| Fat | | -0.29<br>0.63$^{-}$ |
| Group | Cinnamon EO + Citrus fruit EO | |
| Dry matter | 0.41<br>0.56$^{-}$ | 0.36<br>0.49$^{-}$ |
| Fat | | -0.57<br>0.66$^{-}$ |

Note: in each column, the 1st number in the row is the value of the Pearson correlation coefficient ($r$) and the 2nd number in the row is the *p-value*; numeric value marked with a superscript –: no statistically significant difference of the linear relation between 2 variables ($p$ >0.05); EO = essential oil.

In addition, a positive effect on the nutritional and dietary value of products of animal origin has been revealed (**Al-Yasiry et al., 2017; Kiczorowska et al., 2017**). **Ciftci et al.** (**2010**) state that after feeding a diet with cinnamon essential oil, the level of cholesterol in the blood serum is reduced, as well as in the breasts and thighs of broiler chickens. These authors also confirmed that this essential oil manifests hypocholesterolemic properties. But their opinion was not confirmed in our experiment on the thigh muscle. It has been reported that the thymol and carvacrol content of thyme slows down the activity of the cholesterol-synthesizing enzyme hydroxymethyl-glutaryl coenzyme A reductase, and thereby, decreases cholesterol levels (**Elson, 1995**). In agreement with this finding, it has also been reported that thyme significantly decreases cholesterol and triglyceride levels in quails (**Khaksar et al., 2012**) and also in broiler chickens (**Safa and Al-Beitawi, 2009**). The reduction of cholesterol content in the thigh muscle due to thyme essential oil was also found in our experiment.

**Correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the breast and thigh muscles with skin by groups**

***Correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the breast muscle with skin by groups***

The correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the breast muscle with skin by groups are shown in Table 8.

Based on the evaluation of the correlation relationship between the two variables, i.e. different results were found between dry matter and fat, between dry matter and cholesterol, and between fat and cholesterol of breast muscle with skin. Differences in the linear relation between the observed variables were not statistically significant ($p$ >0.05).

A strong positive linear relationship with an r value above 0.5 was found between dry matter and fat in the control group, in the cinnamon essential oil experimental group, in the citrus fruit essential oil experimental group, and the thyme essential oil + citrus fruit essential oil experimental group.

**Table 9** Correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the thigh muscle with skin by groups.

| Indicator | Fat | Cholesterol |
|---|---|---|
| Group | Control | |
| Dry matter | 0.58<br>$0.27^{-}$ | 0.37<br>$0.43^{-}$ |
| Fat | | 0.78<br>$0.17^{-}$ |
| Group | Thyme EO | |
| Dry matter | 0.78<br>$0.04^{+}$ | 0.49<br>$0.37^{-}$ |
| Fat | | 0.64<br>$0.14^{-}$ |
| Group | Cinnamon EO | |
| Dry matter | 0.83<br>$0.02^{+}$ | 0.17<br>$0.77^{-}$ |
| Fat | | 0.21<br>$0.86^{-}$ |
| Group | Citrus fruit EO | |
| Dry matter | 0.75<br>$0.09^{-}$ | 0.82<br>$0.73^{-}$ |
| Fat | | 0.26<br>$0.63^{-}$ |
| Group | Thyme EO + Citrus fruit EO | |
| Dry matter | 0.76<br>$0.65^{-}$ | 0.89<br>$0.84^{-}$ |
| Fat | | 0.41<br>$0.43^{-}$ |
| Group | Cinnamon EO + Citrus EO | |
| Dry matter | 0.95<br>$0.73^{-}$ | 0.27<br>$0.10^{-}$ |
| Fat | | -0.17<br>$0.81^{-}$ |

Note: in each column, the 1st number in the row is the value of the Pearson correlation coefficient ($r$) and the 2nd number in the row is the *p-value*; numeric value marked with a superscript –: no statistically significant difference of the linear relation between 2 variables ($p > 0.05$); numeric value marked with a superscript $^{+}$: statistically significant difference of the linear relation between 2 variables ($p \leq 0.05$); EO = essential oil.

Furthermore, a strong positive linear relationship with an $r$ value above 0.5 was found between fat and cholesterol in the experimental group with thyme essential oil and a strong negative linear relation with a value above 0.5 between fat and cholesterol in the experimental group with cinnamon essential oil + citrus fruit essential oil.

An interesting result of the correlation relation was recorded in the experimental group with citrus fruit essential oil between fat and cholesterol, which manifested itself only in a trivial positive linear relation with a value below 0.1.

Other values of the linear relation between dry matter and fat, between dry matter and cholesterol, and between fat and cholesterol showed a mean value, either positive or negative.

**Correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the thigh muscle with skin by groups**

The correlation between dry matter and fat, dry matter and cholesterol, and between fat and cholesterol in the thigh muscle with skin by groups are shown in Table 9.

Based on the evaluation of the correlation relationship between the two variables, i.e. between dry matter and fat, between dry matter and cholesterol, and between fat and cholesterol of the thigh muscle with the skin, different results were found, which in some relation, only the effect of thyme and cinnamon essential oils were statistically significant ($p \leq 0.05$).

A strong positive linear relationship with an r value above 0.5 was found between dry matter and fat in the control group, which was not statistically significant ($p > 0.05$), in the experimental group with thyme essential oil, which was statistically significant ($p \leq 0.05$), in the experimental group with cinnamon essential oil, which was statistically significant ($p \leq 0.05$), in the experimental group with citrus fruit essential oil, which was not statistically significant ($p > 0.05$), in the experimental group with thyme essential oil + citrus fruit essential oil, which was not statistically significant ($p > 0.05$), and in the experimental group with cinnamon essential oil + citrus fruit essential oil, which was not statistically significant ($p > 0.05$).

Furthermore, a strong positive linear relationship with an $r$ value above 0.5 was found between dry matter and

cholesterol in the citrus fruit essential oil experimental group, which was not statistically significant (p >0.05) and in the thyme essential oil + citrus fruit essential oil experimental group, which was not statistically significant ($p$ >0.05).

Furthermore, a strong positive linear relationship with an $r$ value above 0.5 was found between fat and cholesterol in the control group, which was not statistically significant ($p$ >0.05), and in the experimental group with thyme essential oil, which was not statistically significant ($p$ >0.05).

Other values of linear dependence between dry matter and fat, between dry matter and cholesterol, and between fat and cholesterol showed a mean value, either positive or negative, the difference of which was not statistically significant ($p$ >0.05).

Lipid and cholesterol contents of meat and poultry have been of great research interest for decades. This topic is repeatedly studied and reviewed **(Dinh et al., 2011)**.

**Dinh et al**. **(2008)** reported a positive correlation between intramuscular fat and cholesterol content.

**Sweeten et al. (1990)** further investigated the distribution of cholesterol in adipose tissues of both muscle (intramuscular adipose tissues) and subcutaneous fat (subcutaneous adipose tissues).

The intramuscular adipose tissues had similar cholesterol contents to that of the subcutaneous adipose tissues; however, only 54% of the cholesterol in the intramuscular tissues was from the cytoplasm (cholesterol in storage lipid), compared with a 90% proportion from the cytoplasm in subcutaneous adipose tissues.

Unless muscle type, muscle lipid, and their interactions with each other and with other contributory factors cause a dramatic change in muscle structure and composition, their effects on the cholesterol content of muscle might be limited **(Dinh et al., 2011)**.

## CONCLUSION

We can state from the evaluation and analysis of the results on the effect of thyme, cinnamon, and citrus fruit essential oils and their combinations after feeding in the broiler chickens that:

a) the dry matter content in the breast muscle ranged from 26.73 to 27.89 g.100 $g^{-1}$ compared to 28.61 g.100 $g^{-1}$ of the control group with no statistically significant differences, and in the thigh muscle in the ranged from 27.33 to 28.14 g.100 $g^{-1}$ compared to 28.66 g.100 $g^{-1}$ of the control group with no statistically significant differences,

b) the fat content in the breast muscle ranged from 1.56 to 1.81 g.100 $g^{-1}$ compared to 1.32 g.100 $g^{-1}$ in the control group without statistically significant differences and in the thigh muscle in the range from 2.58 to 2.93 g.100 $g^{-1}$ versus 2.64 g.100 $g^{-1}$ of the control group with no statistically significant differences,

c) the cholesterol content in the breast muscle ranged from 0.028 to 0.042 g.100 $g^{-1}$ compared to 0.035 g.100 $g^{-1}$ of the control group with no statistically significant differences, and in the thigh muscle from 0.034 to 0.045 g.100 $g^{-1}$ vs. 0.037 g. 100 $g^{-1}$ control group with no statistically significant differences,

d) in correlation, a strong positive linear relation between dry matter content and fat content tended to be indicated in the breast muscle due to cinnamon essential oil, citrus fruit essential oil, thyme essential oil in combination with citrus fruit essential oil and in the control group, a strong positive linear relation between fat and cholesterol due to thyme essential oil and a strong negative linear relation between fat and cholesterol due to cinnamon essential oil in combination with citrus fruit essential oil;

in the thigh muscle, a strong positive linear relation between dry matter content and fat content was statistically confirmed by thyme and cinnamon essential oils and tended to be indicated by thyme essential oil in combination with citrus fruit essential oil, cinnamon essential oil in combination with citrus fruit essential oil and in the control group, there was a tendency to indicate a strong positive linear relation between fat content and cholesterol content due to citrus fruit essential oil.

We can conclude that essential oils are one of the alternatives to feed antibiotics. Therefore, a thorough knowledge of their effect and mechanism remains an open question for future research.

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**Conflict of Interest:**

The authors declare no conflict of interest.

**Ethical Statement:**

The use of animals in this research was approved by the Ethics Committee of the Slovak University of Agriculture in Nitra following the legislation of the Slovak Republic.

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# THE CHEMICAL COMPOSITION OF TWO KINDS OF GRAPE JUICE WITH MEDICINAL PLANT ADDITION

***Jakub Mankovecký, Lucia Galovičová, Miroslava Kačániová***

## ABSTRACT

The safety of plant-based food with an herbal origin is a priority for producers and final consumers these days. The interest in the high biological value of the final food products enriched with herbal ingredients is rising. We focused on the study of physico-chemical composition and antioxidant activity of two kinds of grape juice with medicinal plant addition in our study. We used 2 varieties of grapes - Welschriesling and Cabernet Sauvignon, six species of medicinal plants - *Calendula officinalis* L., *Ginkgo biloba*, *Thymus serpyllum*, *Matricaria recutita*, *Salvia officinalis* L., and *Mentha aquatica* var. *citrata* in our experiment. There were14 samples prepared, two of them were control samples and 12 samples were treated with medicinal plants. We tested each of the selected parameters triplicate with an interval of one week. We evaluated the results statistically in 4 levels of significance $p$ <0,01, $p$ <0,001, $p$ <0,0001 and $p$ <0,00001. The content of fructose, glucose, dry matter, density, malic acid, pH, potential alcohol, total acids, and total sugars in the treated samples was significantly lower compared to the control sample, which was probably due to the degree of dilution of grape juice with extracts gained from medicinal plants. The antioxidant effect was demonstrably higher in the samples enriched with medicinal plants than in the control samples. The highest antioxidant effect was measured in the second test in the samples with the addition of *Thymus serpyllum* (80.93 % - white grape must, 82.33 % - blue grape must), *Calendula officinalis* L. (79.29 % - white grape must, 80.49 % - blue grape must) and *Ginkgo biloba* (79.10 % - white grape must, 83.3 % - blue grape must). Generally, we found out that the selected medicinal plants increase the biological quality of grape juice.

**Keywords:** grape juice; *Calendula officinalis* L.; *Ginkgo biloba*; *Thymus serpyllum*; *Matricaria recutita*; *Salvia officinalis* L.; *Mentha aquatica* var. *citrate*; chemical parameters

## INTRODUCTION

Grapes have a high level of moisture and sugar in their fresh form and these aspects can lead to their deterioration **(Farias et al., 2021)**. The grapes belong to fragile fruits therefore the degree of their damage during harvest and the distribution chain is very high **(Huwei et al., 2021)**. In addition, grape berries are sensitive to pathogenic infections, which result in significant economic losses **(Zhao et al., 2021)**. Grape juice mainly consists of water and several metabolites with an emphasis on sugars, organic acids, minerals, phenolic and aromatic compounds **(Dutra et al., 2021)**. Organic acids and sugars are associated with the chemical balance of grape juice and they also have a significant effect on taste balance and sensory properties **(Coelho et al., 2018)**.

Grape juice can be generally divided into fresh, concentrated, and reconstituted juice. Fresh grape juice is undiluted and unfermented pure juice made from the pulp of fresh grapes. Concentrated grape juice is a product that has undergone a physical process to remove water. Reconstituted grape juice is juice obtained by diluting concentrated or dehydrated juice to the original concentration of fresh juice **(Dutra et al., 2021)**.

Grape juices can be prepared from any grape variety (white or purple) as soon as they are properly ripened. Many observational studies suggest that intake of pure (100%) grape juice may reduce the risk of hypertension, cardiovascular disease, and diabetes mellitus, suggesting the importance of these drinks for maintaining health **(Zuanazzi et al., 2019)**.

Consumption of grape juice gradually increases due to factors such as characteristic taste, aroma, color, or refreshment. In addition, grape juice is gaining popularity due to the functional properties related to phenolic compounds which are associated with consumer health **(Dutra et al., 2021).**

The bioactive composition of the juice depends primarily on the grapes used in its preparation. Each variety has an individual phenolic composition. However, the amount of these compounds can vary and this variability depends on several factors, such as the species, the climatic conditions

of the region, or the stage of ripening of the grapes at the time of harvest **(Silva et al., 2019)**.

The most important nutrients in grape juice are vitamins, minerals, proteins, carbohydrates, soluble sugars, organic acids, fatty acids, and amino acids **(Gamboa et al., 2019)**. The health benefits of these nutrients and bioactive substances depend on their level of intake and bioavailability **(Haas et al., 2019)**. Amino acids play a key role in human health because the human body needs them for the synthesis of proteins, peptide hormones, and some neurotransmitters in the body. In addition, amino acids are essential for the catabolism of the intestinal mucosa. The most important amino acids found in grape juice are proline, arginine, glutamine, glutamic acid, and alanine **(Gamboa et al., 2019).**

The chemical composition of the juices is influenced by several factors including processing techniques **(Dutra et al., 2021)**. The processing and preservation of fruit juices need to be gentle and efficient to preserve the original properties of the fruit as much as possible **(Mesquita et al., 2020)**.

Grape juice is rich in flavonols, anthocyanins, procyanidins, and phenolic acids. Therefore, it has beneficial effects on human health **(Azimi et al., 2021)**.

Medicinal plants are used in the food industry to improve appearance and attractiveness. Scientific studies over the last few decades have shown that extracts from plants and herbs can be used as natural preservatives. In addition, it is possible to develop new food products with new and interesting flavor variants by applying various herbs **(Haugaard et al., 2014; Sayed et al., 2019)**.

Our study was focused on the chemical properties and antioxidant activity of some grape juice samples enriched with selected medicinal plants for two weeks.

## Scientific hypothesis

We supposed that the medical plant addition could reduce the chemical parameters of grape juice. We set the hypothesis that the medical plant addition increases the antioxidant activity of grape juice.

## MATERIAL AND METHODOLOGY

### Samples

There were two types of grapes used in the test - white grapes (variety Welschriesling) and blue ones (variety Cabernet Sauvignon), which were obtained from the Slovak University of Agriculture, Institute of Horticulture.

### Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Germany).

### Animal and Biological Material:

Welschriesling, Cabernet Sauvignon (the Slovak University of Agriculture, Institute of Horticulture).

### Instruments

Glomax spectrophotometer (Promega Inc., Madison, USA), MALDI-TOF MS Biotyper, Alpha FT-IR analyzer (Bruker, Daltonics, Bremen, Germany).

### Laboratory Methods

There were analyzed 9 parameters based on a certain calibration by using the Alpha FT-IR analyzer. The calibration is based on more than 1,700 wines from around the world. The determined parameters of grape juice were as follows: fructose ($g.L^{-1}$), glucose ($g.L^{-1}$), dry matter (°Brix), density ($g.L^{-1}$), malic acid ($g.L^{-1}$), pH, potential alcohol (%), total acids ($g.L^{-1}$) and total sugars ($g.L^{-1}$). There were three replicates of measurements on each sample performed.

This used device automatically drew a 10 mL sample of grape juice into a sterile syringe, squeezing about ¾ of the total contents of the syringe into the appropriate hole. The sample of grape juice flowed through the analyzer and it was finally collected through a thin tube into a beaker designated later during this process. The Alpha FT-IR analyzer using Opus software (Bruker Optics, Germany) is checked only with one click. The Alpha FT-IR analyzer was set at a constant temperature of 40 °C, measuring one sample up to 127 times for approximately 2 minutes.

The measurement procedure to determine the antioxidant activity of our samples using the DPPH method was as follows:

- 3.6 mL of DPPH radical (prepared by dissolving 0.025 g in 100 mL of ethanol and then diluting as needed) and 0.4 mL of sample were pipetted into the cuvette.
- the mixture thus formed was mixed quickly and stored in the dark for 10 minutes,
- after 10 minutes, the absorbance at a wavelength of 515 nm on a spectrophotometer was measured

We repeated the measurement 3 times. The results of antioxidant activity were determined as the percentage of radical inhibition.

### Description of the Experiment

**Sample preparation:**

The juice from the grapes was prepared by using the following grape dressing procedures: harvesting, stalking, maceration, pressing, sludge removal, and pasteurization.

The grapes from the Welschriesling variety and the Cabernet Sauvignon variety were collected in October 2020 in the morning in warm and sunny weather. The grapes were hand-harvested in crates with a volume of 25 kg. The grapes were immediately transported to the AgroBioTech research center laboratory of beverages intended for processing to prevent possible steaming of the grapes after harvesting. The sugar content of the Welschriesling grape after harvest was 19.87 ° for Brix and 16.35° for the Cabernet Sauvignon. Then the grapes were put into a mill hopper, where the bunch was separated from the must and berries. The maceration of the grape mash lasted 1 hour. The grape mash was placed in closed containers with a volume of 100 liters during maceration. The pressing was carried out in a gentle way using the maximum pressing pressure - 0.1 MPa. There was discontinuous hydrolysis used for compression. The aim of pressing at a lower pressure is to eliminate damage of the grape seeds and the subsequent transfer of immature phenolic substances to the pressed must. In the case of pressing at a higher pressure than 0.15 MPa, undesirable substances can pass into the pressed must. These substances could negatively affect the character of the must and its sensory properties. The grape must be pumped into a sludge tank using a pump, where static sludge removal was placed without the use of oenological auxiliaries. Subsequently, both experimental musts were pasteurized to eliminate possible contamination by unwanted microcenosis, in particular by fermentation of the must under the influence of wild yeasts.

The extracts were prepared in a ratio of 1:10. We mixed 10 g of the medicinal plant with 100 mL of ethanol. The prepared herbal mixture was stored for 2 weeks. After these 2 weeks, the herbal mixture was treated by evaporation on a vacuum evaporator and subsequent dissolution in distilled water.

We used prepared samples of musts from the varieties of Welschriesling and Cabernet Sauvignon in a volume of 200 mL and extracts from selected medicinal plants in a volume of 20 mL for the sample preparation of grape juice enriched with medicinal plants. We prepared the mixtures of grape juice with herbal extracts in a ratio of 1:10. We used the samples prepared in this way to monitor the microbiological quality and antioxidant activity.

The chemical parameters of the control samples of grape juice, as well as the samples of grape juice enriched with selected medical plant addition, were measured by using Alpha FT-IR is a wine analyzer. The Alpha FT-IR analyzer analyses the sample based on the infrared spectrometry method. It means that the analyzer measures the absorption of infrared radiation that passes through the sample. There were many various changes in the energy states of organic or inorganic molecules during this process which depend on the change in the dipole moment of the molecule. The output of the measurement is a graphical presentation of the infrared spectrum, which is expressed as percent transmittance or absorbance units.

There were used 14 grape juice samples, 2 of them were used as a control (from white and blue grapes) and the 12 samples were enriched with selected medicinal plants (6 juices from white and 6 juices from blue grapes).

**Number of samples analyzed:** 14

**Number of repeated analyses:** 3

**Number of experiment replication:** 2

**Statistical analyses**

We used the basic statistical method using Microsoft Office Excel 2016 (arithmetic mean calculation of 3 measurements) and the statistical program GraphPad Prism 6 (GraphPad Software, San Diego, USA) for the evaluation of individual parameters and test data as well as two-factor analysis of variance ANOVA (Dunnett test) at 4 levels of significance $p < 0.01$, $p < 0.001$, $p < 0.0001$ and $p < 0.00001$.

## RESULTS AND DISCUSSION

There are only a few studies that were focused on the evaluation of the grape juice with the medicinal plant addition. Our work aimed to monitor the chemical parameters of grape juice with the addition of medicinal herbs, as well as to determine the antioxidant activity.

The chemical composition of grape juice depends on the variety of used grapes, the handling and processing of the grapes **(Morris, 1998; Soyer et al., 2003; Camargo, 2004; Camargo and Maia, 2004; Camargo et al., 2005; Yier et al., 2010; Stalmach et al., 2011; Ribeiro et al., 2012; Rizzon and Miele, 2012; Cosme et al., 2018)**.

The following tables process the averages of the measured values of the basic chemical parameters of grape juice (Table 1, Table 2, Table 3 and Table 4). The measurements were repeated after the week because of a better evaluation of the influence of medicinal plants on the chemical parameters of grape juice. Grape juice generally contains a relatively high amount of total sugars **(Fügel et al., 2005; Navarro-Pascual-Ahuir et al., 2015 a, b; Yi et al., 2017; Navarro-Pascual-Ahuir et al., 2017; Nikolaou et al., 2017; Marszałek et al., 2018; Li et al., 2020)**. The amount of total sugar in the control sample was 191.78 $g.1^{-1}$ of white grape juice. In contrast, medicinal plants contain a minimum of sugars, and therefore, after their addition to grape juice, this amount decreased in each sample to the limit of evidence $p < 0.0001$. The lowest measured amount of total sugars was in the sample of grape juice with the addition of *Calendula officinalis* L. (175.21 $g.L^{-1}$). The lowest decrease of glucose content was observed after the addition of *Ginkgo biloba*, where the glucose value of 84.30 $g.L^{-1}$ was measured.

The glucose content of the control sample decreased slightly from 92.15 $g.L^{-1}$ to 91.62 $g.L^{-1}$ after one week. The differences in glucose values between the control sample and the samples with the addition of medicinal plants were at the level of $p < 0.00001$. The highest glucose loss was recorded by the sample with the addition of *Calendula officinalis* and the lowest in the sample with the addition of *Salvia officinalis* in comparison with the second measurement testing. The lowest decrease in dry matter compared to the control sample was achieved by juice with the addition of *Ginkgo biloba* with a value of 84.02 °Brix. Nevertheless, the decrease in dry matter in the samples compared to the indicator reached a high level at the level of significance $p < 0.00001$. The content of potential alcohol decreased slightly one week apart in treated plant samples. On the contrary, its value in the control sample increased slightly from 10.33% to 11.25%. However, these changes were insignificant in terms of statistical significance. Density and pH values did not show any significant differences even after a week.

