

CHARACTERISATION OF LACTOBACILLI FROM EWE'S AND GOAT'S MILK FOR THEIR FURTHER PROCESSING RE-UTILISATION

Miroslav Kološta, Anna Slottová, Maroš Drončovský, Lýdia Klapáčová, Vladimír Kmet', Dobroslava Bujňáková, Andrea Lauková, Gabriel Greif, Mária Greifová, Martin Tomáška

ABSTRACT

Raw ewe's and goat's milk is a good source for isolation of wild lactobacilli which are able to bring unique processing properties in development of dairy products - cheeses or fermented dairy products. 34 strains of lactobacilli were isolated, purified and identified from fermented ewe's and goat's dairy products. These products were processed without thermal treatment and without using of any commercial starters. After preliminary selection, the final collection of 5 strains was established. The strains were identified as: *Lbc. plantarum* (2), *Lbc. paraplantarum* (1), *Lbc. paracasei* (1) and *Lbc. johnsonii* (1). Except two strains, all were able to coagulate milk. After hydrolysis of lactose in milk, two strains were able to form sensorial attractive coagulate too. All of the strains were homofermentative, they produced lactic acid but they did not produce CO₂. Their ability to produce diacetyl was low. They did not show strong proteolytic activity. All strains grew at 30 °C and 37 °C, however *Lbc. johnsonii* much slower at 30 °C than the others. Except *Lbc. johnsonii*, all strains tolerated 2% concentration of NaCl and even in presence of 5% concentration of NaCl their growth was inhibited only moderately. All of characterized strains can be provisionally used as starter or starter adjuncts in dairy technology, during production of cheeses or fermented milk products from pasteurised milk. These results will be used in further processing studies of isolated strains and will be supplemented with other properties e.g. safety, probiotic and antimicrobial properties.

Keywords: ewe's milk; goat's milk; lactobacilli; isolation; technological properties

INTRODUCTION

Addition of lactic acid bacteria (LAB) in cheesemaking process has both advantages and disadvantages. It is associated mostly with heat treatment before their application. The advantages include: safety, uniformity of production thus longer period of expiration. The disadvantage of this attitude is decreasing variability of "wild" lactic acid bacteria for customers. Several corporations that produce wide range of starters and starter adjuncts manage the market. However, they are much narrow than those that are found in spontaneous fermented products. Therefore, it is essential to maintain the adequate balance between both of approaches. Isolation of LAB from typical dairy products for certain region and their technological re-utilisation subsequently is a suitable alternative (Ayada et al., 2004).

Ewe's milk is a substance that is used for manufacture of traditional and EU protected dairy speciality in Slovakia, usually without addition of secondary starters. These include: „Ovčí hrudkový syr - salašnícky“ (TSG) (sheep lump cheese, typically produced in chalets) (OA EC 2010/C20/9) and Slovak Bryndza (PGI) as its final product (OA EC 2007/C 232/10), or žinčica. These products are ideal for isolation of „wild“ LAB. Studies performed with bryndza reported several strains of bacteria, yeasts and filamentous fungi (Berta et al., 2009; Sulo et al., 2009). Most of them have potential utilization as starters or starter adjuncts.

Goat's milk has its own specificity. Because of the risk of encephalitis, it is recommended to consume goat's products only from pasteurized milk (Zavadská et al., 2013). From the other point of view, raw goat's milk is an excellent source of wild microflora, because goats graze wide range of vegetation.

Lactobacilli are significant part of LAB, which are used in cheesemaking process and fermented dairy products. LAB cause rapid decrease of pH through production of organic acids, especially lactic acid. They are also important for production of exopolysaccharides, aromatic substances, bacteriocines and other antimicrobially active compounds. It contributes to improve quality, health safety and sensory properties of dairy products (Leroy and De Vuyst, 2004).

The aim of this study is to isolate of lactobacilli from raw ewe's and goat's milk, followed by characterization of their processing properties: ability to grow in milk, production of organic acids and sensory attractive coagulate, CO₂, diacetyl, optimal growth temperature, resistance to different concentrations of NaCl and proteolysis. These properties are essential for characterization of collection of process-wise suitable lactobacilli from regional specialities that are typical for Slovakia.

MATERIAL AND METHODOLOGY

Presumptive lactobacilli were isolated from products based on raw ewe's and goat's milk by using de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany), after cultivation at 37 °C for 24 h to 72 h. Typical colonies were purified several times and then they were identified by matrix-assisted laser desorption ionization-time-of flight mass spectrometry (MALDI-ToF MS). Bacterial identification MALDI-ToF MS was performed on a Microflex LT instrument (Bruker Daltonik, Leipzig, Germany) as described **Bessedé et al., (2011)**.

