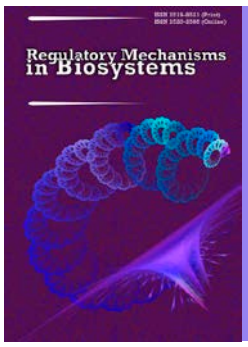

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Strains of lactic acid bacteria isolated from traditional Carpathian cheeses

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Traditional national fermented products and cheeses are a source for the search for species and strains of lactic acid bacteria (LAB) which are not within the range of bacterial agents used in the dairy industry. Classical and modern genetic-molecular methods are used to identify LAB isolated from such products. The purpose of our work was isolation and identification of LAB from traditional Carpathian cheeses made from ewe's milk and the study of their technological properties. Three samples of cheese were selected for our research – one sample of brine cheese bryndza and one sample of budz (bryndza before salting), produced in the highlands of the Carpathians and one sample of buts, produced in the foothills zone. 106 cultures were isolated from the samples of cheese. Genus and species identification was completed using classical microbiological and molecular genetics methods. Based on the complex of tinctorial, cultural, physiological and biochemical indices, the LAB isolated were assigned to the following genera and species: *Lactococcus* spp. (26 cultures), including *L. lactis* (13 cultures) and *L. garvieae* (13 cultures); *Lactobacillus* spp. – *L. plantarum* (31 cultures); *Enterococcus* spp. – *E. faecium* (25 cultures); *Leuconostoc* spp. – *L. mesenteroides* (24 cultures). These results were confirmed by molecular genetics methods. The largest range of species was found in a sample of bryndza from the Carpathian highlands. The isolated cultures were studied according to technological properties – milk-coagulation activity, acid-forming ability and resistance to different concentrations of kitchen salt. Most strains of *L. lactis* ssp. *lactis*, *L. plantarum* and *L. mesenteroides* were active acid-forming agents and coagulated milk in 3–9 hours, while *L. garvieae* and *E. faecium* coagulated milk after more than 24 hours. More than 80% of cultures showed resistance to 4% of kitchen salt solution, *E. faecium* was observed to have the highest salt tolerance. The results of RAPD typing showed significant intra-species heterogeneity, which indicates the need for further research on identification of individual strains. In all samples of cheese, *L. lactis*, *L. garvieae*, *E. faecium* were detected, which shows that they are typical representatives within the traditional Carpathian bryndza. Particular attention was paid to *E. faecium*, as many researchers have indicated probiotic properties of individual strains, as well as the ability to synthesize volatile substances that enrich the flavor bouquet of cheeses. Today strains of *E. faecium* are involved in the bacterial composition of starter cultures for cheeses.

Keywords: cheese bryndza; cheese buts; tinctorial properties; polymerase chain reaction; RAPD-typing

Introduction

Traditional dairy products are made from raw milk using non-industrial methods, and contain specioes and strains of lactic acid bacteria (LAB), which are currently not included in fermentation starter preparations used in the dairy industry. These bacteria may be capable of valuable technological (Zhong et al., 2016) and probiotic properties (Natarajan and Parani, 2015; Zhang et al., 2016, 2017). Therefore, in recent years, researchers have focussed on these bacteria, first of all, as strains which are promising for industrial use (Ortakci et al., 2015; Oberg et al., 2016; Culumber et al., 2017). Strains with probiotic properties are included in bacterial preparations for producing dairy products with functional properties, and also for producing medical preparations (Chaharovskyy & Zholkevskaya, 2003; Kigel', 2003; Diduh et al., 2008). Microbiota of traditional dairy products are a subject of interest for research from the perspective of natural populations, which were formed over a prolonged period in certain geographical regions (Bao et al., 2012; Liu et al., 2012). Between the cultures in such populations, symbiotic interrelations occur (Kimoto-Nira et al., 2012); this can be a model for constructing new microbial compositions. It is important not to lose such natural populations. Unfortunately, the composition and properties of the bacteria of domestic traditional fermented milk products and cheese, made in non-industrial conditions in Ukraine remain unresearched, and the number of such products continues to decrease.

One of the most popular national dairy products is bryndza, brined cheese made in the Carpathians from raw sheep milk directly in the pastures where the sheep are grazed from May to October. Creating bacterial preparations for industrial production of bryndza out of pasteurized milk would allow inoculation of bryndza using pure cultures, which are typical for raw milk. This, in turn, would allow one to obtain a safe product while maintaining the taste and the aroma of the traditional cheese (Neviani et al., 2009; Zhong et al., 2016), which is an daily product of consumption for the inhabitants of the Carpathians and a favorite product for many consumers. We should emphasize the fact that using an industrial bacterial preparation is a precondition for controlling the technological processes and receiving the product with the desired properties.