Blue grape juice with the addition of medicinal plants showed a significant difference compared to the control sample in almost all tested chemical parameters. The fructose content varied approximately equally between the individual treated samples. The differences between the treated samples in the glucose content were minimal, only by 0.6 $g.L^{-1}$. The measured values of density, pH, and total acids did not show statistically significant differences compared to the control sample. Nevertheless, the best-preserved amount of total acids was in the sample of grape juice with the addition of *Salvia officinalis* and at least in the sample of grape juice with the addition of *Mentha aquatica* var. *citrata*.

The measured values of density, pH, and total acids did not show significant differences between the control sample of grape juice and the treated samples of juice even one week after the first measurement. The fructose content of the control sample was 79.32 $g.L^{-1}$. The fructose content with a statistical significance of $P<0.00001$ was observed in other samples treated with medicinal plants. The loss of glucose amount compared to the control sample was in all treated samples at approximately the same level, around 8.54% with a level of significance $p < 0.0000.1$. The differences in the dry matter content of the samples enriched with medicinal plants and the control sample ranged at 2 levels of significance. The potential alcohol did not significantly change compared to the first test, it decreased slightly. After the second measurement, it ranged from 8.62 to 8.88% in the samples enriched with medicinal plants and 9.40% in the control sample.

**Table 1** Chemical parameters of white grape after first week.

| Sample | Fructose ($g.L^{-1}$) | Glucose ($g.L^{-1}$) | Dry matter (°Bx) | Density ($g.L^{-1}$) | Malic acid ($g.L^{-1}$) | pH | Potential alcohol (%) | Total acids ($g.L^{-1}$) | Total sugars ($g.L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **CW** | 101.85 | 92.15 | 19.87 | 1.080080 | 5.18 | 3.19 | 10.33 | 7.58 | 191.78 |
| **W1** | 92.13** | 83.34**** | 18.23*** | 1.073160 | 4.47 | 3.25 | 10.22 | 6.49**** | 176.67*** |
| **W2** | 93.31** | 84.30**** | 18.47*** | 1.074427 | 4.36* | 3.26 | 10.30 | 6.37**** | 178.95*** |
| **W3** | 90.86*** | 82.80**** | 18.29*** | 1.073723 | 4.25* | 3.25 | 10.26 | 6.44**** | 177.84*** |
| **W4** | 90.89*** | 82.65**** | 18.60*** | 1.072107 | 4.32* | 3.25 | 10.17 | 6.44**** | 175.21*** |
| **W5** | 92.43** | 83.61**** | 18.29*** | 1.073530 | 4.51 | 3.25 | 10.26 | 6.65*** | 177.17*** |
| **W6** | 92.32** | 83.60**** | 18.29*** | 1.073800 | 5.16 | 3.20 | 10.32 | 7.34 | 176.39*** |

**Note:** CW - white grape juice control sample, W1 - white grape juice with the addition of *Calendula officinalis* L., W2 - white grape juice with the addition of *Ginkgo biloba*, W3 - white grape juice with the addition of *Thymus serpyllum*, W4 - white grape juice with the addition of *Matricaria recutita*, W5 - white grape juice with the addition of *Salvia officinalis* L., W6 - white grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * $p$ <0.01, ** $p$ <0.001, *** $p$ <0.0001.

**Table 2** Chemical parameters of white grape after second week.

| Sample | Fructose ($g.L^{-1}$) | Glucose ($g.L^{-1}$) | Dry matter (°Bx) | Density ($g.L^{-1}$) | Malic acid ($g.L^{-1}$) | pH | Potential alcohol (%) | Total acids ($g.L^{-1}$) | Total sugars ($g.L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **CW** | 101.23 | 91.62 | 19.82 | 1.079677 | 5.27 | 3.21 | 11.25 | 7.77 | 190.71 |
| **W1** | 91.82** | 82.58**** | 18.28*** | 1.073113 | 4.63 | 3.25 | 10.21 | 6.81*** | 176.41*** |
| **W2** | 92.95** | 84.02**** | 18.43*** | 1.073580 | 4.51 | 3.25 | 10.33 | 6.53**** | 177.05*** |
| **W3** | 90.28*** | 82.52**** | 18.33*** | 1.073937 | 4.25* | 3.24 | 10.29 | 6.63**** | 178.38*** |
| **W4** | 92.27** | 82.71**** | 18.29*** | 1.073147 | 4.57 | 3.24 | 10.27 | 6.73*** | 176.95*** |
| **W5** | 91.04*** | 82.67**** | 18.36*** | 1,.73120 | 4.65 | 3.25 | 10.25 | 6.89*** | 176.09*** |
| **W6** | 90.78*** | 82.71**** | 18.25*** | 1.073327 | 4.85 | 3.24 | 10.30 | 7.43* | 174.97*** |

**Note:** CW - white grape juice control sample, W1 - white grape juice with the addition of *Calendula officinalis* L., W2 - white grape juice with the addition of *Ginkgo biloba*, W3 - white grape juice with the addition of *Thymus serpyllum*, W4 - white grape juice with the addition of *Matricaria recutita*, W5 - white grape juice with the addition of *Salvia officinalis* L., W6 - white grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * $p$ <0.01, ** $p$ <0.001, *** $p$ <0.0001.

The highest decrease in total sugars compared to the control sample was in the sample of grape juice with the addition of *Ginkgo biloba*, namely by 7.68%.

**Burini et al. (2009)** focused on the study of grape juice in terms of physicochemical aspects of grape juice. They used 12 samples of grape juice for the research, of which 7 were commercial, 2 of them were organic juice samples, and 3 samples were homemade. All tested parameters were performed in 3 series and the results were expressed as the average of individual measurements. The pH of the samples ranged from 3.30 to 3.64, which is a slightly higher pH than in our samples, where the pH values did not exceed 3.28. The measured dry matter content was varied in the individual samples. The highest amount of dry matter was observed in homemade grape juice, up to 21 °Brix, and the lowest amount of dry matter was contained in commercial grape juice with the lowest measured value of 9.5 °Brix. Compared to our samples of dark grape juice, samples of organic grape juice with a value of 15 °Brix achieved a similar amount of dry matter. In contrast, the values of our white grape juice samples ranged similarly to **Burini et al. (2009)** home-made juices, around 19 °Brix. The total titratable acids in the samples ranged from 5.60 to 9.84 $g.L^{-1}$.

**Table 3** Chemical parameters of blue grape after first week.

| Sample | Fructose ($g.L^{-1}$) | Glucose ($g.L^{-1}$) | Dry matter (°Bx) | Density ($g.L^{-1}$) | Malic acid ($g.L^{-1}$) | pH | Potential alcohol (%) | Total acids ($g.L^{-1}$) | Total sugars ($g.L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **CB** | 79.77 | 71.43 | 16.30 | 1.067480 | 10.47 | 3.28 | 9.39 | 13.37 | 152.94 |
| **B1** | 73.82**** | 65.14**** | 15.12*** | 1.062827 | 9.42*** | 3.28 | 8.69**** | 12.60 | 142.34**** |
| **B2** | 74.26**** | 65.36**** | 15.11*** | 1.062470 | 9.43*** | 3.29 | 8.69**** | 12.42 | 142.63**** |
| **B3** | 73.44**** | 65.74**** | 15.49** | 1.064340 | 9.34*** | 3.28 | 8.96**** | 12.65 | 147.15*** |
| **B4** | 73.96**** | 65.32**** | 15.12*** | 1.062270 | 9.41*** | 3.28 | 8.70**** | 12.63 | 142.75**** |
| **B5** | 73.58**** | 65.39**** | 15.80* | 1.062293 | 9.60** | 3.28 | 8.70**** | 12.75 | 141.83**** |
| **B6** | 74.13**** | 65.14**** | 15.60** | 1.062640 | 9.47** | 3.27 | 8.70**** | 12.45 | 142.05**** |

**Note:** CB - blue grape juice control sample, B1 - blue grape juice with the addition of *Calendula officinalis* L., B2 - blue grape juice with the addition of *Ginkgo biloba*, B3 - blue grape juice with the addition of *Thymus serpyllum*, B4 - blue grape juice with the addition of *Matricaria recutita*, B5 - blue grape juice with the addition of *Salvia officinalis* L., B6 - blue grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * $p$ <0.01, ** $p$ <0.001, *** $p$ <0.0001.

**Table 4** Chemical parameters of blue grape after second week.

| Sample | Fructose ($g.L^{-1}$) | Glucose ($g.L^{-1}$) | Dry matter (°Bx) | Denstity ($g.L^{-1}$) | Malic acid ($g.L^{-1}$) | pH | Potential alcohol (%) | Total acids ($g.L^{-1}$) | Total sugars ($g.L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **CB** | 79.32 | 70.98 | 16.25 | 1.066927 | 10.26 | 3.26 | 9.40 | 12.74 | 152.54 |
| **B1** | 73.13**** | 64.79**** | 15.05*** | 1.062210 | 9.51** | 3.26 | 8.62**** | 12.56 | 141.88**** |
| **B2** | 73.72**** | 64.88**** | 15.04*** | 1.061960 | 9.50** | 3.26 | 8.65**** | 12.54 | 140.82**** |
| **B3** | 73.09**** | 65.43**** | 15.46** | 1.063993 | 9.35** | 3.25 | 8.88**** | 12.40 | 147.17** |
| **B4** | 73.51**** | 64.85**** | 15.05*** | 1.061830 | 9.47** | 3.26 | 8.63**** | 12.65 | 142.40**** |
| **B5** | 73.05**** | 64.88**** | 15.52** | 1.061833 | 9.51** | 3.25 | 8.63**** | 12.43 | 140.83**** |
| **B6** | 73.82**** | 64.67**** | 15.36** | 1.062030 | 9.57** | 3.25 | 8.64**** | 11.98 | 141.46**** |

**Note:** CB - blue grape juice control sample, B1 - blue grape juice with the addition of *Calendula officinalis* L., B2 - blue grape juice with the addition of *Ginkgo biloba*, B3 - blue grape juice with the addition of *Thymus serpyllum*, B4 - blue grape juice with the addition of *Matricaria recutita*, B5 - blue grape juice with the addition of *Salvia officinalis* L., B6 - blue grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * $p$ <0.01, ** $p$ <0.001, *** $p$ <0.0001.

In contrast, the values of total acids in samples of blue grape juice ranged from 11.98 - 12.74 $g.L^{-1}$. On the contrary, samples from white grapes ranged in similar values as **Burini et al. (2009)** samples, specifically from 6.63 - 7.77 $g.L^{-1}$.

**Nassur et al. (2014)** investigated the number of chemical components contained in grape juice, specifically the content of dry matter, anthocyanins, and total sugars. The number of total sugars ranged from 24.19 gl-1 to 30.85 $g.L^{-1}$, which is a significantly smaller amount than we measured in our samples, where we measured the values of pure juice immediately after processing 191.78 $g.L^{-1}$ in the sample from white grapes and 152.94 $g.L^{-1}$ in a sample of blue grapes. The measured dry matter content in the tested samples was approximately the same (**Nassur et al. (2014**) as in our samples of pure blue grape juice sample (16.30 °Brix). We found out that the lowest dry matter content of samples was 14.10 °Brix and the highest dry matter content was 15.83 °Brix. On the contrary, the dry matter content of our sample of pure white grape juice was higher in the first measurement with the measured value of 19.87 °Brix than in the second measurement.

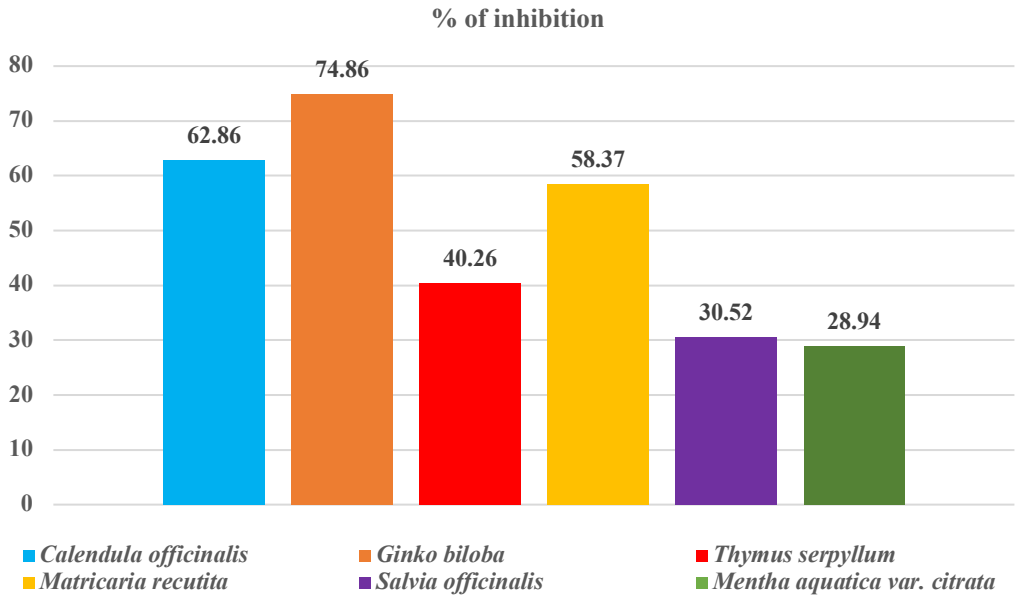


**Figure 1** Antioxidant activity of medicinal plants extract.

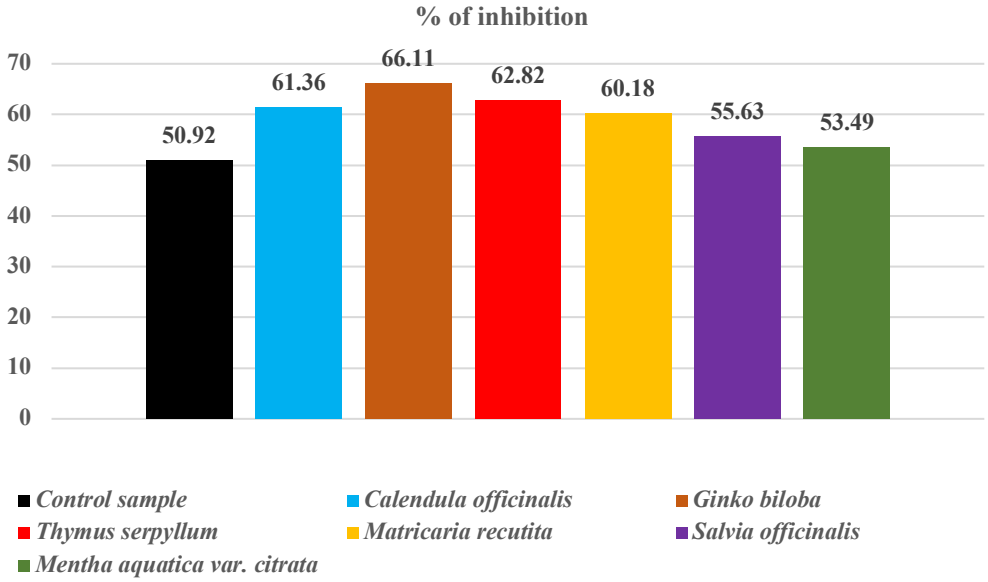


**Figure 2** Antioxidant activity of white grape juice with addition of medicinal plants after one week.

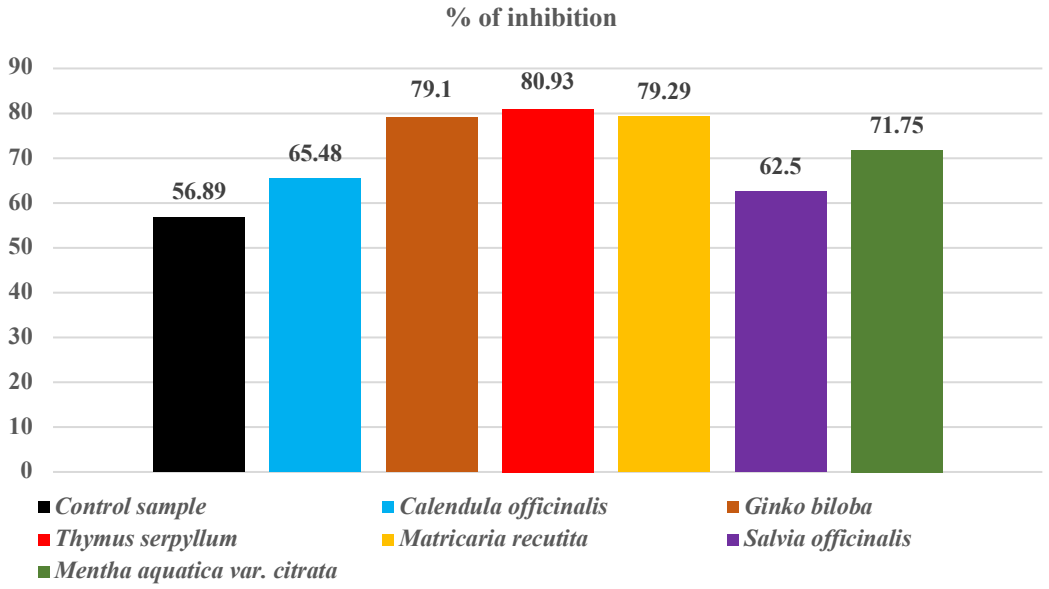


**Figure 3** Antioxidant activity of white grape juice with addition of medicinal plants after two weeks.

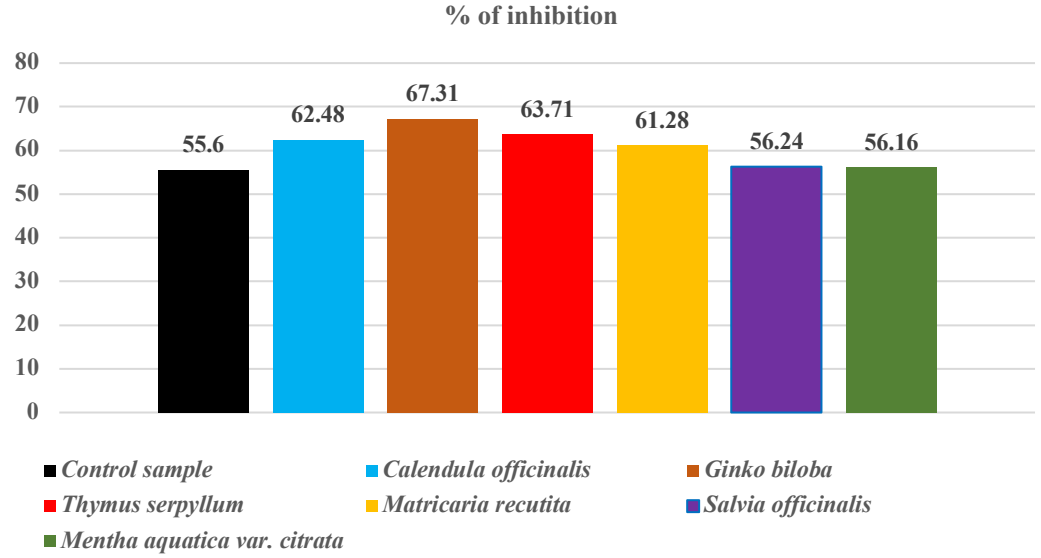


**Figure 4** Antioxidant activity of blue grape juice with addition of medicinal plants after one week.

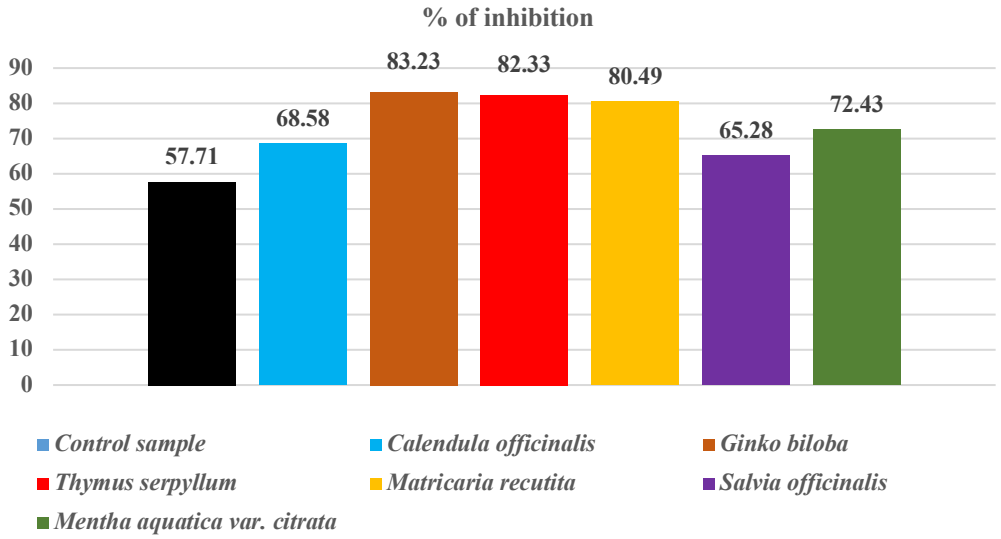


**Figure 5** Antioxidant activity of blue grape juice with addition of medicinal plants after two weeks.

**Costa et al. (2019)** focused their research on the physicochemical aspects of grape juice produced in Brazil in the Cmpanha Gaucha region. They got a type of grape *Vitis labrusca* (blue grapes) from two different places - Dom Pedrito and Santana do Livramento. The study aimed to determine juice density, pH, dry matter, and color intensity. In general, grape juice obtained from both places reached a significant color intensity, Dom Pedrito juice 8.65 and Santana do Livramento juice 10.76. The pH values were the same as in the study by **Burini et al. (2009)** slightly higher than ours, namely 3.59 to 3.67 for Dom Pedrito and 3.45 to 3.63 for Santana do Livramento. The measured density of grape juice samples from *Vitis labrusca* grapes ranged from 1049.25 g.ml$^{-1}$ to 1055.75 g.ml$^{-1}$ (Dom Pedrito) and 1053.25 g.ml$^{-1}$ by 1054.25 g.ml$^{-1}$. Our samples of blue grapes reached density values slightly higher, in particular, these values ranged from 1.06227 g.L$^{-1}$ to 1.06748 g.L$^{-1}$. In addition to these parameters, **Costa et al. (2019)** devoted another important parameter - dry matter content. The Dom Pedrito samples measured an average of 12.37 °Brix and the Santana do Livramento samples measured an average of 13.14 °Brix. Compared to our research, the dry matter content in individual samples was lower by up to 20-25%.

**Gurak et al. (2010)** focused their research on the quality of grape juice, which was concentrated by reverse osmosis. The research was performed at different pressures (40, 50, and 60 bar) and different temperatures (20, 30, and 40 °C). The following parameters were tested to measure the quality of individual samples of grape juice: pH, dry matter, acidity, the concentration of phenolic compounds, anthocyanin content, and color index. However, none of the measured results achieved significant differences in the basic chemical-physical characteristics of grape juice samples. The concentration of phenolic acids averaged 1.98 g.L$^{-1}$, the concentration of anthocyanins averaged 110.86 mg.L$^{-1}$. The color based on absorbance averaged 2,088 ABS.