MRS medium or raw cow's milk sterilised by autoclaving was used in tests that deal with bacterial growth and production of acids. Lactose was hydrolysed by commercial lactase Maxilact (DSM, Heerlen, The Netherlands) according to the recommendation of the enzyme producer.

Reference HPLC assays of DL-lactic acid and lactose were run on a DeltaChrom liquid chromatograph (Watrex, Bratislava, Slovakia) equipped with Applied Biosystems 759A Absorbance Detector and simultaneously connected to WellChrom K-2301 refractive index detector (Knauer, Berlin, Germany). The analytical conditions were as follows: a column, Polymer IEX in H⁺ form, 250 mm × 8 mm, 5 µm (Watrex, Bratislava, Slovakia); a guard column, Polymer IEX in H⁺ form, 10 mm × 4 mm, 8 µm (Watrex, Bratislava, Slovakia); a column thermostat: DeltaChrom™ Temperature Control Unit (50.0 ± 0.1) °C; a mobile phase, H₂SO₄, 1 mM in demineralized water; flow rate, 1.0 mL·min⁻¹; data were collected and processed by Clarity chromatography station (DataApex, Prague, Czech Republic). The samples were clarified prior to analysis by Carrez I and Carrez II and then they were diluted in mobile phase and filtered through 0.2 µm Chromafil AO filters (Macherey-Nagel, Düren, Germany).

Enzymatic activities were measured by API ZYM assay (bioMérieux, Marcy l'Etoile, France). It was performed according to the manual of the producer.

Effect of NaCl on growth of the strains was evaluated by measuring of the optical density at 600 nm of MRS media with various concentrations of NaCl cultivated at 37 °C. The media were inoculated with 1% overnight cultures. Specific growth rate (μ_m) and lag phase (λ) were calculated from Gompertz function using software TableCurve 2D for Windows (Jandel Scientific, California, USA) and Origin 8.1 (Microcal Software, Northampton, USA) (**Zwietering et al., 1990**).

Production of CO₂ was estimated by observation of gas in Durham tubes, during cultivation of the strains in MRS medium at 37 °C for 24 h.

Production of diacetyl was observed by visual check in milk which was fermented by tested strains at 30 °C and 37 °C for 24 h. 1 mL of fermented milk was than mixed with 0.5 mL 16% KOH (Merck, Darmstadt, Germany) and 0.5 mL 1% α -naphthol (Merck, Darmstadt, Germany) and was incubated at 30 °C for 10 minutes. Colour of the mixture was observed. Pink colour of the mixture indicated production of diacetyl.

Proteolytic activity was estimated by measuring of clear zones around 1.3 cm holes filled with 100 µL concentrated tested strains, in M 17 agar medium (Merck, Darmstadt,

Germany) with addition of 10% of skimmed milk, cultivated at 37 °C for 24 h.

RESULTS AND DISCUSSION

Presumptive lactobacilli were isolated from several milk and dairy products. The products were made from unpasteurised (except „Žinčica“) and only spontaneously fermented sheep and goat milk. After preliminary selection on the elective MRS medium, the strains were identified by MALDI-ToF MS. The results are shown in the Table 1. Besides phenotype identification by MALDI-ToF MS, also further genotype identification of strains is necessary. Therefore classification of strains by 16S rRNA PCR is in the progress nowadays.

The enzymatic activities of strains were characterised by API-ZYM. They are discussed later, mostly from technological point of view. Of course many enzymes are significant also for safety criteria (**Delgado et al., 2008**), but this will be mentioned in a following study.

All strains grew at both temperatures 30 °C and 37 °C, however *Lbc. johnsonii* KB2-1 much slower at 30 °C (Figure 1). The 37 °C is the optimal temperature for growth of lactobacilli, although their metabolic activity can be higher at different temperatures (**Hujanen and Linko, 1996**).

The most important processing properties of lactic acid bacteria, which can be used as starters, is their ability to precipitate milk to the sensory pleasant coagulate in adequate time and temperature. With exception of two strains (*Lbc. casei* 21L10 and *Lbc. paraplantarum/plantarum* 25/1 L) all strains were able to coagulate milk at 37 °C. The explanation is that these two strains showed (according to API ZYM) very low lactase activity. After addition of external lactase, they fermented glucose and galactose well. Production of lactic acid after 48 hours fermentation on sterile cow milk is given in Table 3. The achieved amounts of lactic acid were a bit lower as described (**Zimanová, 2011**), which resulted in good, typical acid-milk taste and smell of coagulates, without any significant foreign flavour. Of course addition of lactase enhanced sweet taste of coagulates.