The pH of the samples was significantly more acidic lower in all differently treated samples in comparison with other studies **(Costa et al. 2019**; **Burini et al., 2009)**. The pH values ranged from 2.95 (at 30 °C and 50 bar) to 2.98 (at 20 °C and 40 bar). In contrast, our pH values were around 3.25. The amount of total acids was also not so different due to the process of concentrating grape juice. The average amount of total acids was 7.0 g.L$^{-1}$. In contrast, our samples showed more varied values of total acids, namely the values in the samples of grape juice from white grapes ranged between 6.37 and 7.77 g.L$^{-1}$ and from 8.62 and 9.40 g.L$^{-1}$ in the samples of grape juice from blue grapes. The dry matter content had the highest value in samples of grape juice at a temperature of 20 °C and a pressure of 60 bar, as well as at a temperature of 30 °C and a pressure of 50 bar, namely 14.3 °Brix. The lowest value of dry matter was found at a temperature of 20 °C and a pressure of 40 bar, namely 13.5 °Brix. Our samples of blue grape juice reached slightly higher dry matter values (on average 15.45 °Brix) and samples of white grape juice contained on average up to 18.56 °Brix dry matter.

The antioxidant activity of individual types of food is based on the amount of antioxidants **(Athukorala et al.,2003; Shahidi and Zhong, 2005; Liyana-Pathirana et al., 2006; Cumby et al., 2008; Pereira and Abreu, 2020).** The function of antioxidants is to slow down or cancel unwanted oxidative reactions in the body and thus protect it from unwanted radicals that can harm it **(Young and Woodside, 2001; Lobo et al., 2010)**.

The antioxidant activity of substances can be determined physically or chemically. In our case, we chose a chemical method using the DPPH method to determine the antioxidant potential of grape juice enriched with medicinal plants. We also used a control sample prepared for a more thorough determination of the antioxidant activity of enriched grape juices with medicinal plants. We repeated the testing of individual samples after one week.

The antioxidant activity of the extracts from medicinal herbs determines the extent of their inhibitory effects on radicals (Figure1). The results show that the most significant antioxidant effect was available in *Ginko biloba* with a value of 74.86%. *Calendula officinalis* and *Matricaria recutita* also reached relatively high values, namely 62.86% (*Calendula officinalis*) and 58.37%

(*Matricaria recutita*). *Mentha aquatica* var. *citrata* and *Salvia officinalis* showed a significantly lower antioxidant effect in comparison with *Ginkgo biloba*. *Thymus serpyllum* showed a medium effect and the least significant antioxidant effect was measured with *Mentha aquatica* var. *citrata* from all 6 extracts.

The antioxidant activity of juice samples after one and two weeks is shown in Figure 2-5. A control sample of white grape juice achieved an antioxidant activity of 50.92%. The antioxidant potential increased after the addition of the extracts. The highest activity was achieved by the juice with the addition of *Ginkgo biloba*, namely 66.11%. We measured a mean percentage of radical inhibition, which ranged from 60.18% to 62.82% in the samples of grape juices with the addition of *Calendula officinalis*, *Thymus serpyllum* and *Matricaria recutita*. The lowest values among the treated samples with medicinal plants were measured in *Salvia officinalis* and *Mentha aquatica* var. *citrata*.

It can be seen after the second testing of the samples, that the antioxidant activity of the individual samples increased. There was an increase of the percentage of radical inhibition noticed in the control sample, in percentage 5.97% compared to the first test. The lowest antioxidant activity among the samples treated with medicinal plants was achieved by *Calendula officinalis* and *Salvia officinalis*, which percentage of radical inhibition did not exceed 65.48%. The antioxidant activity of the juice with the addition of maternal sip significantly improved, namely by 18.11% after a week of storage, making this sample also the most effective among all samples of white grape juice. The samples with the addition of *Ginkgo biloba* (79.10%) and *Matricaria recutita* (79.29%) were approximately at the same level of efficiency. There was also a significant increase in antioxidant activity occurred in the sample with the addition of *Mentha aquatica* var. *citrata*, where the antioxidant effect increased by up to 18.26%. This recorded increase was the highest in comparison to the other samples. On the contrary, the lowest increase since the first testing was measured in the sample treated with *Calendula officinalis*, where the antioxidant effect achieved a 77.44% smaller increase than the sample with the addition of *Mentha aquatica* var. *citrata*.

We can say based on the results of testing samples that blue grapes have better antioxidant activity than white ones. Therefore, other measured values of samples treated with medicinal plants also achieved higher efficiency with samples from blue grapes. The most significant antioxidant effect was shown in a sample of grape juice from blue grapes with the addition of *Ginkgo biloba* (67.31%). The minimum difference compared to the control sample was achieved by samples of juices with the addition of *Mentha aquatica* var. *citrata* and *Salvia officinalis*, where the increase in efficiency was only 0.6%. On the contrary, relatively significant differences compared to the control sample were achieved by samples of juices with the addition of maternal sap (difference 8.11%), *Calendula officinalis* (difference 6, 88%), and *Matricaria recutita* (difference 5.68%).

**Nile et al. (2013)** focused in their study on the phenol content and antioxidant activity of various grape species. They used 20 types of grapes, which were received from Gyeonggi-do province located in Korea and processed into grape juice. They extracted the juice from the pulp and the skins of individual grapes. Research shows that, in general, juice made from grape pulp has greater antioxidant activity. The results of antioxidant activity were different, ranging from 35% to 87%. However, the most of samples showed high antioxidant activity, from 62% up to 17 samples of grape juice. Our samples of grape juice also showed a relatively high value of antioxidant activity from 56.89% to 80.93% for samples of grape juice from white grapes and from 57.71% to 83.23% for samples of grape juice from blue grapes.

The most significant increase in efficiency was achieved in the samples with the addition of *Ginkgo biloba* (83.23%) and *Thymus serpyllum* (82.33%), which is up to 30.27% more compared to the control sample. The weakest inhibitory effect of radicals among all treated samples with medicinal plants was shown by the sample with the addition of *Salvia officinalis* (65.28%). There was obtained a very significant antioxidant effect in the blue grape juice samples in the sample with the addition of *Matricaria recutita*, which percentage of radical inhibition increased to 19.21% compared to the first test in contrast to the white grape juice samples. The smallest difference between the first and second testing occurred in the control sample, where the value increased by 2.11%, which is 17.11% less compared to the increase in the sample with the addition of *Matricaria recutita*.

## CONCLUSION

Grape juice is becoming more and more popular with consumers every day, not only for its unique taste, aroma, or clear color but especially for its rich content of minerals, vitamins, and antioxidants, which have a positive effect on the body. We evaluated 9 parameters - fructose, glucose, total sugar, dry matter, density, malic acid, total acids, potential alcohol, and pH. In general, the pH and density values were not different. We consider the storage temperature, sludge removal during storage, and dilution of grape juice by medicinal plants as the reasons for the reduction of the values of chemical parameters in the treated samples. The larger molecules settle to the bottom during storage entraining some compounds in certain amounts and therefore there is an increase in loss. We consider the dilution of grape juice by medicinal plant extracts to be the greatest factor that influences the decrease of measured chemical parameters. We suppose that if we add a larger amount of medicinal plant extract to grape juice, the loss of individual chemicals will be higher and vice versa. We tested the antioxidant activity of grape juice samples using the DPPH method. The antioxidant activity of individual samples gradually increased, as is clear from the results of the second testing. In general, the antioxidant activity was higher in the samples of grape juice from the Cabernet Sauvignon variety than in the samples of grape juice from the Welschriesling variety. The best antioxidant results in juice samples from both varieties were found in juice samples with the addition of *Thymus serpyllum*, *Matricaria recutita,* and *Ginkgo biloba*. It is clear from obtained test data that the enrichment of grape juice with medicinal plants increases, resp. improves their biological properties. Medical plants have a high potential to be bioactive compounds therefore a deeper study focused on the improvement of the food functionality is needed.

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This article does not contain any studies that would require an ethical statement.

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# ANTIBIOFILM AND ANTIOXIDANT ACTIVITY OF *ROSMARINUS OFFICINALIS* ESSENTIAL OIL

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## ABSTRACT

The aim of the work was to explore the antioxidant potential and antibiofilm activity of the *Rosmarinus officinalis* essential oil. The DPPH method was used to determine the antioxidant activity. The agar microdilution method was used to determine the minimum biofilm inhibiting concentration (MBIC). The MALDI-TOF MS Biotyper was used to evaluate the antibiofilm activity on the wood and glass surface. Vapor phase antimicrobial analysis was used to determine the effect on the food model. The antioxidant activity was 28.76% ±2.68%. The MBIC for *Stenotrophomonas maltophilia* was 25 µL.mL$^{-1}$ and for *Bacillus subtilis* 12.5 µL.mL$^{-1}$. Analysis of the mass spectra of *S. maltophilia* revealed an inhibitory effect from the 5$^{th}$, which persisted until the end of the experiment. Analysis of the mass spectra of *B. subtilis* showed an inhibitory effect from the 7$^{th}$ of the experiment. The experiments showed an effect on both tested surfaces. The food model showed a more pronounced effect of the *Rosmarinus officinalis* essential oil against *B. subtilis*. We assume that the effect of the essential oil is to disrupt the polysaccharide structure of the biofilm and consequently reduce the resistance of the biofilm. We have established that MALDI-TOF MS Biotyper is a suitable tool for evaluating changes in biofilm structure and could find more significant application for the study of biofilms in food and clinical practice.

**Keywords:** biofilm; *Stenotrophomonas maltophilia*; *Bacillus subtilis*; essential oil; MALDI-TOF MS Biotyper

## INTRODUCTION

In recent years, there has been a growing interest in research into essential oils and their applications in the food and human health. Essential oils are volatile aromatic substances that are obtained from glandular trichomes and other secretory structures of plants. Subsequently, they are distributed mainly on the surface of plant organs, especially flowers, leaves, stems, and roots. Essential oils and their ingredients are a safe alternative to chemical preservatives. They have biological activity that inhibits the growth of microorganisms **(Zhang et al., 2020)**.

Rosemary (*Rosmarinus officinalis*) is a perennial aromatic herb native to the Mediterranean Sea. It belongs to the family Lamiaceae. *R. officinalis* is an important source of volatile and non-volatile bioactive compounds **(Rahbardar et al., 2017)**. The main components of *R. officinalis* essential oil are camphor, camphene, 1,8-cineole, β-thujene, α-thujene, chrysanthantone, and β-cubebene. These compounds show a variety of biological activities, including antimicrobial, antioxidant, anti-inflammatory, and anticarcinogenic properties **(Touazi et al., 2018)**. Due to its therapeutic effects, it was used in the Middle Ages for the treatment of various diseases and it was also used as a preservative and flavouring agent **(Elyemni et al., 2019)**.

Biofilms are defined as complex bacterial communities found in an exopolysaccharide matrix on both biotic and abiotic surfaces. Biofilm formation is usually a cyclic multistage process. It is necessary to understand this process to develop effective strategies to combat pathogenic biofilms **(Khatoon et al., 2018)**. The issue of biofilm formation concerns many food sectors such as the dairy industry, poultry and red meat processing, and fresh products **(Kocot and Olszewska, 2017)**.

*Stenotrophomonas maltophilia* plays an important role in the colonization of biotic and abiotic surfaces, which significantly increases its resistance to antibiotics. This opportunistic pathogen is the originator of many nosocomial infections **(Pompilio et al., 2020)**. It is also often found in the food industry, for example in raw milk, fish products, vegetables, and also in drinking water reservoirs. The presence of *S. maltophilia* in food products causes deterioration and significantly endangers human health **(Zhang et al., 2020)**.

*Bacillus subtilis*, a non-pathogenic, gram-positive bacterium, is one of the most studied biofilm-forming microorganisms. Its importance in the food industry lies in the formation of a colony biofilm at the water-air interface **(Yahav et al., 2018)**. Under industrial conditions, biofilm formation leads to costly regular cleaning, equipment corrosion, and the production of extracellular enzymes by biofilm bacteria. Importantly, endospore-producing biofilm genera such as Bacillus can become a significant source of persistent contamination **(Ranmadugala et al., 2017)**.

The work was aimed to evaluate the antioxidant and antibiofilm activity of the essential oil *Rosmarinus officinalis* against *Stenotrophomonas maltophilia* and *Bacillus subtilis*. To evaluate the molecular profiles of biofilms on glass and wood after the application of *R. officinalis* essential oil using MALDI-TOF MS Biotyper. To evaluate the effectiveness of essential oil against biofilms in a food model (carrot, potato, apple) using the vapor phase antimicrobial analysis.

### Scientific hypothesis

After studying the available literature, we assume the presence of bioactive substances and the antioxidant potential of the essential oil *Rosmarinus officinalis*. Given the available publications, we anticipate an antibiofilm effect against *S. maltophilia* and *B. subtilis*. We believe that the antibiofilm effect could also be manifested in the gas phase in the food model.

## MATERIAL AND METHODOLOGY

### Samples

*Rosmarinus officinalis* essential oil was purchased from the Slovak company Hanus s.r.o (Nitra, Slovakia). It was obtained by steam distillation of a flowering flower. The manufacturer states as the main components of the essential oil 1,8-cineole 38 – 55%, camphor 5 – 15%, α + β pinene 13 – 23%, limonene 1 – 4%, borneol 1 – 5%. The sample was stored in the cold (4 °C) and in the dark throughout the analyses.

### Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Germany), Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK), Muller Hinton agar (MHA, Oxoid, Basingstoke, UK).

### Animals and Biological Material:

Bacterial strains forming the biofilm of *Stenotrophomonas maltophilia* and *Bacillus subtilis* were obtained from the dairy industry in the Czech Republic. They were identified by 16S rRNA sequencing and MALDI-TOF MS Biotyper.

### Instruments

Glomax spectrophotometer (Promega Inc., Madison, USA), MALDI-TOF MS Biotyper (Bruker, Daltonics, Bremen, Germany).

### Laboratory Methods

To determine the antioxidant activity of *Rosmarinus officinalis* essential oil, 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich, Germany) was used according to the method of **Sánchez-Moreno, Larrauri and Saura-Calixto (1998)**. It is a colorimetric method in which the deep purple colour of DPPH changes to yellow after scavenging free radicals. The colour change is detected by a spectrophotometer. For essential oil analysis, a stock solution of DPPH was prepared by weighing 0.0025 g of DPPH into 100 mL of ethanol (96%). 3.9 mL of stock solution was pipetted into the tube and 0.1 mL of *R. officinalis* essential oil was added. The prepared mixture in triplicate was incubated at laboratory temperature in the dark place for 10 minutes. The absorbance of the sample was measured with Glomax spectrophotometer (Promega Inc., Madison, USA) at 515 nm and the average absorbance of the sample was calculated. The percentage of antioxidant activity was calculated according to the formula:

$$AA\% = [(A0 - AAT) / A0 \times 100]$$

Where:
A0 – is the absorbance of the control reaction (DPPH radical); AAT – is the absorbance of tested sample.

Minimal Biofilm Inhibitory Concentration (MBIC) was determined according to **Hassan et al. (2011)**. The bacterial suspensions were incubated in the Mueller Hinton Broth (MHB, Oxoid, Basingstoke, UK) under aerobic conditions for 24 h at 37 °C. After incubation, an inoculum with optical density of 0.5 McFarland standard was prepared. 100 µL MHB and 50 µL inoculum were pipetted into a 96-well microtiter plate. Subsequently, 100 µL of essential oil was added to the first column of the microplate. Mixing with a pipette gave a two-fold dilution with concentrations from 400 $\mu L.mL^{-1}$ to 0.195 $\mu L.mL^{-1}$. MHB with essential oil was used as a negative control and MHB with bacterial inoculum was used as maximal growth control. After culturing for 24 hours at 37 °C in an aerostat, the supernatant was discarded, the wells were washed three times with 250 µL of saline and allowed to dry for 30 minutes at laboratory temperature. After drying, the wells were stained with 200 µL crystal violet (0.1% w/v) for 15 minutes. The plates were repeatedly washed with distilled water and allowed to dry. Subsequently, 200 µL of 33% acetic acid was added to resolubilize the samples. Samples were measured on Glomax spectrophotometer (Promega Inc., Madison, USA) at 570 nm. The concentration at which the absorbance was lower or equal to the negative control was determined as MBIC.

### Description of the Experiment

### Sample preparation:

The analysis of the developmental stages of the biofilm and the evaluation of the molecular differences on the glass and the wood were performed in the same way as in **Kačániová et al. (2020a)** using MALDI-TOF MS Biotyper (Bruker, Daltonics, Bremen, Germany).

The antibiofilm activity of *R. officinalis* in a food model was analysed by a vapor phase antimicrobial assay. The antibiofilm effect was analysed on potato, carrot, and apple. Vegetables and fruit were cut into 5 mm slices and washed with distilled water. A layer of Muller Hinton agar (MHA, Oxoid, Basingstoke, UK) was poured into 60 mm Petri dishes and lids. After the agar solidification, one slice of the sample was placed on the plates. *S. maltophilia* and *B. subtilis* were applied to the samples by stabbing. The essential oil was diluted in ethyl acetate to final concentrations of 500, 250, and 125 $\mu g.mL^{-1}$. A circle of sterile 55 mm diameter filter paper was placed in the lid. 100 µL of the appropriate concentration of essential oil was pipetted onto the surface of the filter paper. The filter paper was allowed to dry for 1 minute to evaporate the ethyl acetate and the dishes were sealed. Petri dishes were incubated for 7 days at 37 °C. Inhibition of bacterial growth by the essential oil was expressed as a percentage of inhibition compared to the control, where the control represented 0% inhibition. Inhibition by more than 50% was considered effective.

**Number of samples analyzed:** biofilm18, food model 24
**Number of repeated analyses:** 3
**Number of experiment replication:** 3

### Statistical analysis

All analyses were performed in triplicate. Statistical variability of data was processed using Microsoft-Excel® software.

## RESULTS AND DISCUSSION

The antioxidant activity of *Rosmarinus officinalis* essential oil was 28.76% ±2.68%. **Wang et al. (2008)** in their work determined a free radical scavenging activity 62.45% ±3.42%. **Gachkar et al. (2007)** found out, that antioxidant activity is 69.30%. **Kasparavičienė et al. (2013)** determined the antioxidant activity of *R. officinalis* at 75.96% ±1.12%. **Hussain et al. (2010)** found out free radical scavenging activity 33.60%. **Okoh, Sadimenko and Afolayan (2011)** detected, that antioxidant activity is 48.80%. **Teneva et al. (2020)** compared the antioxidant activity of essential oils from the leaves and flowers of *R. officinalis*. They recorded antioxidant activity in range 25 – 82%. This variability was due to the different chemical composition of the essential oils. **Mohammed et al. (2020)** set the percentage of free radical scavenging at 44.50%. **Nie et al. (2020)** determined free radical scavenging activity 39.50%. **Amjadi et al. (2020)** determined the antioxidant activity of rosemary essential oil at 24.00% ±3.10%. **Kanth et al. (2018)** examined the antioxidant activity at concentrations range 1250 – 25000 ppm and found free radical scavenging activity 8.16 – 51.8%. The differences between the individual findings may be due to different chemical composition of the essential oils and the different concentrations of the active substances. The authors agree that *R. officinalis* essential oil has increased antioxidant activity, and our findings confirm this.

The minimal biofilm inhibiting concentrations determined by us were 25 µL.mL$^{-1}$ for *Stenotrophomonas maltophilia* and 12.5 µL.mL$^{-1}$ for *Bacillus subtilis*. **Vieira et al. (2017)** determined MBIC for *B. subtilis* 20 µL.mL$^{-1}$. **Elhariry et al. (2013)** determined MBIC of rosemary 12 µL.mL$^{-1}$ for the genera *Bacillus* and *Pseudomonas.* **Jardak et al. (2017)** investigated the antibiofilm activity against *S. epidermidis* and recorded an effect at oncentration 25 µL.mL$^{-1}$. **Kanth et al. (2018)** determined the MBIC for the biofilm of *L. monocytogenes* and *S. aureus* at 1.25 µL.mL$^{-1}$. **Quave et al. (2008)** tested the antibiofilm effect of rosemary on *S. aureus* and determined MBIC 8 µL.mL$^{-1}$. **Ceylan et al. (2014)** evaluated the antibiofilm activity of rosemary on *S. aureus* and determined MBIC 1.25 µL.mL$^{-1}$. **Nasr-Eldin, Abdelhamid and Baraka (2017)** focused on the antibiofilm effect of essential oil on *S. aureus* and found MBIC 10 µL.mL$^{-1}$. **Rahnama et al. (2019)** tested antibiofilm activity on *B. cereus* with MBIC 5 µL.mL$^{-1}$. **Miladi et al. (2016)** determined MBIC 25 µL.mL$^{-1}$ for the genus Salmonella in their work. All authors agree that *Rosmarinus officinalis* essential oil has significant antibiofilm effect. Different minimum inhibitory concentrations are due to different origins of essential oils with different chemical compositions and because they were tested on different strains of bacteria.

Analysis of the mass spectra of *Stenotrophomonas maltophilia* showed that on the third day of the experiment (Figure 1A), the similarity between the experimental spectra (wood and glass) and the control planktonic spectrum was maintained. On the fifth day of the experiment (Figure 1B), we recorded a gradual change in the experimental spectra compared to the control planktonicspectrum. The same trend was observed on the seventh day of the experiment (Figure 1C). On the ninth day of the experiment (Figure 1D) there was a decrease in the number of peaks in the experimental groups compared to the planktonic spectrum. The decrease was maintained on the twelfth day (Figure 1E) as well as the difference compared to the planktonic spectrum. On the last day (Figure 1F) of the experiment, there was an increase in the number of peaks in the experimental group compared to days 9 and 12, while maintaining the difference from the planktonic spectrum. The results show the inhibitory effect of the essential oil *Rosmarinus officinalis* on the structure of the biofilm.

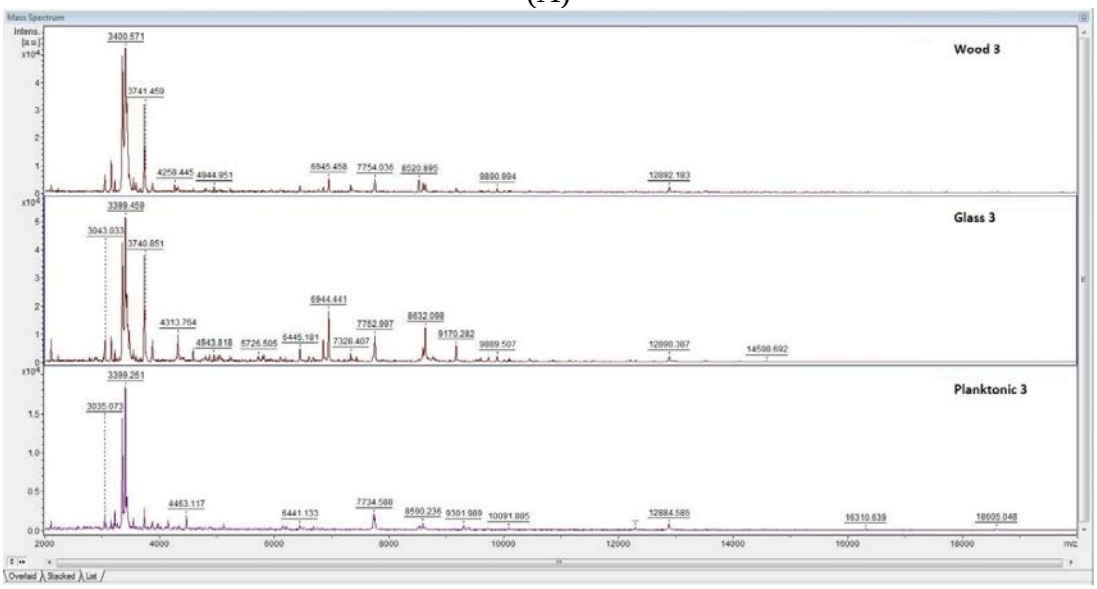
The *S. maltophilia* dendrogram (Figure 2) is divided into two main clusters and 5 subclusters. In the constructed dendrogram, it is possible to observe the grouping primarily to the time point of view of the analysed samples. The planktonic spectrum showed the most significant relationship with the experimental group on day 3 of the experiment in MSP distance. The control groups showed shorter MSP distances, and thus higher similarity of spectra from planktonic cells than the experimental groups in the following days. This finding confirms the inhibitory effect of *R. officinalis* essential oil.

Analysis of the mass spectra of *Bacillus subtilis* showed that on days 3 and 5 of the experiment (Figures 3A and 3B), the similarity between the experimental and planktonic spectra was maintained. On day 7 of the experiment, the difference between the experimental and control groups began to show (Figure 3C). On the ninth and twelfth days of the experiment (Figures 3D and 3E), the change in experimental spectra compared to the control planktonic spectrum continued. On the last day of the experiment (Figure 3F), the most significant difference between the experimental and control groups was noted. These findings confirm the inhibitory effect of the essential oil on the structure of the *B. subtilis* biofilm.

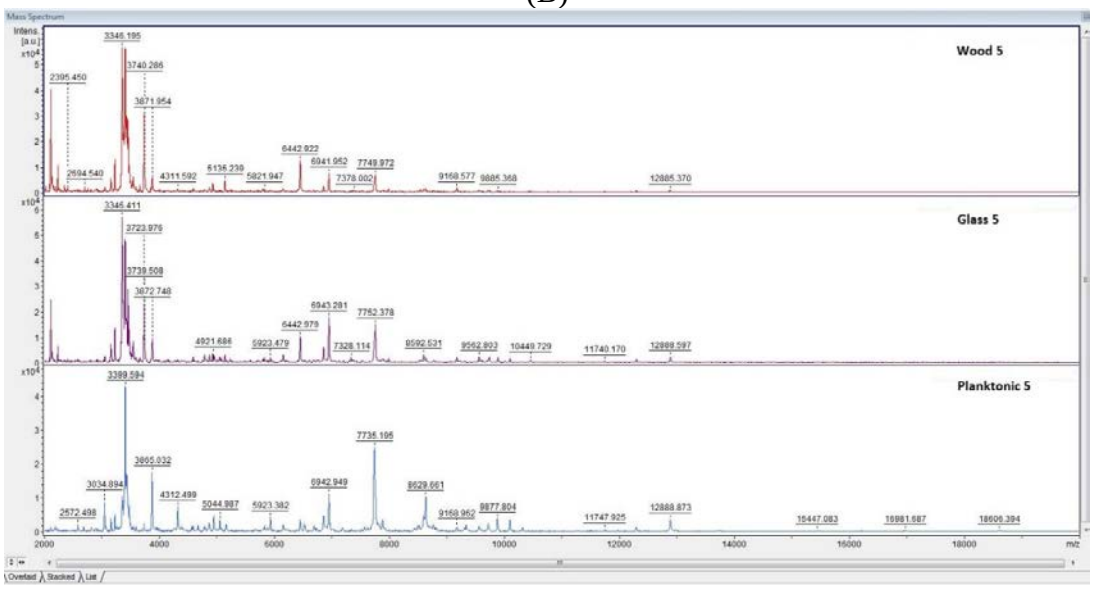
The *B. subtilis* dendrogram (Figure 4) consists of two main clusters, and it is possible to observe that analysed samples were grouped primarily according to time progression. The most significant similarity of the planktonic spectrum with the experimental group was recorded on days 3 and 5 of the experiment. All control groups were in the same cluster as the planktonic cells. Experimental groups from day 7 were divided into a separate cluster. This finding confirms the inhibitory effect of *R. officinalis* from day 7 of the experiment.

**Pereira et al. (2015)** report that profiling with MALDI-TOF MS Biotyper is a very useful tool. Their results showed that the MALDI-TOF MS Biotyper approach is sufficiently sensitive to detect phenotypic changes in biofilm progression and can detect differences in biofilms cultured on different surfaces. **Lo and Chang (2014)** stated that the MALDI-TOF MS Biotyper is suitable for the investigation and identification of clinical isolates, including biofilm-forming ones. **Gaudreau et al. (2018)** found that the MALDI-TOF MS Biotyper is useful for biofilm studies. **Hasan, Gopal and Wu (2011)** confirm the suitability of the MALDI-TOF MS Biotyper method for biofilm analysis. **Kačániová et al. (2020a)** analysed the effect of *Coriandrum sativum* essential oil on the inhibition of *S. maltophilia* and *B. subtilis* biofilm with positive effect.

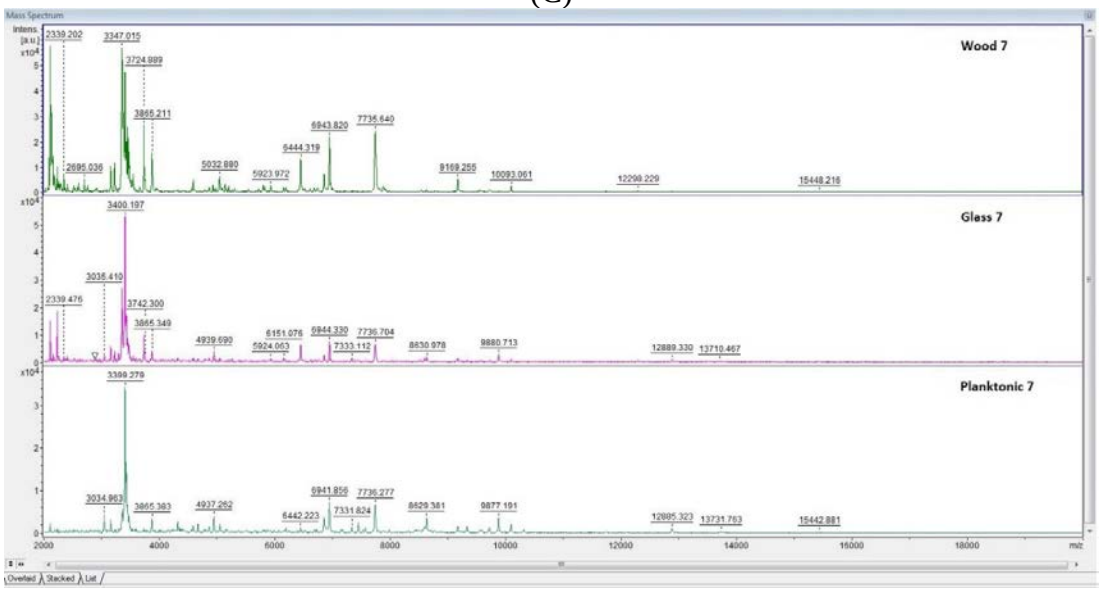
(A)


Wood 3
Glass 3
Planktonic 3

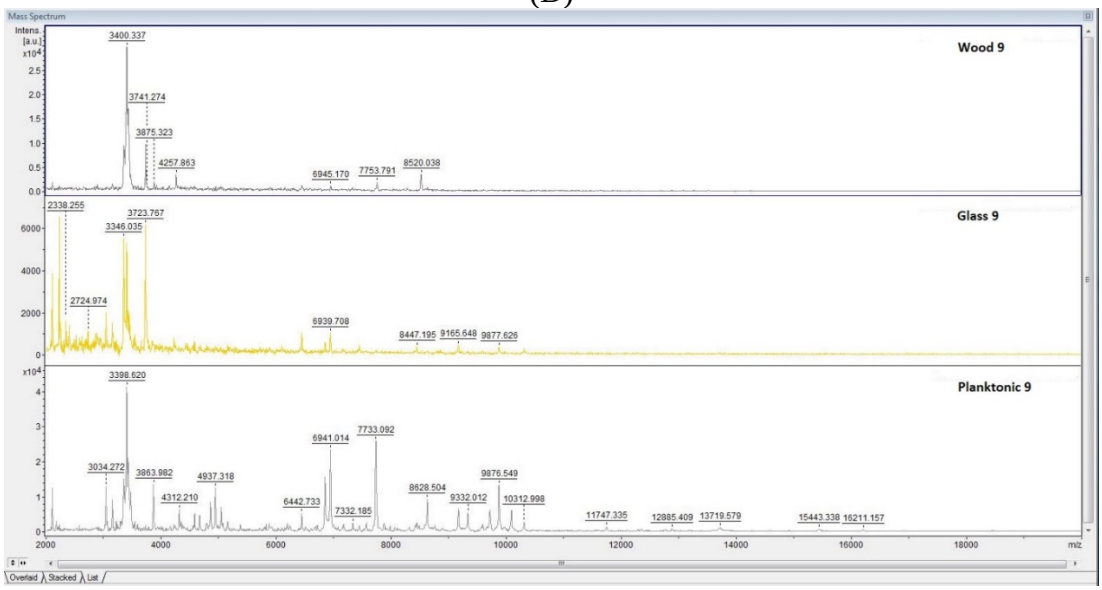
(B)


Wood 5
Glass 5
Planktonic 5

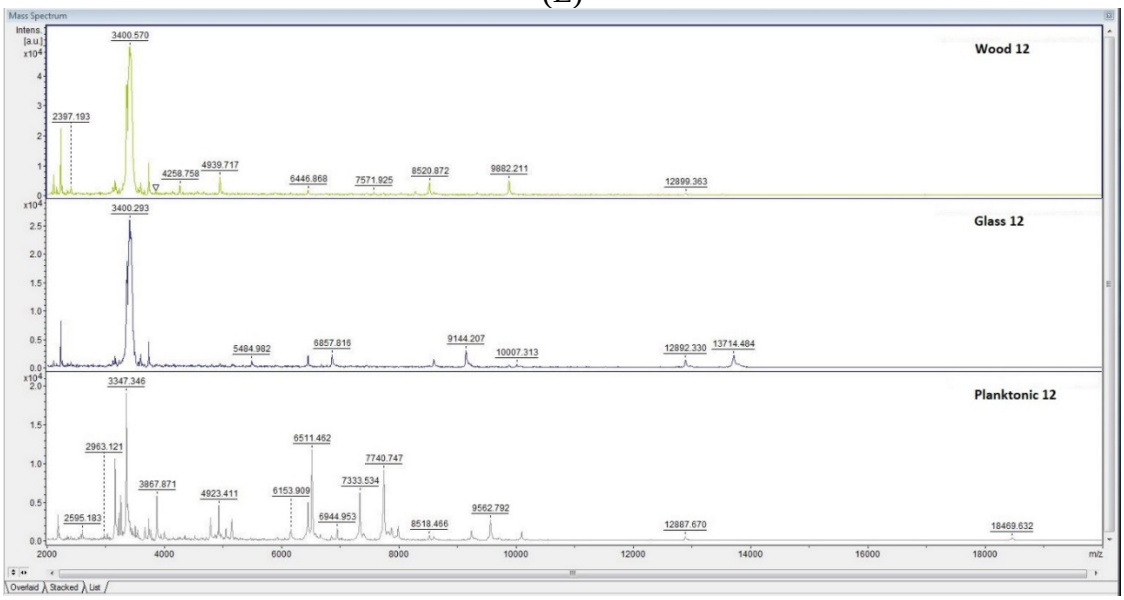
(C)


Wood 7
Glass 7
Planktonic 7

(D)




(E)




(F)

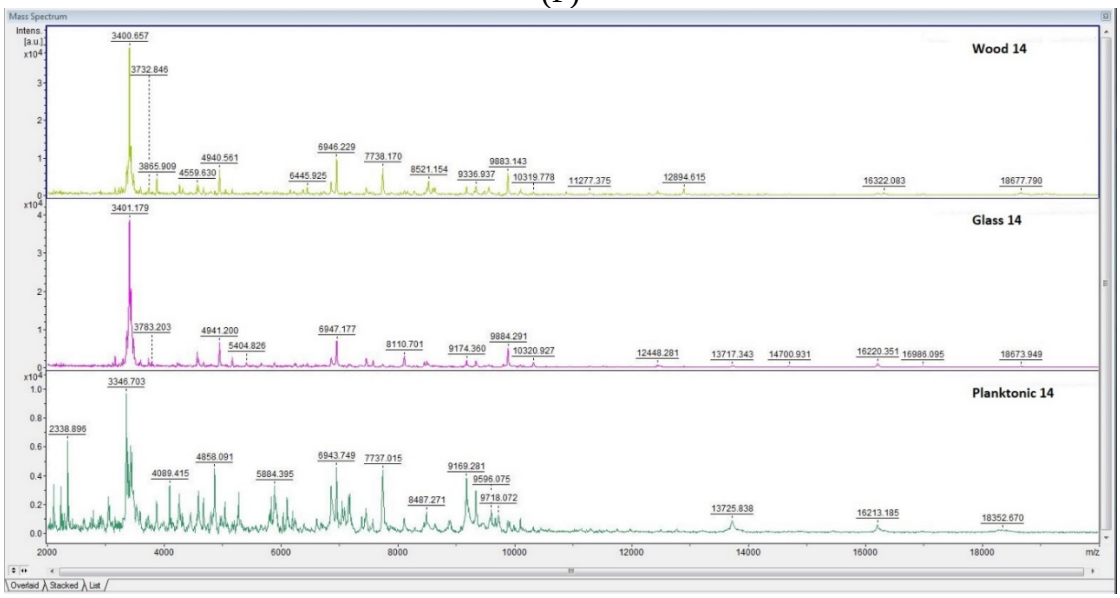



**Figure 1** Representative MALDI-TOF mass spectra of *S. maltophilia*: (A) 3 days; (B) 5 days; (C) 7 days; (D) 9 days; (E) 12 days; and (F) 14 days.

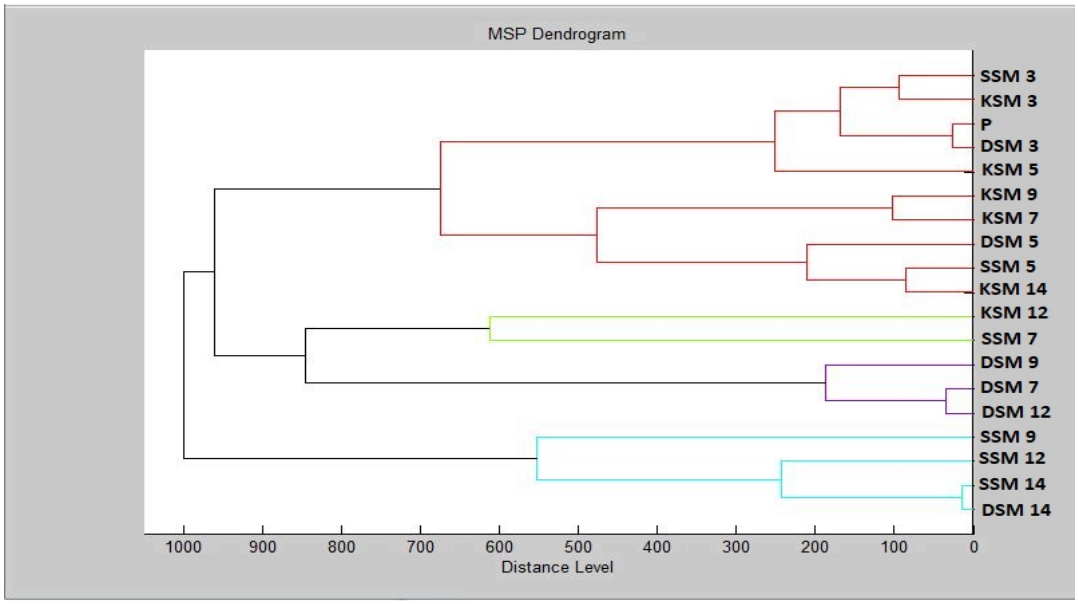
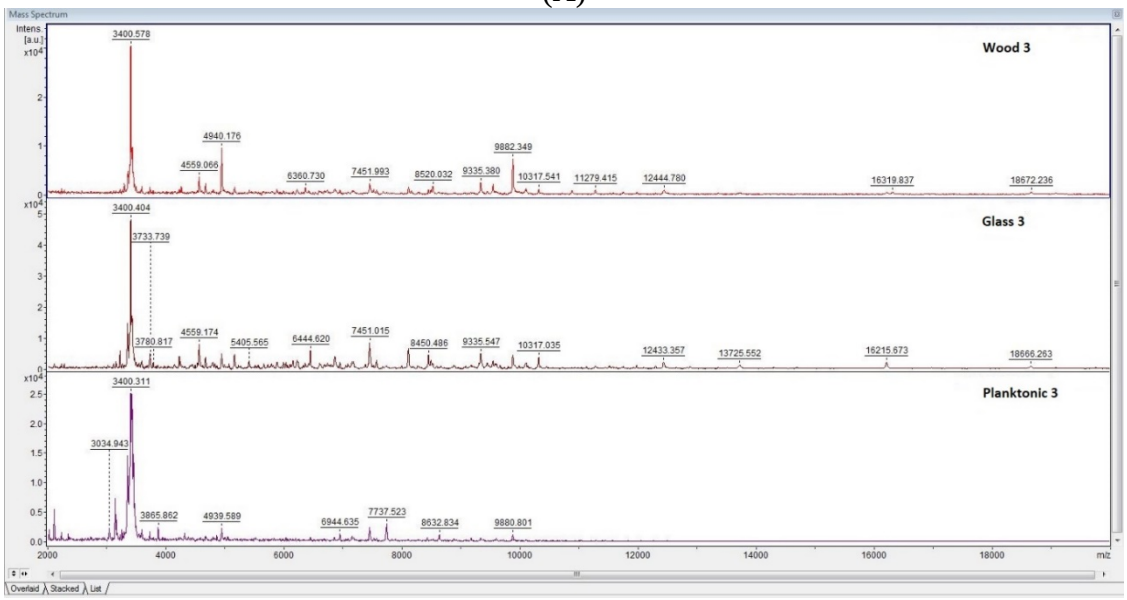



**Figure 2** Dendrogram of *S. maltophilia* generated using the MSPs for all experimental group: SM – *S. maltophilia*; K – control; S – glass; D – wood; P – planktonic cell.

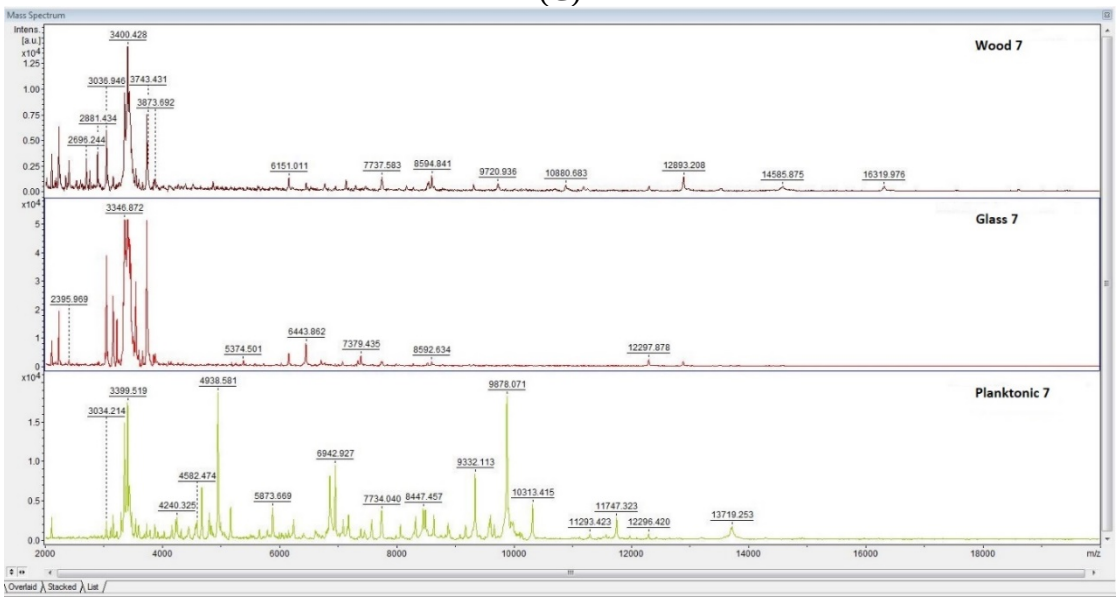
(A)



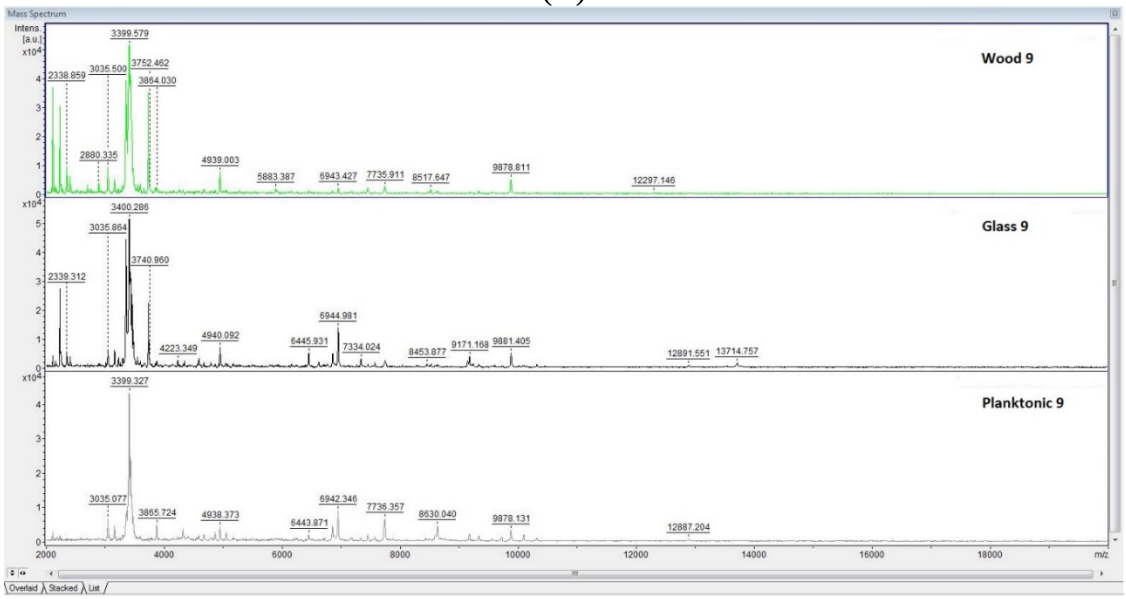

(B)