Diacetyl flavour was not observed, since *Lbc. casei* 21L10 did not produce it and the rest of the strains formed only negligible amounts.

The strains produced only lactic acids, no CO₂. Therefore, they can be considered as homofermentative. These species of lactobacilli are usually facultative heterofermenters (*Lbc. plantarum*, *Lbc. paraplantarum* and *Lbc. casei*) or obligatory homofermenters (*Lbc. johnsonii*) (**Stiles and Holzappel, 1997**).

The tested strains (except *Lbc. johnsonii* KB2-1) were not very sensitive to NaCl in model MRS medium. Although the concentration of 5% NaCl slowed their growth at 37 °C, typical concentration of 2% NaCl in hard and semi-hard cheeses did not inhibit them. Specific growth rates, lag phases according to strains and NaCl concentrations are summarized in Table 4. Similar observations were also published by **Zimanova, 2011**, where some strains were even stimulated at the presence of 2% NaCl.

Table 1 Identification of lactobacilli by MALDI-Tof MS

Strain	Identification 1st match	Identification 2nd match
17L1	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>
18L2	<i>Lactobacillus plantarum</i>	<i>Lactobacillus plantarum</i>
21L10	<i>Lactobacillus paracasei</i>	<i>Lactobacillus paracasei</i>
KB2-1	<i>Lactobacillus johnsonii</i>	<i>Lactobacillus gasseri*</i>
25/1L	<i>Lactobacillus paraplantarum</i>	<i>Lactobacillus plantarum</i>

* Score below 2, it means worse probability of identification

Table 2 Enzymatic characterisation of lactobacilli by API ZYM (strain identification see Table 1)

Activity* / strain	17L1	18L2	21L10	KB2-1	25/1L
Control	0	0	0	0	0
Alkaline phosphatase	0	0	1	1	1
Esterase (C4)	1	1	2	1	2
Esterase Lipase (C8)	1	1	2	2	2
Lipase (C14)	1	0	1	1	1
Leucine arylamidase	5	3	5	5	2
Valine arylamidase	5	3	5	1	1
Cystine arylamidase	1	0	1	1	1
Trypsin	1	1	0	1	1
α-chymotrypsin	1	0	1	1	1
Acid phosphatase	2	2	4	1	2
Naphthol_AS-BI-phosphohydrolase	1	2	3	3	1
α-galactosidase	1	0	0	3	1
β-galactosidase	5	5	1	4	1
β-glucuronidase	0	0	0	0	0
α-glucosidase	4	3	1	2	2
β-glucosidase	2	2	1	3	1
N-acetyl-β-glucosaminidase	2	2	1	3	1
α-mannosidase	0	0	0	0	1
α-fucosidase	2	1	1	0	1

*Values (0-2) are negative, (3-5) are positive

Table 3 Production of lactic acid by fermentation on sterile cow milk at 37 °C (strain identification see Table 1)

Strain	0 (h)		24 (h)		36 (h)		48 (h)	
	Lactic acid (g.100g ⁻¹)	pH						
17L1	0.00	6.59	0.16	5.57	0.31	5.18	0.39	4.84
18L2	0.00	6.59	0.22	5.25	0.36	4.96	0.42	4.76
21L10	0.00	6.59	0.01	6.07	0.03	6.10	0.08	5.84
21L10*	0.00	6.41	0.54	4.53	0.73	4.20	0.82	3.97
KB2-1	0.00	6.59	0.23	5.17	0.34	4.94	0.42	4.90
25/1L	0.00	6.59	0.06	5.95	0.09	5.80	0.17	5.54
25/1L*	0.00	6.41	0.30	5.06	0.50	4.69	0.64	4.56

* After hydrolysis of lactose in milk

Table 4 Growth of lactobacilli in MRS medium with various concentration of NaCl at 37 °C (strain identification see Table 1)

Strain	0% NaCl		2% NaCl		5% NaCl	
	$\mu_m(h^{-1})^*$	$\lambda (h)^{**}$	$\mu_m(h^{-1})^*$	$\lambda (h)^{**}$	$\mu_m(h^{-1})^*$	$\lambda (h)^{**}$
17L1	0.358	5.2	0.305	6.2	0.107	10.0
18L2	0.311	5.2	0.323	6.0	0.059	7.4
21L10	0.228	3.4	0.237	4.2	0.100	6.9
KB2-1	0.298	6.7	0.148	9.2	No growth	No growth
25/1L	0.315	7.4	0.297	8.6	0.158	13.2