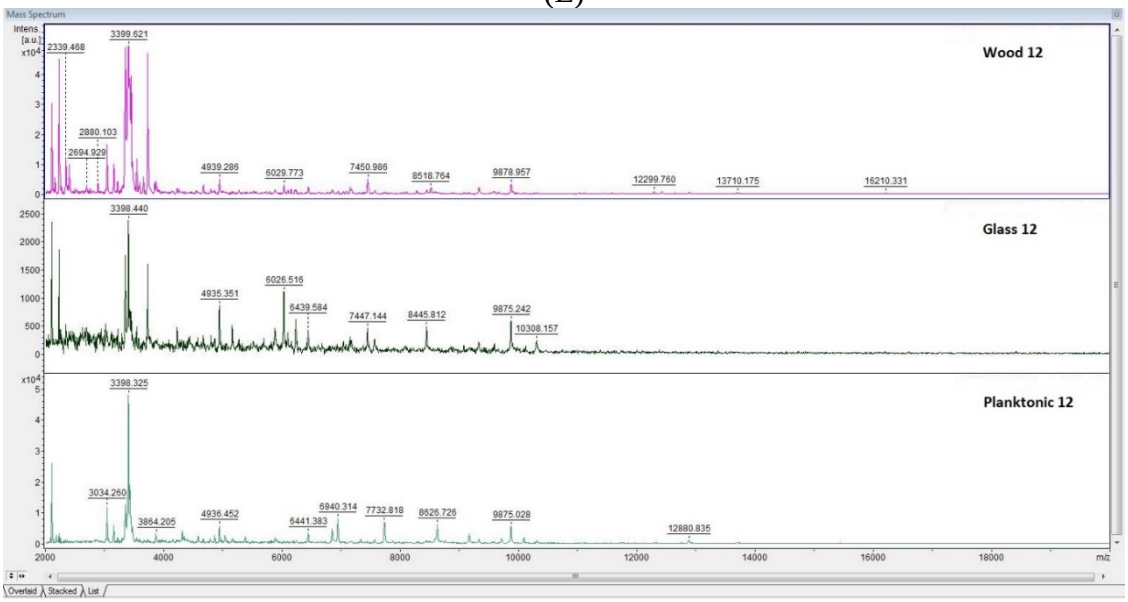
(C)


Mass Spectrum
Wood 7
Glass 7
Planktonic 7
m/z

(D)


Mass Spectrum
Wood 9
Glass 9
Planktonic 9
m/z

(E)


Mass Spectrum
Wood 12
Glass 12
Planktonic 12
m/z

(F)

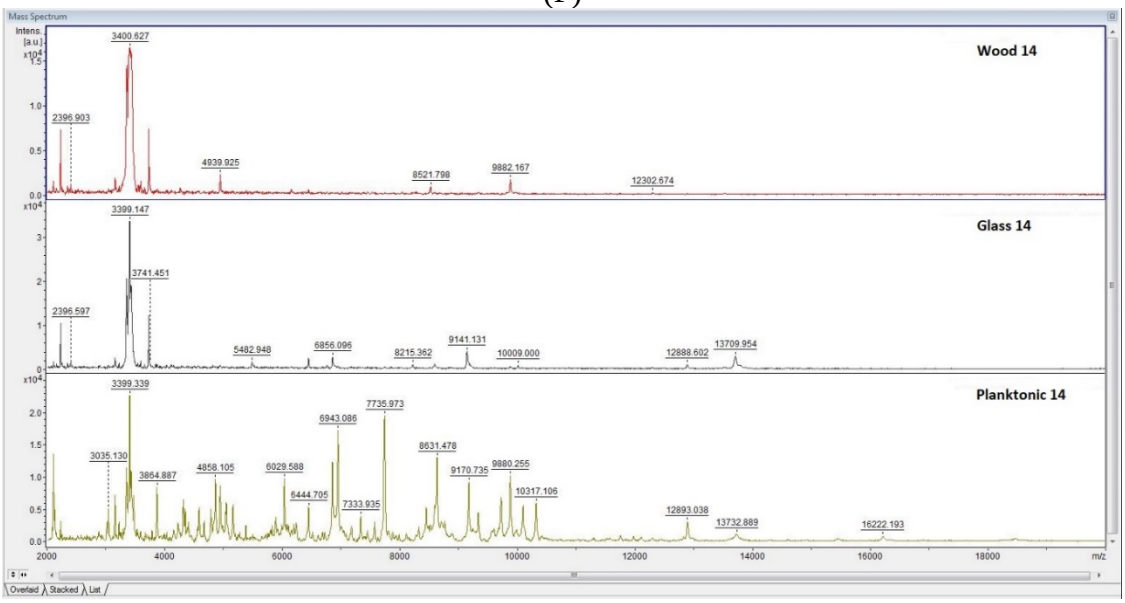



**Figure 3** Representative MALDI-TOF mass spectra of *B. subtilis*: (A) 3 days; (B) 5 days; (C) 7 days; (D) 9 days; (E) 12 days; and (F) 14 days.

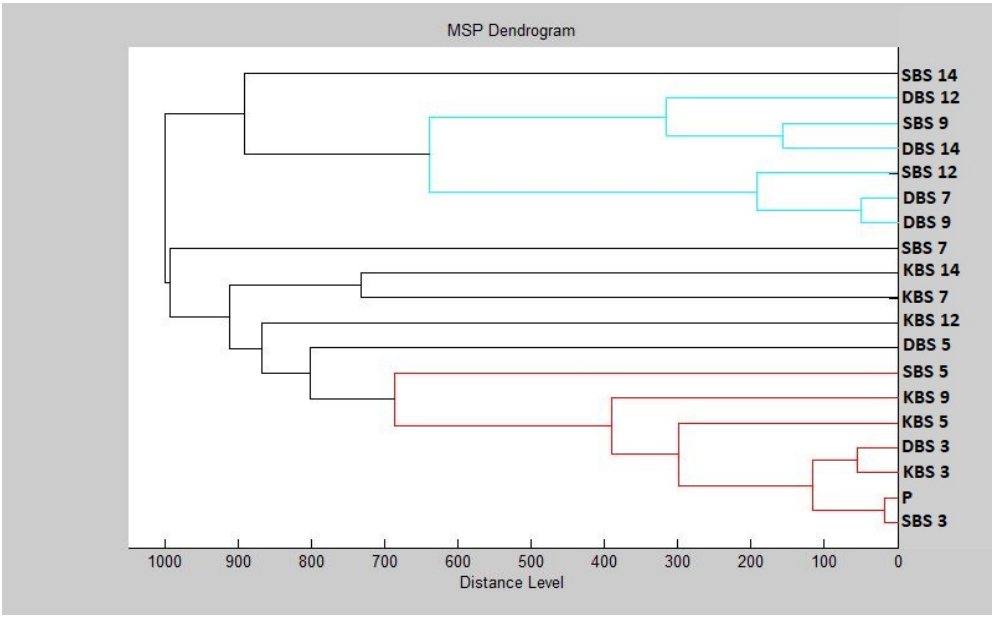



**Figure 4** Dendrogram of *B. subtilis* generated using the MSPs for all experimental group: BS – *B. subtilis*; K. – control; S – glass; D – wood; P – planktonic cell.

**Table 1** In situ antimicrobial analyses of vegetables and fruit with S. maltophilia in vapor phase with essential oil *R. officinalis*.

| Concentration of EO / Food model | 125 µL.L$^{-1}$ | 250 µL.L$^{-1}$ | 500 µL.L$^{-1}$ |
|---|---|---|---|
| Carrot | 50.78 ±2.59 | 69.82 ±3.21 | 87.80 ±1.41 |
| Potato | 24.36 ±2.11 | 47.44 ±3.56 | 79.92 ±1.27 |
| Apple | 42.56 ±2.28 | 53.46 ±1.41 | 85.45 ±1.69 |

**Table 2** In situ antimicrobial analyses of vegetables and fruit with B. subtilis in vapor phase with essential oil *R. officinalis*.

| Concentration of EO / Food model | 125 µL.L$^{-1}$ | 250 µL.L$^{-1}$ | 500 µL.L$^{-1}$ |
|---|---|---|---|
| Carrot | 20.39 ±1.21 | 39.73 ±2.67 | 67.42 ±1.32 |
| Potato | 62.53 ±2.52 | 74.46 ±2.71 | 92.48 ±1.83 |
| Apple | 64.56 ±1.33 | 73.82 ±1.19 | 89.35 ±1.87 |

In another work, **Kačániová et al. (2020b)** addressed the antibiofilm effect of *Citrus aurantium* essential oil by using MALDI-TOF MS Biotyper with positive result.

The results of the analysis of antibiofilm activity in the food model show that the essential oil of *R. officinalis* showed an inhibitory effect. The essential oil inhibited the growth of *S. maltophilia* on carrots by more than 50% (Table 1) at concentration 250 μg.mL$^{-1}$. The effect was observed on potato at 500 μg.mL$^{-1}$ and apple at concentration 250 μg.mL$^{-1}$. The essential oil inhibited the growth of *B. subtilis* by more than 50% (Table 2) on carrots at concentration 500 μg.mL$^{-1}$. The effect on potato and apple was already manifested at concentration 125 μg.mL$^{-1}$. **Laird and Phillips (2011)** report that vapor phase essential oils are effective antimicrobial systems and have advantages over the use of liquid phase essential oils. **Kačániová et al. (2020b)** used this method in their work to determine the antifungal activity of the essential oil *Citrus aurantium*.

## CONCLUSION

The work confirmed the antioxidant potential of *Rosmarinus officinalis* essential oil using the DPPH method. The findings suggest that the essential oil has significant effect on biofilm inhibition. This effect was confirmed by the change in biofilm structure recorded by the MALDI-TOF MS Biotyper and by vapor phase inhibition in a food model. It is believed that the effect of the essential oil is to disrupt the polysaccharide structure of the biofilm and consequently reduce the resistance of the biofilm. MALDI-TOF Biotyper is a suitable tool for evaluating changes in biofilm structure. It could find more significant application for the study of biofilms in the food and clinical practice.

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The authors declare no conflict of interest.

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# THE CONTENT OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF GARLIC (*ALLIUM SATIVUM* L.)

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## ABSTRACT

Garlic (*Allium sativum* L.) is one of the most commonly grown vegetables and thanks to its sensory properties, it has an important place in numerous world cuisines. Garlic is also known for its health-promoting properties, which are attributed to its chemical composition. The health benefits of garlic depending on the content of biologically active compounds, which vary between cultivars and geographical areas. Seven cultivars of garlic, namely Sukoradsky, Zahorsky, Germidour, Mojmir, Karel IV., Arkus, Makoi, were analyzed in this study. This study aimed to determine the total polyphenol content, total flavonoid content, and antioxidant activity of garlic. Studied characteristics were analyzed by the UV-VIS spectrometry method. Total polyphenol content ranged from 430.26 to 640.04 mg GAE.kg$^{-1}$ FW. Total flavonoid content ranged from 10.29 to 60.49 mg CE.kg$^{-1}$ FW. Antioxidant activity measured by ABTS assay ranged from 1.098 to 1.955 mmol TE.kg$^{-1}$ FW. Antioxidant activity measured by FRAP assay ranged from 0.63 to 1.467 mmol.kg$^{-1}$ FW. Highest TPC, TFC, and AA were determined in the cultivar Mojmir. The lowest TPC and TFC were determined in the cultivar Zahorsky. The lowest AA was determined in the cultivar Sukoradsky. High positive correlations were determined between individual parameters.

**Keywords:** *Allium sativum*; garlic; polyphenols; flavonoids; antioxidants

## INTRODUCTION

The genus Allium includes the most widespread and commonly grown vegetables such as onion (*Allium cepa* L.), garlic (*Allium sativum* L.), leek (*Allium porrum* L.), and many others. All these species are very important for agriculture, gastronomy, and the food industry. This genus includes plants with a very specific aroma and taste, for which sulfur-containing phytochemicals are responsible. Substances in these species have a positive effect on human health **(Poojary et al., 2017)**. Allium vegetables have maintained a leading position in cultivation throughout the centuries. All these species are characterized by a content of valuable components with significant beneficial effects on human health. In addition to their popularity in the therapeutic area, they have an important place in almost every kitchen. From a nutritional point of view, it contains important components such as chemoprotective substances, which also include natural plant antioxidants. They also contain a wide range of vitamins and trace elements that are essential for the proper functioning of the body **(Hedges and Lister, 2007; Choi et al., 2011)**. The genus *Allium* is rich in flavonoids, saponins, sapogenins, and volatile sulfur compounds. Due to the content of rare phytochemicals, Allium plants have several positive effects on human health, including antimicrobial, antidiabetic, antioxidant, antiviral, anticarcinogenic, antimutagenic, hepatoprotective, and neuroprotective. Since ancient times, these plants have been used in the treatment of cardiovascular diseases, inflammatory diseases, vascular diseases, elevated blood cholesterol levels, and various degenerative diseases **(Putnik et al., 2019)**. Plant species belonging to the genus Allium are characterized by their pungent taste and significant medicinal properties. Individual plant species differ mainly in morphological structure, taste, and color. In terms of chemical composition, plants belonging to the genus Allium are similar **(Lorigooini et al., 2014)**.

Garlic (*Allium sativum* L.) is a monocotyledonous species belonging to the *Alliaceae* family and is one of the oldest traditional crops in the world **(Khan et al., 2017)**. It is one of the most common types of vegetables and is an important part of many world cuisines. Garlic is used mainly for its sensory properties, but also for its health-promoting properties, which are attributed to its chemical composition. The health benefits of garlic depending on the content of biologically active compounds, which vary between cultivars and geographical areas **(Szychowski et al., 2018)**. The chemical composition of garlic is significantly affected by the cultivar, growing conditions, and cultivation practices. In particular, the fertilization regime and soil properties can have a significant effect on quality properties

such as mineral composition, dry matter, protein content, and total soluble solids content. Garlic is considered a rich source of volatile compounds, which are responsible for the typical taste and bioactive properties of garlic. There is also a high content of non-volatile compounds with well-known medicinal and therapeutic properties, such as amides, nitrogen oxides, proteins, saponins as well as antioxidants, minerals (especially P, K, and Se), vitamins (vitamin C and B vitamins), and phenolic compounds, especially flavonoids and phenolic acids **(Petropoulos et al., 2018)**. Garlic is characterized by a high content of polyphenolic compounds that have a positive effect on the human body. Phenolic acids and flavonoids account for the largest proportion of polyphenolic compounds in garlic. The total content of polyphenols in garlic can be influenced by variety, environmental influences (sun exposure, precipitation, different types of cultivation, fruit yield), but also by the storage and technological processing of garlic **(Srivastava et al., 2013; Rasouli et al., 2017)**.

Polyphenol compounds have been identified as nutrients, plant secondary metabolites, phytonutrients, antioxidants, dietary bioactive agents, and protective factors **(Cory et al., 2018)**. Polyphenols form one of the most important groups of secondary metabolites of plants. They are widespread in the plant kingdom and can be obtained directly from plants and other foods rich in antioxidants, gaining worldwide attention as nutraceuticals in the prevention of multiple diseases. The health and antitumor effects associated with regular consumption of foods rich in polyphenols are an effective therapy for maintaining health against reactive oxygen species **(Losada-Barreiro and Bravo-Díaz, 2017)**. Polyphenol compounds are partly responsible for determining the sensory and nutritional characteristics of foods. They represent a wide variety of different pigments, are involved in attracting pollinators, performing structural functions, protecting against ultraviolet radiation, and protecting plants from microbial invasion and herbivores **(Cutrim and Cortez, 2018)**. The availability of polyphenols depends primarily on their bioavailability. The rate of absorption in the intestines depends on their chemical structure **(Abbas et al., 2017)**.

Polyphenol compounds are potent antioxidants. Unlike several sulfur compounds in garlic, polyphenols are more stable and can be extracted from fresh, frozen, or dried plant samples. The content of biologically active compounds in garlic varies between cultivars grown in different geographical areas **(Szychowski et al., 2018)**. Given the unique combination of bioactive compounds, representatives of the genus *Allium* should be a regular part of our diet. Garlic has a wide range of applications as an antioxidant, antifungal, antithrombotic and hypoglycemic agent. It reduces glucose metabolism in diabetics, slows the development of arteriosclerosis, and reduces the risk of the development of various types of cancer. In addition, it reduces the possibility of myocardial infarct in patients and could also improve the function of the immune system **(Suleria et al., 2015)**.

## Scientific hypothesis

Garlic is a natural source of polyphenols and antioxidants. The concentration of polyphenols in garlic is cultivar dependant.

## MATERIAL AND METHODOLOGY

### Samples

Seven cultivars of garlic, namely Sukoradsky, Zahorsky, Germidour, Mojmir, Karel IV., Arkus, and Makoi, were conventionally cultivated and harvested in full ripeness.

### Chemicals

Folin-Ciocalteau reagent (Merck, Germany), anhydrous natrium carbonate p.a. (Sigma-Aldrich, USA), monohydrate of gallic acid p.a., (Sigma-Aldrich, USA), aluminium chloride p.a., (Sigma-Aldrich, USA), sodium nitrite p.a., (Sigma-Aldrich, USA), sodium hydroxide p.a., (Sigma-Aldrich, USA), catechin hydrate 98%, (Sigma-Aldrich, USA), methanol p.a., 80% (Sigma-Aldrich, USA), Trolox (2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich, USA), ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic) acid, Sigma-Aldrich, USA), potassium persulfate p.a. (Sigma Aldrich, USA), acetic acid p.a. (CH3COOH, Sigma Aldrich, USA), sodium acetate p.a. (COONa, Sigma Aldrich, USA) , TPTZ (2,4,6-tri (2-pyridyl) -s-triazine, Sigma-Aldrich, USA), hydrochloric acid (HCl, Sigma Aldrich, USA) , ferric chloride p.a. ($FeCl_3$), distilled water

### Biological material

Garlic (*Allium sativum* L.)

### Instruments

Unimax 2010 Horizontal Shaker (Heidolph Instrument, GmbH, Germany).

Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer (Shimadzu, Japan).

### Laboratory Methods

Folin – Ciocalteau method was modified by **Lachman et al. (2003)**.

Aluminium chloride method was modified by **Chang et al. (2002)**.

ABTS radical scavenging assay **(Re et al., 1999)**.

FRAP assay by **(Pedersen et al., 2000)**.

### Description of the Experiment

**Sample preparation:** To prepare extracts, 25 g of homogenized peeled garlic bulbs were shaken in 50 mL of 80% methanol for 16 hours on the Unimax 2010 horizontal shaker (Heidolph Instrument, GmbH, Germany), and filtered through Munktell No. 390 filtrating paper (Munktell and Filtrac, Germany).

**Number of samples analyzed:** 7

**Number of repeated analyses:** 4

**Number of experiment replication:** 1

## Total polyphenol content

Total polyphenol content was determined by the method of **Lachman et al**. **(2003**), using Folin – Ciocalteau phenol reagent, 20% $Na_2CO_3$, and distilled water. 0.1 mL of extract was pipetted into a 50 mL volumetric flask and diluted with distilled water. Then, 0.85 mL of Folin – Ciocalteau reagent was added, and after 3 minutes, 5 mL of 20% $Na_2CO_3$ was added. Volume was made up to 50 mL with distilled water. and left at laboratory temperature for 2 hours. Absorbance was measured against blank solution at 765 nm, using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer, Shimadzu, Japan.

Total polyphenol content was expressed as mg of gallic acid equivalent in 1 kg of fresh garlic, based on the calibration curve ($R^2 = 0.9948$).

### Total flavonoid content

Total flavonoid content was determined by the aluminium chloride method **(Chang et al., 2002)**, using 5% $NaNO_2$, 10% $AlCl_3$, 1M NaOH, and distilled water. 1 mL of extract was pipetted into a 10 mL volumetric flask and diluted with 5 mL of distilled water. Then, 0.3 mL of 5% $NaNO_2$ was added. After 6 minutes, 0.6 mL of 10% $AlCl_3$ was added. After 5 minutes, 2 mL of 1M NaOH was added. After mixing on vortex, volume was made up to 10 mL with distilled water and left at laboratory temperature for 15 minutes. Absorbance was measured against blank solution at 510 nm, using Shimadzu UV-1800 UV/ Visible Scanning Spectrophotometer, Shimadzu, Japan.

Total flavonoid content was expressed as mg of catechin equivalent in 1 kg of fresh garlic, based on the calibration curve ($R^2 = 0.9981$).

### Antioxidant activity using ABTS assay

Antioxidant activity was determined by the ABTS assay **(Re et al., 1999)**, using $ABTS^{\bullet+}$ radical cation - (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate ($K_2S_2O_8$), and acetate buffer (pH = 4.3) Working ABTS solution was produced by the reaction of $ABTS^{\bullet+}$ solution, $K_2S_2O_8$ solution, and acetate buffer. 0.05 mL of extract was pipetted into 3 mL of working ABTS solution, stirred, and left at laboratory temperature for 20 minutes. Absorbance was measured against blank solution at 734 nm, using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer, Shimadzu, Japan.

Antioxidant activity was expressed as mmol of Trolox equivalent in 1 kg of fresh garlic, based on the calibration curve ($R^2 = 0.9931$).

### Antioxidant activity using FRAP assay

Antioxidant activity was determined by the FRAP assay **(Pedersen et al., 2000)**, using TPTZ - (2,4,6-tris(2-pyridyl)-S-triazine), ferric chloride ($FeCl_3$), and acetate buffer (pH = 3.5) Working FRAP solution was produced by the reaction of TPTZ solution, $FeCl_3$ solution, and acetate buffer. 0.05 mL of extract was pipetted into 3 mL of working FRAP solution, stirred, and left at laboratory temperature for 20 minutes. Absorbance was measured against blank solution at 593 nm, using Shimadzu UV-1800 UV/Visible Scanning Spectrophotometer, Shimadzu, Japan.

Antioxidant activity was expressed as mmol of Trolox equivalent in 1 kg of fresh garlic, based on the calibration curve ($R^2 = 0.9956$).

### Statistical Analysis

The **RStudio (2020)** software package was used to perform statistical analysis. A nonparametric Kruskal-Wallis test was performed to obtain statistically significant information about the differences among the tested samples ($p$ <0.05). Each variety was compared with the median value using the Wilcoxon test. To determine the relationship between individual parameters, linear regression and Spearman's correlation test ($\alpha = 0.05$) were performed.

## RESULTS AND DISCUSSION

Total polyphenol content, total flavonoid content, and antioxidant activity of garlic cultivars are given in Table 1 and Table 2.

### Total polyphenol content

The total polyphenol content in studied garlic cultivars ranged from 430.26 to 640.04 mg GAE.kg$^{-1}$ FW (966.01 to 1365.56 mg GAE.kg$^{-1}$ DM), with a mean value of 505.32 mg GAE.kg$^{-1}$ FW (1099.83 mg GAE.kg$^{-1}$ DM). Highest TPC was determined in the cultivar Mojmir, while the lowest TPC was determined in the cultivar Zahorsky.

Based on their TPC, an order for garlic cultivars could be as following: Zahorsky< Sukoradsky< Germidour< Arkus< Karel IV.< Makoi< Mojmir.

Similar values were determined by other authors. **Lenková et al. (2016)** reported TPC in garlic cultivar Mojmir 1051 mg GAE.kg$^{-1}$ FW. **Lenková et al. (2017)** reported TPC in garlic cultivars in the range from 621.13 to 763.28 mg GAE.kg$^{-1}$ FW. (In Zahorsky – 621.13, in Makoi – 678.18, in Mojmir – 698.82 mg GAE.kg$^{-1}$ FW). **Lenková et al. (2018)** reported TPC in garlic cultivars in the range from 566.01 to 612.23 mg GAE.kg$^{-1}$ FW. (In Mojmir – 612.23, in Makoi – 612.21, in Zahorsky – 577.68, in Lukan – 566.01 mg GAE.kg$^{-1}$ FW). **Kavalcová et al. (2014)** reported TPC in garlic cultivars in the range from 260.62 to 279.74 mg GAE.kg$^{-1}$ FW. (In Mojmir – 260.62 mg GAE.kg$^{-1}$ FW). **Kovarovič et al. (2017)** determined TPC in garlic cultivar Dukat 600.30 mg GAE.kg$^{-1}$ FW. **Micová et al. (2019)** reported TPC in garlic cultivars in the range from 635.1 to 742.0 mg GAE.kg$^{-1}$ FW. (In Zahorsky – 665.2, in Makoi – 635.1, in Mojmir – 742.0 mg GAE.kg$^{-1}$ FW). **Bystrická et al. (2018)** determined TPC in garlic cultivars in the range from 401.25 to 595.00 mg GAE.kg$^{-1}$ FW. **Najman et al. (2020)** reported TPC in garlic in the range from 562 to 728 mg GAE.kg$^{-1}$ DM. **Khalid et al. (2014)** reported 390 mg GAE.kg$^{-1}$ FW. **Khan et al. (2016)** reported 408 mg GAE.kg$^{-1}$ FW.

Higher TPC was determined by **Beato et al. (2011)** – 6500 mg GAE.kg$^{-1}$ DM, and **Zhou et al. (2017) –** 2719.3 mg GAE.kg$^{-1}$ FW. **Škrovánková et al. (2018)** determined TPC in garlic cultivars in the range from 922 to 1196 mg GAE.kg$^{-1}$ FW. **Kim et al. (2013)** determined lower TPC in garlic – 105.73 mg GAE.kg$^{-1}$ DM.

### Total flavonoid content

The total flavonoid content in studied garlic cultivars ranged from 10.3 to 60.49 mg CE.kg$^{-1}$ FW (23.12 to 129.05 mg CE.kg$^{-1}$ DW), with a mean value of 24.75 mg CE.kg$^{-1}$ FW (53.65 mg CE.kg$^{-1}$ DW).

Highest TFC was determined in the cultivar Mojmir, while the lowest TFC was determined in the cultivar Zahorsky.

Based on their TFC, an order for garlic cultivars could be as following: Zahorsky< Germidour< Sukoradsky< Arkus< Karel IV.< Makoi< Mojmir.

Similar values were determined by other authors. **Chun et al. (2005)** reported 54.3 mg CE.kg$^{-1}$ FW in garlic. **Priecina and Karlina (2013)** determined 89 mg CE.kg$^{-1}$ DM in garlic. **Bhandari et al. (2014)** determined TFC in garlic in the range from 100 to 219 mg CE.kg$^{-1}$ DM, with a mean value of 161 mg CE.kg$^{-1}$ DM.

**Table 1** Total polyphenol content, total flavonoid content, and antioxidant activity of garlic (in fresh weight).

| Cultivar | TPC (mg GAE.kg$^{-1}$ ±SD) | TFC (mg CE.kg$^{-1}$ ±SD) | AA ABTS (mmol TE.kg$^{-1}$ ±SD) | AA FRAP (mmol TE.kg$^{-1}$ ±SD) |
|---|---|---|---|---|
| **Sukoradsky** | 456.15 ±5.93 | 12.73 ±0.61 | 1.098 ±0.021 | 0.63 ±0.017 |
| **Zahorsky** | 430.26 ±3.65 | 10.29 ±0.37 | 1.23 ±0.037 | 0.737 ±0.018 |
| **Germidour** | 461.22 ±5.91 | 10.86 ±0.43 | 1.148 ±0.011 | 0.72±0.025 |
| **Mojmir** | 640.04 ±5.75 | 60.49 ±0.72 | 1.955 ±0.015 | 1.467 ±0.018 |
| **Karel IV.** | 506.40 ±4.90 | 26.78 ±0.72 | 1.36 ±0.013 | 0.888 ±0.21 |
| **Arkus** | 503.76 ±4.32 | 20.60 ±0.43 | 1.203 ±0.018 | 0.791 ±0.016 |
| **Makoi** | 539.41 ±5.36 | 31.46 ±0.61 | 1.463 ±0.022 | 1.001 ±0.025 |
| **Mean** | 505.32 ±66.06 | 24.75 ±16.74 | 1.351 ±0.278 | 0.891 ±0.266 |

Note: TPC – total polyphenol content, TFC – total flavonoid content, AA ABTS – antioxidant activity measured by ABTS assay, AA FRAP – antioxidant activity measured by FRAP assay.

**Table 2** Total polyphenol content, total flavonoid content, and antioxidant activity of garlic (in dry matter).

| Cultivar | TPC (mg GAE.kg$^{-1}$ ±SD) | TFC (mg KE.kg$^{-1}$ ±SD) | AA ABTS (mmol TE.kg$^{-1}$ ±SD) | AA FRAP (mmol TE.kg$^{-1}$± SD) |
|---|---|---|---|---|
| **Sukoradsky** | 1002.08 ±13.03 | 27.97 ±1.34 | 2.412 ±0.046 | 1.383 ±0.039 |
| **Zahorsky** | 966.01 ±8.20 | 23.12 ±0.84 | 2.762 ±0.083 | 1.655 ±0.040 |
| **Germidour** | 1010.55 ±12.96 | 23.80 ±0.95 | 2.516 ±0.024 | 1.578 ±0.055 |
| **Mojmir** | 1365.56 ±12.26 | 129.05 ±1.53 | 4.17 ±0.031 | 3.13 ±0.039 |
| **Karel IV.** | 1132.63 ±10.96 | 59.89 ±1.6 | 3.043 ±0.028 | 1.986 ±0.047 |
| **Arkus** | 1026.20 ±8.80 | 41.96 ±0.88 | 2.45 ±0.037 | 1.611 ±0.034 |
| **Makoi** | 1195.77 ±11.87 | 69.74 ±1.36 | 3.244 ±0.048 | 2.219 ±0.054 |
| **Mean** | 1099.83 ±134.67 | 53.65 ±35.72 | 2.942 ±0.591 | 1.937 ±0.563 |

Note: TPC – total polyphenol content, TFC – total flavonoid content, AA ABTS – antioxidant activity measured by ABTS assay, AA FRAP – antioxidant activity measured by FRAP assay.

**Soto et al. (2015)** determined TFC in garlic cultivars in the range from 70 to 110 mg CE.kg$^{-1}$ DM. **Park and Kim (2015)** determined higher TFC in garlic – 334.27 mg CE.kg$^{-1}$ DM.

## Antioxidant activity using ABTS assay

Antioxidant activity in analyzed garlic cultivars measured by ABTS assay ranged from 1.098 to 1.955 mmol TE.kg$^{-1}$ FW (2.41 to 4.17 mmol TE.kg$^{-1}$ DM), with a mean value of 1.35 mmol TE.kg$^{-1}$ FW, (2.94 mmol TE.kg$^{-1}$ DM). The highest AA was determined in the cultivar Mojmir, while the lowest AA was determined in the cultivar Sukoradsky. Based on their TPC, an order for garlic cultivars could be as following: Sukoradsky< Germidour< Zahorsky< Arkus< Karel IV. < Makoi< Mojmir.

**Azzini et al. (2014)** reported AA of garlic in the range from 4.42 to 6.27 mmol TE.kg$^{-1}$ DM. **Boonpeng et al. (2014)** determined 7.62 mmol TE.kg$^{-1}$ FW. Other authors reported higher AA in garlic. **Lu et al. (2011)** reported 57.86 to 65.22 mmol TE.kg$^{-1}$ FW. **Gorinstein et al. (2006)** determined 21.4 mmol TE.kg$^{-1}$ FW. **Gorinstein et al. (2009)** 23.71 to 37.02 mmol TE.kg$^{-1}$ DM. **Zhou et al. (2017)** determined lower antioxidant activity in garlic – 0.49 mmol TE.kg$^{-1}$ DM.

## Antioxidant activity using FRAP assay

Antioxidant activity in studied garlic cultivars measured by FRAP assay ranged from 0.63 mmol TE.kg$^{-1}$ FW to 1.467 mmol TE.kg$^{-1}$ FW (1.383 to 3.13 mmol TE.kg$^{-1}$ DM), with a mean value of 0.89 mmol TE.kg$^{-1}$ FW (1.94 mmol TE.kg$^{-1}$ DM). The highest AA was determined in the cultivar Mojmir, while the lowest AA was determined in the cultivar Sukoradsky. Based on their AA, an order for garlic cultivars could be as following: Sukoradsky < Germidour< Zahorsky<Arkus< Karel IV. < Makoi< Mojmir.

**Nencini et al. (2011)** determined 0.38 mmol TE.kg$^{-1}$ in aged garlic extracts. **Bhatt and Patel (2013)** determined 2.3 mmol TE.kg$^{-1}$ FW. **Lu et al. (2011)** reported 7.62 to 11.45 mmol TE.kg$^{-1}$ FW in garlic. **Gorinstein et al. (2009)** reported 6.9 to 10.8 mmol TE.kg$^{-1}$ DM in garlic. **Boonpeng et al. (2014)** reported lower AA of garlic – 0.01 mmol TE.kg$^{-1}$ FW.

As shown in Figure 1., a significantly higher content of total polyphenols and total flavonoids was observed in the cultivar Mojmir. In this cultivar, significantly higher antioxidant activity was also observed. Significantly lower total polyphenol content was observed in cultivar Zahorsky. Significantly lower total flavonoid content was also observed in cultivar Zahorsky, and cultivar Germidour. Significantly lower antioxidant activity was observed in cultivar Sukoradsky.

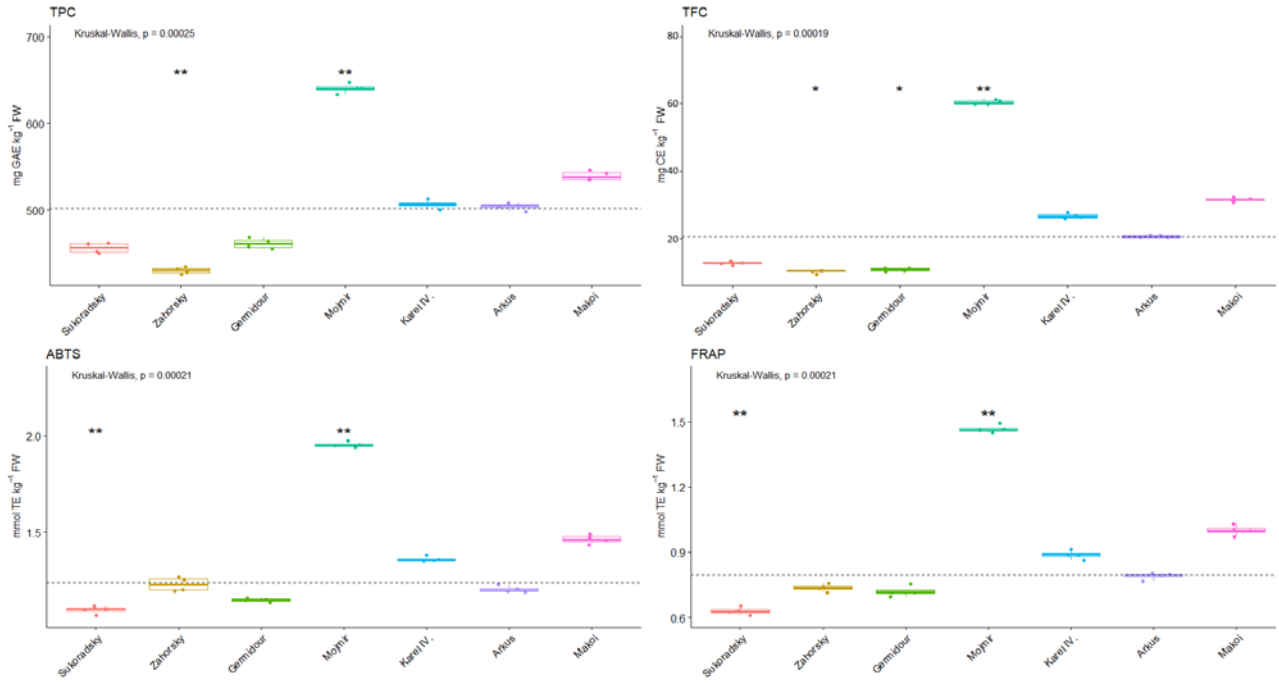


**Figure 1** Differences in the content of bioactive compounds in selected garlic cultivars.

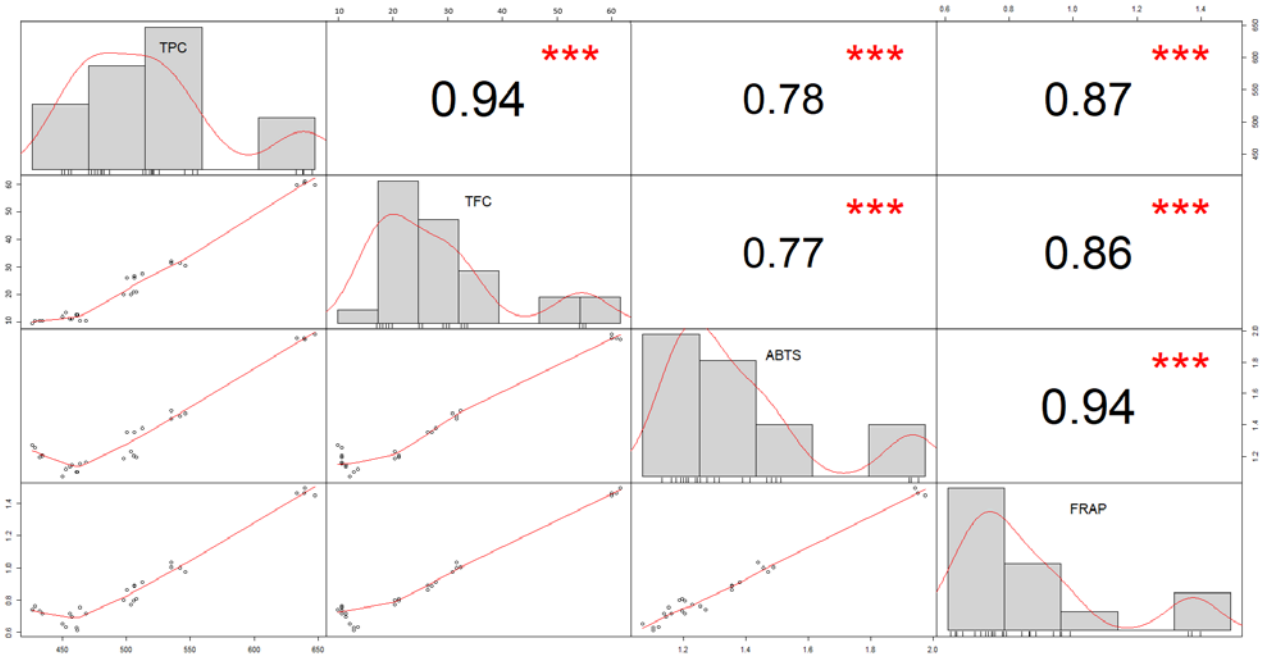


**Figure 2** The relationships between the analysed parameters.

The relationships between the parameters are shown as the Spearman correlation matrix. Statistical evaluation of results confirmed strong positive linear correlation between TPC and TFC (r= 0.94), TPC and ABTS (r= 0.78), TPC and FRAP (r= 0.87), TFC and ABTS (r= 0.77), TFC and FRAP (r= 0.86), and ABTS and FRAP (r= 0.94). **Bhandari et al. (2014)** also determined a positive correlation between TPC and TFC of garlic. **Škrovánková et al. (2018)**, and **Locatelli et al. (2017)** also determined **a** positive correlation between TPC and ABTS of garlic. **Locatelli et al. (2017)** and **Chen et al. (2013)** also determined a positive correlation between TPC and FRAP of garlic. **Soto et al. (2015)** also determined a positive correlation between ABTS and FRAP of garlic.

## CONCLUSION

Total polyphenol content, total flavonoid content, and antioxidant activity of 7 garlic cultivars were determined in this study. Based on the results of the study, we can state that garlic is a natural source of polyphenols and antioxidants. Significantly higher TPC, TFC, and AA were determined in cultivar Mojmir. Significantly lower TPC and TFC were determined in cultivar Zahorsky. Significantly lower AA was determined in cultivar Sukoradsky.

Based on statistical evaluation strong positive correlations were determined between individual studied parameters.

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# THE *CITRUS RETICULATA* ESSENTIAL OIL: EVALUATION OF ANTIFUNGAL ACTIVITY AGAINST PENICILLIUM SPECIES RELATED TO BAKERY PRODUCTS SPOILAGE

***Veronika Valková, Hana Ďúranová, Lucia Galovičová, Jana Štefániková, Nenad Vukovic, Miroslava Kačániová***

## ABSTRACT

Fungal food spoilage plays a key role in the deterioration of food products, and finding a suitable natural preservative can solve this problem. Therefore, antifungal activity of green mandarin (*Citrus reticulata*) essential oil (GMEO) in the vapor phase against the growth of *Penicillium* (*P.*) *expansum* and *P. chrysogenum* inoculated on wheat bread (*in situ* experiment) was investigated in the current research. The volatile compounds of the GMEO were analyzed by a gas chromatograph coupled to a mass spectrometer (GC–MS), and its antioxidant activity was determined by testing free radical-scavenging capacity (DPPH assay). Moreover, the disc diffusion method was used to analyze the antifungal activity of GMEO in *in vitro* conditions. The results demonstrate that the *Citrus reticulata* EO consisted of α-limonene as the most abundant component (71.5%), followed by γ-terpinene (13.9%), and β-pinene (3.5%), and it displayed the weak antioxidant activity with the value of inhibition 5.6 ±0.7%, which corresponds to 103.0 ±6.4 μg TEAC.mL$^{-1}$. The findings from the GMEO antifungal activity determination revealed that values for the inhibition zone with disc diffusion method ranged from 0.00 ±0.00 (no antifungal effectiveness) to 5.67 ±0.58 mm (moderate antifungal activity). Finally, exposure of *Penicillium* strains growing on bread to GMEO in vapor phase led to the finding that 250 μL.L$^{-1}$ of GMEO exhibited the lowest value for mycelial growth inhibition (MGI) of *P. expansum* (-51.37 ±3.01%) whose negative value reflects even supportive effect of the EO on the microscopic fungus growth. On the other hand, GMEO at this concentration (250 μL.L$^{-1}$) resulted in the strongest inhibitory action (MGI: 54.15 ±1.15%) against growth of *P. chrysogenum*. Based on the findings it can be concluded that GMEO in the vapor phase is not an effective antifungal agent against the growth of *P. expansum* inoculated on bread; however, its antifungal potential manifested against *P. chrysogenum* suggests GMEO to be an appropriate alternative to the use of chemical inhibitors for bread preservation.

**Keywords:** *Citrus reticulata*; volatile compound; DPPH assay; antifungal properties; bread

## INTRODUCTION

Essential oils (EOs) are complex mixtures of water-vapor aromatic substances (mainly terpenoids, less frequently aromatic and aliphatic compounds) derived from diverse parts of plants in which they determined their pleasant aroma **(Denkova-Kostova et al., 2021)**. They possess a broad range of various biological activities such as antimicrobial, antioxidant, anti-inflammatory, and anticancer activities **(Sharifi-Rad et al., 2017)** which were documented in many preclinical studies. In addition, their antifungal properties have been screened on a global scale as potential sources of novel antimicrobial compounds, promoting food preservation **(Chouhan, Sharma and Guleria, 2017)**.

Citrus essential oils (EOs) have a volatile fraction usually >90% in which monoterpenes and sesquiterpenes are found with limonene being the major compound **(Raspo et al., 2020)**. From them, mandarin EO is extracted from *Citrus reticulata* of the *Rutaceae* family and has some great properties to help relieve stress and digestive problems. Also, it is used to increase circulation to the skin, reducing fluid retention, and to help prevent stretch marks **(Fayed, 2009)**. In general, there are three kinds of mandarin EO, i.e., green, yellow, and red, all derived from the same fruit, but at different stages of maturity. Green mandarin essential oil (GMEO) is generally sharper and with more of a "peel" note compared to red mandarin **(Boughendjioua, Mezedjeri and Idjouadiene, 2020)**. From a chemical profile point of view, the mandarin EO contains α-pinene, β-pinene,

camphene, citral, citronellal, γ-terpinolen, geraniol, citric, linalool, methyl myrcene, sabine, and terpineol **(Denkova-Kostova et al., 2021)**. It is well-known for its antibacterial and antifungal actions in a wide spectrum. Indeed, its strong activity against some microorganisms related to food spoilage and food safety including *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Aspergillus flavus*, and *P. digitatum* was reported **(Gao et al., 2010; Tao et al., 2009; Wang et al., 2012)**.

The major purpose of our study was to evaluate the antifungal potential of GMEO against selected *Penicillium* (*P.*) species (*P. expansum* and *P. chrysogenum*) using the contact vapor method. Moreover, the chemical composition of the GMEO, as well as its antioxidant properties and antifungal effect in *in vitro* conditions were established. The obtained findings of the studied GMEO would give a reason for their inclusion in the development of biopreservation strategies for the food industry.

## Scientific hypothesis

Since *Citrus reticulata* essential oil represents a rich source of bioactive monoterpenes, its antifungal potential against *Penicillium* spp. could be expected.

## MATERIAL AND METHODOLOGY

### Samples

Green mandarin essential oil (GMEO; *Citrus reticulata*) was purchased from Hanus Company (Ltd, Hrochoť, Slovakia).

Wheat bread was obtained from Laboratory of Cereal Technologies (AgroBioTech Research Centre, Slovak University of Agriculture in Nitra).

### Chemicals

All chemicals were analytical grade, and were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (Schnelldorf, Germany).

Sabouraud Dextrose Agar (SDA; Schnelldorf, Germany).

### Animals and Biological Material

The fungi *P. expansum* and *P. chrysogenum* were isolated from grape and bread samples, respectively. Then, they were identified with the MALDI-TOF MS Biotyper and 16S rRNA sequencing.

### Instruments

Mass spectrophotometer (MALDI-TOF MS Biotyper, Bruker, USA).

Spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, USA).

Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA).

Quadrupole mass spectrometer 5975B (Agilent Technologies, Santa Clara, CA, USA).

Spiral mixer (Diosna SP 12 D, Diosna, Germany).

Fermentation cabinet (MIWE cube, Pekass s.r.o., Plzeň, Czech Republic).

Steamy oven (MIWE cube, Pekass s.r.o., Plzeň, Czech Republic).

### Laboratory Methods

#### *Determination of volatile compounds*

The chemical profile of GMEO was performed using an Agilent 6890N gas chromatograph coupled to quadrupole mass spectrometer 5975B as reported by **Valková et al. (2021)**. The individual volatile constituents of the injected EO samples were identified based on their retention indices **(Adams, 2007)** and compared with reference spectra (Wiley and NIST databases). The retention indices were experimentally determined using the standard method **(Van Den Dool and Kratz, 1963)** which included retention times of n-alkanes (C6-C34), injected under the same chromatographic conditions. The percentages of the identified compounds (amounts higher than 0.1%) were derived from their GC peak areas.

#### *Determination of radical scavenging activity*

The radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to measure the antioxidant activity (AA) of GMEO described previously by **Galovičová et al. (2021)**. The AA was expressed as the percentage of DPPH inhibition, and was calculated according to the formula: (A0 – A1)/A0 × 100; where A0 was the absorbance of DPPH and A1 was the absorbance of the sample. The AA values increased in the following manner: weak (0 – 29%) < medium-strong (30 – 59%) < strong (60 and more %). Moreover, the value of total AA was expressed according to the calibration curve as 1 μg of standard reference Trolox to 1 mL of the GMEO sample (TEAC).