* Specific growth rate, ** Lag phase

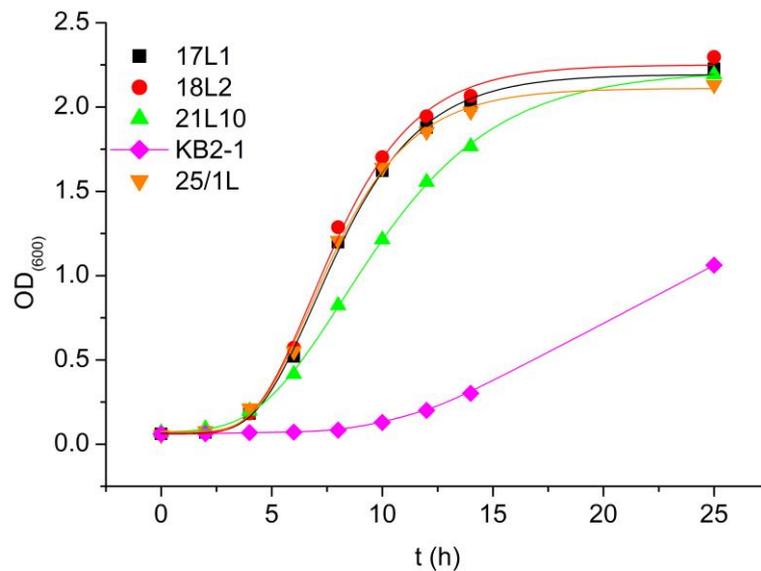


Figure 1 Growth of lactobacilli strains at 30°C in MRS medium (strain identification see Table 1)

The screening for the proteolytic activity showed only negligible proteolysis of the strains. The diameters of clear zones were from 1.4cm to 1.7cm.

Based on API ZYM result, (see Table 2) activities of trypsin and α -chymotrypsin were very low. However all strains, except *Lbc. paraplantarum/plantarum* 25/1L, exhibited positive leucine arylamidase activity and the strains *Lbc. plantarum* 17L1, *Lbc. plantarum* 18L2 and *Lbc. paracasei* 21L10 also exhibited positive valine arylamidase activity. Cystine arylamidase was negative. Proteolytic activity should be confirmed during ripening of model cheese production, using a suitable method e.g. OPA (reaction of amino groups from amino acids with o-phthaldialdehyde).

CONCLUSION

Five strains of lactobacilli isolated from goat's and ewe's milk showed different technological properties.

All the strains (with exception of *Lbc. johnsonii* KB2-1) grew well at typical temperatures used in dairy technology: 30 °C and 37 °C.

Lbc. johnsonii KB2-1 is suitable for production of fermented dairy drinks because his growth was

most affected by addition of NaCl. The rest of the strains tolerated NaCl up to concentration of 5%. Therefore, they are also suitable for cheesemaking when higher salt concentration is expected.

Lactase activity of two strains – *Lbc. casei* 21L10 and *Lbc. paraplantarum/plantarum* 25/1L was a bit lower. This handicap can be eliminated either by external addition of lactase (in production of fermented dairy drinks) or by appropriate co-cultivation with other starters like lactococci (in process of cheesemaking).

No one of the strains produced CO₂. From among organic acids, they only produced lactic acid.

If typical diacetyl flavour is requested, it will be necessary to use beside these cultures also other strains – e.g. leuconostocci.

Lower proteolytic activity of all strains can be an advantage during cheesemaking because sensory problematic bitter peptides may not be formed.

Created collection of five strains of lactobacilli can be used as starters or starter's adjuncts in manufacture of dairy products made from pasteurised milk.

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Contact addresses:

Miroslav Kološta, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina Slovakia, E-mail: kolosta@vumza.sk

Anna Slottová, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina Slovakia, E-mail: slottova@vumza.sk

Maroš Drončovský, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina Slovakia, E-mail: chemia@vumza.sk.

Lýdia Klapáčová, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice Slovakia, E-mail: klapacol@gmail.com

Vladimír Kmeť, Institute of Animal Physiology Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice Slovakia, E-mail: kmetv@saske.sk

Dobroslava Bujňáková, Institute of Animal Physiology Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice Slovakia, E-mail: dbujnak@saske.sk

Andrea Lauková, Institute of Animal Physiology Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice Slovakia, E-mail: laukova@saske.sk

Gabriel Greif, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina Slovakia, E-mail: gabriel.greif@stuba.sk

Mária Greifová, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina Slovakia, E-mail: maria.greifova@stuba.sk

Martin Tomáška, Dairy Research Institute, JV, Dlhá 95, 010 01 Žilina Slovakia, E-mail: tomaska@vumza.sk