#### *Evaluation of antifungal activity*

The evaluation of the antifungal activity of the GMEO was performed using the agar disc diffusion method according to the **Valková et al. (2021)** with minor modifications. For this purpose, an aliquot of 0.1 mL of fungal in distilled water was inoculated on SDA (60 mm). Subsequently, the discs of filter paper (6 mm) were impregnated with 10 μL of GMEO samples (in four concentrations: 62.5, 125, 250, and 500 $\mu L.L^{-1}$), then applied on the SDA surface, and incubated at 25 °C for 5 days. The disks impregnated with ethanol served as negative controls. After incubation, the diameters of the inhibition zones in mm were measured. Each test was repeated three times (one repeat reflected one separate plate). The values of inhibitory activity increased in the following manner: weak antifungal activity (5 – 10 mm) < moderate antifungal activity (5 – 10 mm) < very strong antifungal activity (zone > 15 mm).

#### *Bread making procedure*

Wheat bread, as a substrate for fungal growth, was made according to the procedure by **Kačániová et al. (2020a)** in the Laboratory of Cereal Technologies (AgroBioTech Research Centre, the Slovak University of Agriculture in Nitra).

#### *Antifungal analyses on bread loaves model*

First, the bread samples were cut into slices with 150 mm height and placed into 0.5 L sterile glass jars (Bormioli Rocco, Fidenza, Italy). The fungal spores were used for bread inoculation. The GMEO in concentrations of 62.5, 125, 250, and 500 $\mu L.L^{-1}$ (diluted in ethyl acetate) were evenly distributed in a volume of 100 μL on a sterile paper–filter disc (6 cm), which was inserted into the cover of the jar, except for the control group. The jars were hermetically closed and kept at 25 ±1 °C for 14 days in the dark **(Kačániová et al., 2020a)**. The size of the microfungal colonies with visible mycelial growth and visible sporulation was evaluated using stereological methods. In this concept, the volume density of the colonies was firstly assessed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), counting the points of the stereological grid hitting the colonies and those falling to the reference space (growth substrate used, i.e., bread). The

antifungal activities of the EOs were expressed as the percentage of mycelial growth inhibition (MGI), which was calculated using the formula: MGI = [(C − T)/C] × 100, where C = volume density of the fungal colony in the control group and T = volume density of that in the treatment group **(Sempere-Ferre et al., 2021)**.

**Description of the Experiment**

**Sample preparation:** 42
**Number of samples analyzed:** 42
**Number of repeated analyses:** 4
**Number of experiment replication:** 3

### Statistical Analysis

The obtained data were statistically evaluated using Prism 8.0.1 (GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) followed by Tukey's test was used to evaluate the significance of differences between analyzed groups of samples. The level of significance was set at $p < 0.05$.

## RESULTS AND DISCUSSION

### Chemical composition of GMEO

Although the mode of action of EOs has not been fully elucidated it is well-known that their primary effects are related to their chemical composition **(Burt, 2004; Cox and Markham, 2007)**. Indeed, these compounds, typically monoterpenes or phenylpropanoids, often exhibit antioxidant capacity and also antimicrobial activity against a wide range of bacterial and fungal species **(Sikkema, de Bont and Poolman, 1995; Behbani et al., 2017)**. Moreover, the interaction between the various compounds of EOs can lead to antagonistic, additive, or synergistic effects **(Burt, 2004)**. Therefore, the identification of individual volatile substances may be a useful tool for the characterization of EOs, and may help to understand the key points in their antioxidant and antimicrobial activity, thus being possible to employ either alone or in combination with other additives in food preservation techniques **(Viuda-Martos et al., 2007; Miladinovic et al., 2015)**.

GC-MS analysis was also used in our study to determine the chemical composition of GMEO. It revealed that a total of 28 compounds, accounting for 99.7% of the whole constituents, were identified in the EO chemical profile. The major compound was shown to be α-limonene (71.5%), and other major ones included γ-terpinene (13.9%), β-pinene (3.5%), p-cymene (3.1%), α-pinene (2.6%), and β-myrcene (2.2%), as presented Table 1.

Our results are in agreement with the previous findings reported by **Viuda-Martos et al. (2009)**, who consider α-limonene (74.7%) and γ-terpinene (15.7%) as the major oil compounds of the mandarin EO. The same observation was also demonstrated by **Espina et al. (2011)** and **Denkova-Kostova et al. (2021)**, who found that the main compound of *C. reticulata* EO was limonene which comprised 74.4% and 84.88% of the EO, respectively. In contrast to our findings, **Yabalak, Eliuz and Nazlı (2021)** detected eucalyptol (7.2%), methyl palmitate (3.8%), and α-terpineol (3.7%) as the most represented substances in their *C. reticulata* EO, whereby α-limonene (71.5%) or γ-terpinene were absent in its chemical profile. However, we assume that the differences in the EO chemical composition between these studies might be due to various factors such as genetic factors (genotype or variety), geographical locations, environmental conditions, season, cultivation practices, fertilizer application, stress during growth or maturity, harvesting time, stage of maturity, as well as processing methods which strongly influenced it **(Burt, 2004; Sandeep, Sanghamitra and Sujata, 2015; Srinivasan et al., 2016)**.

**Table 1** Main volatile compounds of green mandarin essential oil.

| Compounds | (%) |
|---|---|
| α-limonene | 71.5 |
| γ-terpinene | 13.9 |
| β-pinene | 3.5 |
| *p*-cymene | 3.1 |
| α-pinene | 2.6 |
| β-myrcene | 2.2 |
| α-terpineol | 1.0 |
| α-terpinolene | 0.7 |
| α-thujene | 0.5 |
| n-decanal | 0.4 |
| sabinene | 0.3 |
| (*E*)-β-ocimene | 0.1 |
| trans-limonene oxide | tr |
| 4-terpinenol | tr |
| α-copaene | tr |
| (*E*)-caryophyllene | tr |
| (*E*,*E*)-α-farnesene | tr |
| δ-cadinene | tr |
| **TOTAL** | 99.7 |

Note: tr – compounds identified in amounts less than 0.1%.

Summary, based on the findings we can propose that the high proportion of monoterpenes (mainly α-limonene, γ-terpinene, and β-pinene) in our analyzed GMEO can predict its promising use as a preservative in the food industry as it was stated in the study by **Badawy, Lotfy and Shawir (2020)**.

### Antioxidant activity of GMEO

It is generally known that EOs have antioxidant properties, which are subjected to analyses performed in many scientific papers **(Diniz do Nascimento et al., 2020; Kačániová et al., 2020b; Boudiba et al., 2021)**.

The DPPH method, also used in our study, is considered a simple and sensitive technique applicable to most plant extracts including EOs **(Noipa et al., 2011)**. DPPH is a stable free radical with deep violet color, which accepts an electron or hydrogen radical to create a stable diamagnetic molecule with discoloration. The degree of discoloration indicates the free radical-scavenging potential of the sample **(Schaich, Tian and Xie, 2015)**. Interestingly, employing this assay we have found that values for AA of the EO of *C. reticulata* were 103.0 ±6.4 μg TEAC.mL$^{-1}$, with 5.6 ±0.7% free radical-scavenging inhibition linked to a weak AA. Dissimilar to our results, **Boudries et al. (2017)** reported a higher value of % of inhibition associated with a stronger AA of mandarin EO which was even the highest between all analysed citrus fruit EOs (mandarin, clementin, wilking).

Also, the highest DPPH activities (78.0%; 73.32%) of EO from *Citrus reticulata* were found in the studies by **Denkova-Kostova et al. (2021)** and **Ishfaq et al. (2021)**, respectively. Most probably, different chemical compositions and amounts of individual constituents (depending on various aforementioned factors) of the mandarin EOs used in our and all mentioned studies could be responsible for the discrepancies in AA of the EOs demonstrated by their results.

### *In vitro* antifungal properties of GMEO

Data from the inhibitory effects of GMEO against two tested *Penicillium* spp. fungi (*P. expansum*, and *P. chrysogenum*) assessed by the disc diffusion method are shown in Table 2. Our results revealed that the growth inhibition of fungi strains depends on the concentration of the GMEO applied. In effect, moderate antifungal activity (inhibition zone: 5.67 ±0.58 mm) was observed at the highest concentration (500 μL.L$^{-1}$) of GMEO against the growth of *P. chrysogenum*. This effect was statistically different ($p$ <0.05) from those exhibiting at the lowest concentration (62.5 μL.L$^{-1}$) of the GMEO which showed no inhibitory activity (inhibition zone: 0.00 ±0.00 mm) against this fungus strain. On the other hand, the growth of *P. expansum* was only weakly inhibited (inhibition zone: 1.67 ±0.58 mm) by GMEO in the concentration of 500 μL.L$^{-1}$, and this inhibition even significantly differ from those (inhibition zone: 0.00 ±0.00 mm) displayed by remaining concentrations (62.5, 125, 250 μL.L$^{-1}$) of GMEO.

Several scientific studies have demonstrated that citrus EOs have been shown to reduce or completely inhibit the growth of microscopic fungi depending on their concentration **(Sharma and Tripathi, 2006; Sharma and Tripathi, 2008)**. **Droby et al. (2008)** observed that citrus EOs (mandarin, lemon, grapefruit, and orange) in concentration of 20.0 μL.mL$^{-1}$ stimulated the growth of *P. italicum* and *P. digitatum*. On the other hand, grapefruit EOs (in concentration of 40.0 μL.mL$^{-1}$) had moderate antifungal activity against *P. digitatum*. Moreover, it was found that some microscopic fungi can catalyze the conversion of antifungal compounds in various plant extracts (including those obtained in EOs). Such conversion has also been reported for instance in *P. digitatum* which was able to convert limonene to α-terpineol, cis- and trans-p-menth-2-en-1-ol, neodihydrocarveol and limonene oxide **(Tan, Day and Cadwallader, 1998; Demyttenaere, Van Belleghem and De Kimpe, 2001; Badee, Helmy and Morsy, 2011)**, i.e., to less effective substances related to antifungal activity. Therefore, we assume that a similar type of conversion may explain why GMEO was more powerful against *P. chrysogenum* as compared to *P. expansum*, as was reported in our study.

### *In situ* antifungal vapor contact assay of GMEO

In general, antifungal agents are used in the food industry for the preservation of food (control natural spoilage processes) or their safety (prevent or control the growth of microorganisms; **Shaaban, 2020)**. As spoilage of bakery products is often caused by microscopic fungi including *Penicillium* spp. **(Salas et al., 2017)** the antifungal properties of GMEO in the vapor phase on bread as a substrate for the growth of these species were evaluated in our study. The relative volatilities of the EOs compounds determine the characteristics of their vapors which have impacts on antimicrobial potential **(Tullio et al., 2007)**. Several studies have shown that the vapor phases of EOs are more effective than liquid ones **(Soylu, Soylu and Kurt, 2006; Mondello et al., 2009; Tyagi and Malik, 2010)**. This fact can be attributed to the free adhesion of EOs to the substrate (vapor phase), while in the aqueous phase the lipophilic molecules combine to form micelles suppressing the adhesion of EOs to the substrate **(Inouye et al., 2003)**.

The inhibitory effects of our GMEO on the growth of the *Penicillium* spp. inoculated on wheat bread are demonstrated in Table 3 and Figure 1. From the results, it

**Table 2** Antifungal activity of EOs (*in vitro*).

| Fungal strains | The inhibition zones (mm) | | | |
|---|---|---|---|---|
| | GMEO (μL.L$^{-1}$) | | | |
| | 62.5 | 125 | 250 | 500 |
| *P. expansum* | 0.00 ±0.00 $^{a}$ | 0.00 ±0.00 $^{a}$ | 0.00 ±0.00 $^{a}$ | 1.67 ±0.58 $^{b}$ |
| *P. chrysogenum* | 0.00 ±0.00 $^{a}$ | 2.67 ±0.58 $^{b}$ | 4.00 ±1.00 $^{b}$ | 5.67 ±0.58 $^{b}$ |

Note: Means ± standard deviation. Values followed by different superscript within the same row are significantly different ($p$ <0.05). GMEO – Green mandarin essential oil; 00.00 – total growth.

**Table 3** Mycelial growth inhibition of the GMEO.

| Fungal strains | MGI (%) | | | |
|---|---|---|---|---|
| | GMEO (μL.L$^{-1}$) | | | |
| | 62.5 | 125 | 250 | 500 |
| *P. expansum* | 3.49 ±1.23 a | -11.77 ±0.40 b | -51.37 ±3.01 c | -15.35 ±0.99 d |
| *P. chrysogenum* | 14.17 ±0.53 a | 20.02 ±2.13 b | 54.15 ±1.15 c | 45.61 ±0.88 d |

Note: Means ± standard deviation. Values followed by different superscript within the same row are significantly different ($p$ <0.05). MGI – Mycelial growth inhibition; GMEO – Green mandarin essential oil; 00.00 – total growth. The negative values indicate a profungal activity against *Penicillium* strains.

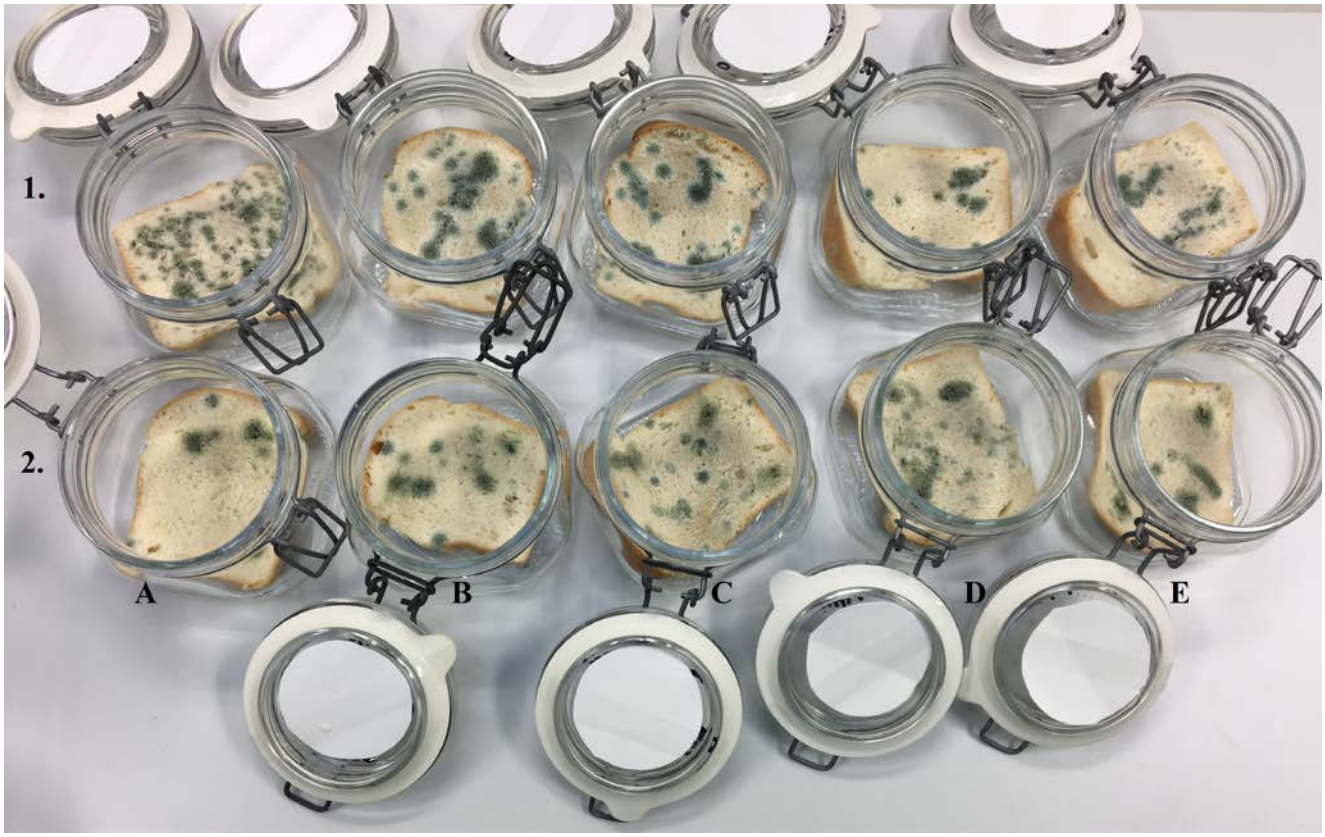


**Figure 1** *In situ* antifungal analyses of bread with *Penicillum expansum* and *Penicillium chrysogenum* in vapor phase. Note: 1 – *P. chrysogenum*; 2 – *P. expansum* and after their treatment with different GMEO concentrations (A – control; B – 62.5 µL.L$^{-1}$; C - 125 µL.L$^{-1}$; D – 250 µL.L$^{-1}$ and E – 500 µL.L$^{-1}$).

can be evident that all GMEO concentrations analyzed (62.5, 125, 250, and 500 µL.L$^{-1}$) exhibited antifungal potential against *P. chrysogenum*, whereby the concentration of 250 µL.L$^{-1}$ showed the best efficiency (54.15 ±1.15%). Moreover, between all concentrations used, statistically significant differences ($p$ <0.05) were noted. Interestingly, the growth of *P. expansum* was only very weak (3.49 ±1.23%) inhibited by the lowest concentration (62.5 µL.L$^{-1}$) of GMEO. On the other hand, the remaining concentrations used (125, 250, and 500 µL.L$^{-1}$) acted proactively on the growth of this fungus which was indicated by the negative values of -11.77 ±0.40, - 51.37 ±3.01, and -15.35 ±0.99%, respectively.

We hypothesize that the weak antifungal activity of GMEO against *P. expansum* may be due to the high resistance of this strain **(Adams, 2007)**. Therefore, we assume that GMEO used can be more effective against other species of microscopic fungi as was shown in the case of *P. chrysogenum*. Interestingly, *P. chrysogenum* was the most sensitive to the concentration of 250 µL.L$^{-1}$ of the EO. For this reason, in our further research activities, we will deal with the optimization of GMEO concentration to obtain its highest possible efficiency. Moreover, the results are following our previous researches, in which the antifungal properties of other EOs, such as *Citrus aurantium* EO or **(Kačániová et al., 2020a)** coriander EO **(Kačániová et al., 2020b)**, against the fungi of *Penicillium* spp. analysed were confirmed. These findings are of particular relevance for food production because by introducing GMEO, the contamination with pathogens could be avoided, and the growth of the microscopic fungi could be inhibited, which is primarily important for the food industry **(Denkova-Kostova et al., 2021)**.

## CONCLUSION

The aim of the present research was to assess the antifungal activity of GMEO in the vapor phase on the growth of selected *Penicillium* species inoculated on wheat bread. The chromatography analysis has shown that the major chemical compounds of the EO were α-limonene (71.5%), γ-terpinene (13.9%) and β-pinene (3.5%). The mandarin EO exhibited only weak AA comparable with Trolox as a standard with a value of 103.0 ±6.4 µg.mL$^{-1}$ for DPPH, reflecting 5.6 ±0.7% of free radical-scavenging inhibition. The antifungal activity of GMEO against the tested fungi was shown to be not effective or moderate *in vitro*. Similar trend was also found for *in situ* analysis in which a maximum value for MGI (54.15 ±1.15%) was observed against *P. chrysogenum* using 250 µL.L$^{-1}$ of GMEO. Thus, our results pointed to the fact that GMEO has weak both AA and inhibitory action against the growth of *P. expansum*. However, against *P. chrysogenum* it can be considered an appropriate alternative to use synthetic inhibitors for the preservation of bakery products such as wheat bread.

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# THE EFFECT OF SOIL BIOSTIMULANT AGRIFUL ON THE SELECTED QUANTITATIVE AND QUALITATIVE PARAMETERS OF CARROT (*DAUCUS CAROTA* SUBSP. *SATIVUS* (*HOFFM.*) *ARCANG.*)

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## ABSTRACT

The research task was focused on an important type of root vegetable – carrot (*Daucus carota* L.), cultivar ´Romance F1´ (family: *Apiaceae*). The main goal was to verify the application of soil biostimulant Agriful (based on humic and fulvic acids) and its impact on the yield potential and quality of carrot roots. Nowadays, it is the common need to intensify and increase crop production because of the growing human population and look for environmentally friendly cultivation methods. In general, biostimulants are biologically based products and their purpose is to stimulate the natural nutritional processes in cultivated crops. In the realized experiment, Agriful (treatment of 5 L/300 L water/ha) was applied twice per vegetation period by spraying over the pivot. The increase of average root weight after the application of Agriful was found, concretely about +4.47% compared to the control variant. The higher root weight after Agriful application resulted in a higher total yield of carrot about +2.84% compared to the control variant. The quality of consumable parts of carrots were evaluated based on the classification of roots into quality classes according to the valid standard for fresh carrot marketing (UNECE FFV-10). In the ´Extra class´, there was an increase in the average yield after the application of Agriful about +5.4%; The average carrot yield in ´Class I´ was decreased about -1.0% and the root ratio in the ´Class II´ decreased about -1.9%. The ratio of ´Non-standard´ carrot roots was lower about -2.5%. Based on evaluating qualitative substance content (total carotenoids, refractometric dry matter), the positive influence of the effect of Agriful application was found. The content of total carotenoids was higher about +8.7% compared to the control variant. The refractometric dry matter was higher about +4.1% compared to the control variant. The obtained results can be used in further research on biostimulants and it is possible to create clear recommendations for using Agriful for small growers. It should be useful to verify these results in another vegetation period for recommendation to large-scale producers of carrots.

**Keywords:** biostimulant; carrot; yield; carotenoid; refractometric dry matter

## INTRODUCTION

The carrot (*Daucus carota* L.) is one of the most popular root vegetables grown in the world. Worldwide, the average carrot production was 1.16 mil. tons within period 2015 – 2018. China is the major carrot-producing country in the world **(FAO, 2021)**. In Slovakia **(Meravá, 2021)**, the situation in carrot growing has a growing character. In 2019, carrot production (14 292 tons) was more than multiple compared to 2017 (13 596 tons).

The consuming part of carrots is the storage root. The carrot root contains almost 88% water, 1% protein, 7% carbohydrates, 0.2% fat, and 3% fiber. Carbohydrates are represented mostly by simple sugars (sucrose, glucose, xylose, and fructose) with a negligible starch content **(Arscott and Tanumihardjo, 2010)**. The carrot root is characterized by accumulating high amounts of carotenoids, mainly β-carotene **(Oleszkiewicz et al., 2018; Tang, 2010)**. Further, carrot root is a good source of dietary fiber, minerals, and phytochemicals, e. g. vitamin C, phenolic compounds, polyacetylenes **(Que et al., 2019; Ahmad et al., 2019)**.

With increasing of health awareness, carrot, or vegetables and fruits generally, becomes more attractive in the human diet due to their health-promoting effect and antioxidant activity. These effects are closely related to the strengthening of the human immune system and preventing degenerative diseases such as cardiovascular disease, stroke, cancer, skin diseases etc. **(Sharma et al., 2012; Zaheer, 2017; Durec et al., 2019; Sabolová and Kouřimská, 2020)**.

Many agricultural research studies are currently focused on the application and importance of biostimulants preparations, which have been shown to have an impact on the quantitative and qualitative parameters of various fruits and vegetables **(Adamec et al., 2019; Mikhov, 2012; Staneva, Akova and Kornov, 2020; Caruso et al., 2019; Soppelsa et al., 2018)**.

Biostimulants are biologically based products, which stimulate plant physiological processes of nutrition in cultivated crops independently on their nutrient content. They offer a new approach to the regulation of natural processes, from which the response to stress factors, increased efficiency of fertilizer use, and total crop yield are the most important. Biostimulants are produced from a wide range of biological and inorganic materials. The main sources are microbial fermentation of animal or plant raw materials, live microbial cultures, macro and microscopic algae, protein hydrolysates, humic and fulvic substances, composts, manure, food, and industrial waste prepared by very specific industrial production processes **(Yakhin et al., 2017)**.

Humic substances belong to the most established biostimulant used in agriculture because they have been shown to significantly improve plant growth (directly or indirectly), as well as improve soil properties and fertility **(Nardi et al., 2016)**. One of the main results of their use is a better utilization of nutrient uptake **(Jindo et al., 2020)** and modulating of nutrient acquisition pathway of primary and secondary metabolism and hormonal and reactive oxygen balance **(Vujinović et al., 2020)**. The use of humic substances relates to a positive impact on changes in the architecture in the root system **(Canellas et al., 2015)**.

## Scientific hypothesis

The effect of different soil biostimulants has been investigated on various crops, where its positive effect has been demonstrated. However, the root vegetables have not yet been well-examined and each year more and more biostimulant preparations are coming to the market. Thus, this study was focused on the hypothesis about the possible positive impact of Agriful application on the yield and quality of carrot roots.

## MATERIAL AND METHODOLOGY

The field experiment was realized in 2020, in the company Agromačaj s.r.o. in the cadastral area of village Kostolná pri Dunaji in The west of Slovakia. The carrot culture was grown in 20 hectares.

**Samples**

The carrot cultivar (*Daucus carota* subsp. *Sativus*) ´Romance F1´ was used in the experiment. This variety is characterized by a homogeneous shape of the root, which is not inclinable to cracking. It is suitable for storage, direct consumption as well as processing. The seed producer indicates that the period of the growing season is 110 days. The carrot seed sowing was done on 5$^{th}$ July 2020. The carrot root harvest was carried out on 9$^{th}$ November 2020.

Within the experimental field, two variants were tested: C – control (without Agriful application); A – Agriful application. Five repeatings of each variant were diagonally arranged in carrot culture. The soil biostimulant Agriful is mainly based on humic and fulvic substances. Its composition is the following: humic acids (25%), fulvic acids (25%), N (4.5%), P (1%), $K_2O$ (1%), and other organic substances (45%). The application dose of Agriful (5 L/300 L water/ha) was realized by spraying, as a part of irrigation, on 2$^{nd}$ July 2020 and 14$^{th}$ August 2020.

After harvest, the average weight of root, total yield, and quality classification of carrot roots was evaluated. The classification of carrot roots to the class quality was determined according to the standard UNECE FFV-10 concerning the marketing and commercial quality control of carrots. Within mentioned standard carrot quality classes are the following:

'Extra class' - carrot roots must be of superior quality. The roots must be smooth, fresh in appearance, regular in shape, free of fissures, free of bruises and cracks, free of damage due to frost, free of green or violet/purple tops.

'Class I' - carrot roots must be of good quality. The roots must be fresh in appearance. Slight defects may be allowed; however, defects must not effect on the general appearance of produce, the quality, the keeping quality, and presentation in the package. Slight defects are allowed in shape, coloring, healed cracks, slight cracks or fissures due to handling or washing, green or violet/purple tops up to the length of 1 cm for roots not exceeding 10 cm in length and 3 cm for other roots.

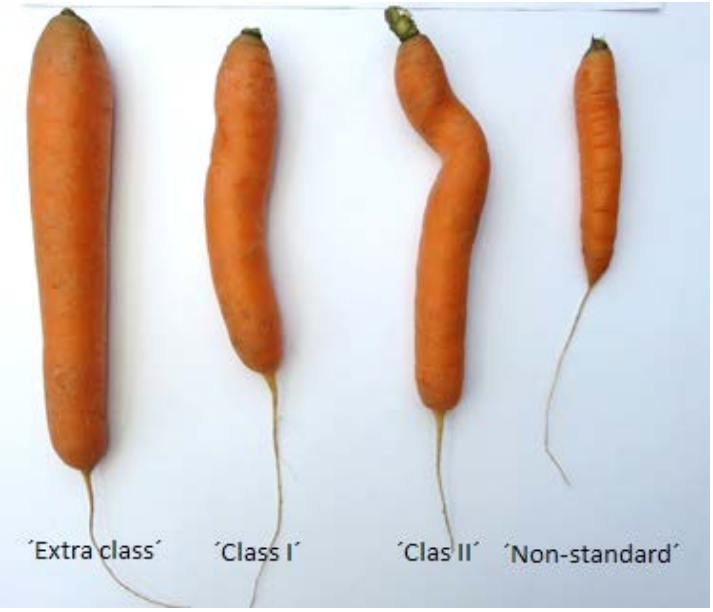


**Figure 1** Sorting the consumer part according to quality classes based on the shape of the root.

'Class II' - this class includes carrots which can not be classified in higher quality classes but the minimal criteria for carrot root must be filled. Root defects may be allowed, provided the carrots retain their essential characteristics as regards the quality, the keeping quality, and presentation: defects in shape, defects in coloring, healed cracks not reaching the heart, cracks or fissures due to handling or washing, green or violet/purple tops up to the length of 2 cm for roots not exceeding 10 cm in length and 3 cm for other roots.

**Laboratory Methods**

Qualitative laboratory analyses were realized at the Institute of Horticulture, the Slovak University of Agriculture in Nitra immediately after harvest. The content of total carotenoids and refractometric dry matter were analyzed in the fresh weight of carrot roots.

The average sample of tested variants for analyses was prepared from carrot roots of 'Extra class' or 'Class' I. All roots were quartered, and opposite quarters were used for qualitative analyses.

The analysis of total carotenoid content was realized by spectrophotometric measurement of substance absorbance in petroleum ether extract on the spectrophotometer PHARO 200 at the wavelength of 445 nm. As an extraction reagent, acetone was used **(Hegedűsová et al., 2018)**.

Within analysis of refractometric dry matter content, the juice from homogenized carrot was squeezed on the dry block of the digital hand-held refractometer (Kern ORD 45BM, Balingen, Germany). The value of soluble solids was directly read. Measurement was performed at room temperature according to **Hegedűsová et al. (2018)**.

## Statistical Analysis

Statistical analysis was performed by using of Microsoft Excel. The obtained results were evaluated by analysis of variance (ANOVA) and paired T-test at the significance level of 95%.

# RESULTS AND DISCUSSION

## The average weight of root (AW)

The AW value of the carrot root in the control variant (C) was 59.50 g. In the variant with Agriful (A), an increase of AW to the value of 62.16 g was found (+4.5% compared to the control). The difference in AW of carrot roots between variant C and A was evaluated by multifactor analysis of variance (ANOVA) as statistically non-significant.

Due to the lack of experimental studies focused on the carrot and Agriful, gained results were confronted and compared with results of studies focused on the effect of Agriful in different crops. The field experiment focused on potatoes was realized by the company **Organix s.r.o. (2017)**, distributor of the soil biostimulant Agriful in Slovakia. In the experiment, the increase of potato tuber AW from 79 g (control variant) to 88 g (Agriful variant) was found (+11.4%). The field experiment oriented on the onion and Agriful was realized by **Kandil, Sharief and Fathalla (2013)**. The AW of one onion bulb was increased from 120.1 g to 125.6 g (+4.6%) after Agriful application. **Adamec et al. (2019)** found that the average weight of bell pepper fruits (Capsicum annum L.) after Agriful application was higher about +5.4% ('Esperansa F1') or 22.6% ('Kapirex F1') compared to the control variant. Results of this study indicate that genotype is also a very important factor influencing the reaction of plants to the application of Agriful, or humic and fulvic acids generally.

One of the main Agriful substances are humic (HA) and fulvic acids (FA) and their impact on the AW of the consumable part was monitored in experiments with carrot and some other crops. **Alhariri and Boras (2020)** tested the application of HA in the field experiment with carrots. The value of carrot root AW was increased from 45 g (control) to 70 or 111 g, in dependency on the HA dose. **El-Helaly (2018)** realized experiment focused on the impact of HA and FA application on the AW of carrot roots. Its values were increased from 41 g (control) to 54 (HA) or 44 g (FA). Results of this study showed a stronger effect of HA compared to the carrot root AW than FA application. The beneficial effect of HA or FA application to AW was also found in studies focused on potato tubers **(Suh, Yoo and Suh, 2014)** or chili pepper fruits **(Jan et al., 2020)**.

## Total carrot yield

The total carrot yield was evaluated and converted to the area of 1 hectare ($t.ha^{-1}$). The application of Agriful resulted in the increase of total carrot yield from 63.46 $t.ha^{-1}$ (C) 65.27 $t.ha^{-1}$ (A). In percentage expression, this difference represented a value of 2.9% (Table 1). The difference in total yield of carrot roots between variants C and A variant was evaluated as statistically non-significant by using of multifactor analysis of variance (ANOVA).

Several studies have also shown that organic soil biostimulant Agriful tended to a higher yield of vegetable crops. In the experiment of company **Organix s.r.o. (2014)**, potato tuber yield was increased from 59.2 $t.ha^{-1}$ (C) to 61.5 $t.ha^{-1}$ (A). After Agriful application, a higher yield of potatoes about +3.89% was reached compared to the control variant. **Kandil, Sharief and Fathalla (2013)** found that Agriful application resulted in the increase of total onion bulb yield 36.7 $t.ha^{-1}$ to 38.5 $t.ha^{-1}$ (+4.9%) compared to the control variant. The positive impact of Agriful on the crop yield was also presented in the study with bell pepper (*Capsicum annum* L.) realized by **Adamec et al. (2019)**. In the variant with Agriful, authors found a higher yield of bell pepper about +22% ('Esperansa F1') and 31.5% ('Kapirex F1') compared to the control variant. On the contrary, **Arnaoudov, Boteva and Dintcheva (2019)** found a slight decrease of cucumber yield about -0.4% after Agriful application, compared to the control, in the greenhouse experiment.

The effect of HA and FA, the main components of Agriful, was tested in experiments with carrots and other vegetable crops. **El-Helaly (2018)** found a different impact of HA and FA application on the total carrot yield. The application of HA resulted in the increase of total carrot yield from 13.93 $t.ha^{-1}$ to 16.98 $t.ha^{-1}$ (average value of 4 cultivars) in comparison with control variant (+21.9%). On the contrary, FA application (13.19 $t.ha^{-1}$) was shown by a decrease of total carrot yield of about -5.3% compared to the control (13.93 $t.ha^{-1}$). Authors indicate that cultivar of carrot, or vegetables generally, is a very important factor determining the resulting effect of HA or FA acid application. **Suh, Yoo and Suh (2014)** tested effect of different doses of HA and FA on the total yield of potato tubers. The authors found statistically non-significant changes of total potato yield after the application of HA or FA as it was similarly found

in this study with Agriful. The application of HA resulted in a slight increase of total potato yield from +2.4% to +3.6% compared to the control variant. From the aspect of FA application, a slight increase of total carrot yield was found in two variants with FA (+1.0% or +2.8%) and a slight decrease of carrot yield was found in one FA variant (-2.5%). Non-significant impact of FA application was also presented in the study with safflower **(Moradi, Pasari and Fayyaz, 2017)**. On the contrary, **Jan et al. (2020)** found a statistically significant increase of yield after HA application in the field experiment with chilli pepper. In individual HA variants, fruit yield increase was ranged from +7.3% to +19.1% compared to the control variant. These results are inconsistent with results of other studies realized on red bean **(Mohajerani et al., 2016)**, bell pepper **(Karakurt et al., 2009; Maraei, Eliwa and Aly, 2019)**, strawberries **(Shehata et al., 2011)** of sugar beet **(Wilczewski, Szczepanek and Wenda-Piesik, 2018)**, at which significant higher yield of crops was found after HA application. Results of these studies were showed the more significant influence of HA on the yield of grown crops.

## Quality classes of carrot roots

The quality classes of roots were evaluated according to the valid marketing standard for fresh carrots (FFV-10), published by **UNECE (2017)**. The total yield of carrot roots was divided into three quality classes: 'Extra class', 'Class I' and 'Class II'. Carrot roots, which do not meet the criteria of the above-mentioned class, are included in the class called 'Non-standard'. In Table 2, the percentage share of individual quality classes for both variants is mentioned. The percentage share of individual carrot quality classes in the control (C) variant was following: 'Extra class' = 60.4%, 'Class I' = 19.8%, 'Class II' = 2.5% and 'Non-standard' = 17.3%. In the variant with Agriful application (A), values of percentage share of carrot quality classes were following: 'Extra class' = 65.8%, 'Class I' = 18.8%, 'Class II' = 0.6% and 'Non-standard' = 14.8%.

From gained data, the percentage difference between variants C and A for individual carrot quality classes were counted. Within the experiment, the share of 'Extra class' from total carrot yield was increased from 60.4% to 65.8% after Agriful application (+5.4%). In other quality classes of carrot roots, its share was decreased about -1.0% (Class I), -1.9% (Class II) and -5.5% (Non-standard). The difference of root share in 'Class II' between variants A and C were evaluated as statistically highly statistically significant. Differences of root share in other root quality classes between experimental variants were evaluated as statistically non-significant.

According to market requirements in the Slovak Republic, carrot quality classes 'Extra class' and 'Class I' are only marked as a marketable share. Carrot roots of the other two quality classes ('Class II' and 'Non-standard') are marked as a non-marketable share. From mentioned classification, the marketable share of carrot roots (Extra class + Class I) was increased from 80.2% (C) to 84.6% (A). On the contrary, the non-marketable carrot share was decreased from 19.8% (C) to 15.4% (A). The difference of marketable and non-marketable share value between variants C and A was evaluated as statistically non-significant.

Gained results showed that the application of soil biostimulant Agriful has a positive effect on the higher share of marketable carrot roots. This finding is very interesting and important to producer from the aspect of economical profit.

The effect of Agriful to the marketable share of fruits was tested by **Adamec et al. (2019)** in the experiment with two bell pepper cultivars. Authors found more than 20% increase in total fruit yield while only a minimum of fruits was classified in the 'Non-standard class'. In the average of two cultivars, an increase of 'Extra class' (+16%) fruits and a decrease of 'Non-standard class' (-17.5%) were found after Agriful application, compared to the control. The share of pepper fruits in 'Class I' and 'Class II' was relatively comparable between tested variants. Differences between quality classes were like in the study realised with Agriful and carrot.

**El-Helaly (2018)** tested effect of humic and fulvic acids, main components of Agriful, to the marketable yield of carrot roots. Different effect of HA and FA was found. The application of HA resulted in the increase of marketable carrot yield about +20.0% compared to the control variant. On the contrary, application of FA was showed by lower, non-significant decrease of marketable carrot yield about -2.0%. Results of this study were pointed on the more significant effect of HA as a part of biostimulant preparations, compared to the FA. Similar findings were presented in the study with potatoes **(Suh, Yoo and Suh, 2014)**. Within mentioned study, HA application tended to the increase of marketable potato yield about 5.6% compared to the control; on the contrary, no effect of FA application to the marketable yield of potato tubers was found.

## Total carotenoid content

Total carotenoid content (TCC) in carrot roots from both experimental variants is mentioned in the Table 3. After Agriful application, average TCC was at the level of 124.06 $mg.kg^{-1}$ of fresh weight. In the control variant, TCC was 114.07 $mg.kg^{-1}$ f.w. Value of TCC was increased after application of Agriful about 9.99 $mg.kg^{-1}$ f. w. (+8.7%) compared to the control variant. The difference between measured data was evaluated by a paired T-test to the mean value as a statistically non-significant. **Adamec et al. (2019)** studied the impact of Agriful on the TCC in fruits of two bell pepper (*Capsicum annuum* L.) cultivars. In the 'Esperansa F1' cultivar, a decrease of TCC was observed after application of Agriful (-17.03% compared to the control). On the contrary, a positive effect of Agriful application to the value of TCC was found in the 'Kapirex F1' cultivar (+12.2% compared to the control).

There is lack of studies oriented on the effect of Agriful directly; thus, gained results could be compared with studies focused to the effect of humic (HA) and fulvic (FA) acids, which are the main components of Agriful, to the carotenoid content of vegetables. **El-Helaly (2018)** found different effect of HA and FA application to TCC in four cultivars of carrot. Average value of TCC was increased by application of FA (+3.7%) and decreased by application of HA (-23.6%). On the contrary, **Aminifard et al. (2012)** found a positive effect of different HA and FA doses on the content of ß-carotene, dominant carotenoid in bell pepper ('Red Chili') fruits.

**Table 1** Average weight of carrot root and total carrot yield.

| Variant | Average weight of carrot root (g) | Rel. %* | Total yield ($t.ha^{-1}$) | Rel. %* |
|---|---|---|---|---|
| **C – control** | 59.50 ±3.02[a] | 100 | 63.46 ±5.53[a] | 100 |
| **A – Agriful** | 62.16 ±3.09[a] | 104.47 | 65.27 ±3.54[a] | 102.85 |

Note: Same letters (a) within the same column means statistically non-significant difference (at 95.0% confidence level). *Rel. %. – relatively in %.

**Table 2** Classification of carrot roots into quality classes.

| Quality class | C – control (%) | A – Agriful (%) | Yield difference in individual classes (C-A) (%) |
|---|---|---|---|
| **Extra class** | 60.4[a] | 65.8[a] | +5.4 |
| **Class I** | 19.8[a] | 18.8[a] | -1.0 |
| **Class II** | 2.5[b] | 0.6[a] | -1.9 |
| **Non-standard** | 17.3[a] | 14.8[a] | -2.5 |

Note: Different letters (a; b) within the same column means statistically significant difference (at 95.0% confidence level).

**Table 3** Content of total carotenoids and refractometric dry matter.

| Variant | Total carotenoids ($mg.kg^{-1}$) | Rel. %* | Refractometric dry matter (°BRIX) | Rel. %* |
|---|---|---|---|---|
| **C – control** | 114.07 ±18.51[a] | 100 | 9.84 ±0.54[a] | 100 |
| **A – Agriful** | 124.06 ±11.12[a] | 108.76 | 10.24 ±0.28[a] | 104.07 |

Note: Same letters (a) within the same column means statistically non-significant difference (at 95.0% confidence level). *Rel. %. – relatively in %.

Within this study, the increase of ß-carotene content, compared to the control, was ranged from +10.7% to +109.4% (+81.1% on average). In the variant with FA application, the increase of ß-carotene content was ranged from 25.5% to 61.3% (+46.6% on average). Results of this study are focused on the significant effect of HA and FA from the aspect of carotenoid content in bell pepper fruits, or vegetables generally. A stronger effect of HA than FA was also detected. The slight increase of TCC after FA application was also shown and confirmed by another study with bell pepper **(Akladious and Mohamed, 2018)**. On the contrary, a slight decrease of carotenoid due to the FA application was found in the study with wheat **(Akcin, Akcin and Yildirim, 2020)**. **Gholami et al. (2018)** found that HA application was shown by an increase of total carotenoid content from 8.2 $mg.g^{-1}$ (control) to 9.5 $mg.g^{-1}$ (+16.4%) in chicory. Results of mentioned studies were pointed to the strong effect of species and cultivar reaction to the application of HA or FA from the aspect of carotenoid content in consumable parts of grown crops.

## Refractometric dry matter

**Cejpek (2012)** indicates that the method for analysis of refractometric dry matter (RDM) content is used for the determination of sugar content in fruit and vegetable juices, dairy products, and total concentration of monosaccharides and disaccharides in any solutions. According to **Hegedűsová et al. (2018)**, RDM is defined as additive quantity expressing the content of dissolved substances, mostly sugars, in vegetable of fruit extracts. The Brix degree (°BRIX) is used as the unit of RDM content.

Within the realised study, an increase of RDM in carrot roots from 9.84 °BRIX (C - control) to 10.24 °BRIX (A - Agriful) was reached (+4.1%). The difference in RDM content between variants C and A was evaluated by multifactor analysis of variance (ANOVA) as statistically non-significant.

The effect of Agriful biostimulant on RDM content was also tested and demonstrated in the experiment with peach 'Glohaven' **(Staneva, Akova and Kornov, 2020)**. After Agriful application, authors found an increase of RDM, compared to the control, about +37.4%, concretely from 8.3 °BRIX (C) to 10.24 °BRIX (A).

The effect of humic and fulvic acids, basic components of Agriful, was studied in the experiment with bell pepper 'Red Chili'. After HA application, an average value of RDM, dependent on the HA dose, was higher about +5.1% in comparison with the control variant. The application of FA was shown by an increase of RDM in bell pepper about +7.8% compared to the control. Results of this study were pointed to the positive impact of HA and FA application, both important Agriful substances, for increased accumulation of RDM, or sugars generally, in bell pepper fruits. This fact was also confirmed in other studies with different crops. **Yildirim (2007)** found the higher RDM after HA application in the experiment with tomato plants. The increase of its value, compared to the control, was ranged from +11.2% to 18% in dependency on the HA dose and method of its application. **Tejada and Gonzalez (2003)** found an increase of RDM in the asparagus as an effect of foliar HA application. The non-significant increase of sugar content after HA application (+0.6%) and FA application (1.6%) was shown in experiments with sugar beet **(Wilczewski, Szczepanek and Wenda-Piesik, 2018; Braziene, Paltanavicius and Avizienytė, 2021)**. On the contrary, **Shehata et al. (2011)** found a statistically non-significant decrease of RDM in strawberry fruits after HA application.

## CONCLUSION

The results obtained in this experimental work indicate that the application of biostimulant Agriful has a positive effect on important quantitative and qualitative parameters of carrots. The increase of total carrot yield (+2.9%) and an average weight of roots (+4.5%) was evaluated as statistically non-significant. The total yield of carrot roots can be classified into four quality groups based on UNECE standards. According to market requirements in the Slovak Republic, carrot quality classes 'Extra class' and 'Class I' are only marked as a marketable share. Carrot roots of the other two quality classes ('Class II' and 'Non-standard') are marked as a non-marketable share. The marketable share of carrot roots (Extra class + Class I) was increased, statistically non-significantly, from 80.2% (C) to 84.6% (A). This finding is very interesting and important to the producer from the aspect of economical profit. The content of most important bioactive substances in carrot – carotenoids (+8.7%) and content of refractometric dry matter (+4.1%) were also increased, statistically non-significantly, after Agriful application. The obtained research results can be used in further research on biostimulants and it is possible to create a clear recommendation for using Agriful for small growers. It could be more profitable to apply Agriful on carrot plants more times (3 or 4 times) during the vegetation period for an expected higher increase of yield and quality of carrot roots. It should be useful to verify these results in another vegetation period for recommendation to large-scale producers of carrots.

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**THE BIOCHEMICAL CHANGES IN LEGUMES DURING HIGH-TEMPERATURE MICRONIZATION**
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**THE EFFECT OF ANTIOXIDANTS ON XANTHINE OXIDASE ACTIVITY IN FRESH OVINE MILK**
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**CHARACTERISTICS OF GELATIN FROM SKIN AND BONE OF SNAKEHEAD (*CHANNA STRIATA*) EXTRACTED WITH DIFFERENT TEMPERATURE AND TIME**
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**THE EFFECT OF SORGHUM TEMPEH (*SORGHUM BICOLOR* L. *MOENCH*) ON LOW-DENSITY LIPOPROTEIN (LDL) AND MALONDIALDEHYDE (MDA) LEVELS IN ATHEROGENIC DIET-INDUCED RATS**
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**JUSTIFICATION OF THERMODYNAMIC EFFICIENCY OF THE NEW AIR HEAT PUMP IN THE SYSTEM OF REDISTRIBUTION OF ENERGY RESOURCES AT THE ENTERPRISE**
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