Until now, the selection of strains for bacterial preparations have used mostly classical microbiological methods. The identification and assessment of the natural ecological niches isolated from the microbiological cultures were made studying their morphological, cultural, physical-chemical and technological properties (Ao et al., 2012). The

achievements of the past decades in microbiology, genetics and molecular biology allow the genetic diversity of certain species of microorganisms to be characterised. The development of the systematics of microorganisms on the basis of molecular-genetic methods oriented towards studying the bacterial genome, and accumulation of experimental data enable a number of controversial problems to be solved regarding the taxonomical position of particular groups of microorganisms (Kovalenko & Lashhevskyj, 2003; Giraffa et al., 2010; Vasyljuk et al., 2014). Modern biotechnology is inextricably linked to using new approaches to selecting the natural strains of microorganisms for the composition of bacterial preparations (Podgorskyj & Kovalenko, 2002; Naumenko, 2005; Mayo et al., 2014). The new approaches include the selection of strains, for the strains possess features which are essential for industrial use in food products. These features, first of all, include safety, i.e. no pathogenic factors and no signs of resistance to antibiotics (Amaral et al., 2016; Zhang et al., 2016). Some features of the strains are related to the probiotic properties, i.e. ability to synthesize the bacteriocins (Ao et al., 2012; Goh & Philip, 2015), survival in the conditions of the gastrointestinal tract and manifestation of adhesive properties (Amaral et al., 2016) and decrease in the level of cholesterol in the blood (Zhang et al., 2017). The objective of our research was the isolation and identification of LAB in traditional Carpathian cheese, made of sheep milk, and study of their properties.

Materials and methods

The article analyzes LAB, isolated from three samples of traditional sheep cheese, bryndza and budz, selected in different geographical regions of the Carpathians and made from milk of different sheep breeds (Table 1).

Table 1
Origin of cheese samples

Kind of cheese	Designations of samples hereinafter	Location of cheese selection	Sheep breed
Bryndza	A	Putyla, Chernivtsi Oblast (highland)	Ukrainian Carpathian
Budz*	B	Putyla, Chernivtsi Oblast (highland)	Ukrainian Carpathian
Budz*	C	Dana farm, Koteleve village, Chernivtsi Oblast (pre-mountain area)	Bucovina type of Karakul breed

Note: * – Budz sheep cheese according to the technology is Bryndza before salting.

For isolating the bacteria, we selected one gram of cheese from each sample, and homogenized it in 9 ml of sterile saline solution. The bacteria were inoculated onto solid growth mediums M_{17} and MRS (Himedia, India) using the method of ten-fold serial dilutions (10^{-1} to 10^{-6}) of the analyzed material. The inoculations were carried out in three parallels. They were cultivated in a thermostat at the temperatures of 30, 42 ºC over 48 hours. All studied cultures were inoculated at least twice into MRS and M_{17} agar. The strains of lactate bacteria were maintained in 0.5 ml of MRS or M_{17} nutrient broth, which included LAB cultures, which were mixed with 50% sterile glycerol and frozen to –80 °C. The numberof lactate microorganisms were calculated using the standard method according to GOST 104444.11-89. Food products. Methods for identification of the lactic acid bacteria.

The study of morphological characteristics of the LAB cultures used preparations stained using Gram's method. Microscopy was made using immersion oil and ×1350 zoom. We determined the size, pattern of staining, cell position, absence of unfavourable (external) microflora and changed forms in the smear.

We chose only gram-positive and catalase-negative isolates, which were maintained at the temperature –80 °C in sterile MRS broth with addition of 15% glycerine. Frozen bacterial cultures were used for further identification. The working cultures were revived from frozen into working cultures by making two inoculations in MRS broth at the temperature of 45 °C.

The differential characteristics for determination of genus were conducted using the complex of tinctorial, cultural and physical-biochemical features of the studied LAB strains. The main criteria for the differentiation were: the ability to release CO_2 when the media is cultivated with glucose, fermentation of a particular range of carbohydrates, hydrolysis of arginine (Harrigan & McCance, 1976), growth at different temperature regimes 10, 15 and 45 °C, and also capability of growth in the presence of 2.0, 4.0, 6.5% NaCl (Kvasnikov & Nesterenko, 1975).

Biochemical properties of the LAB were studied in accordance with a range of fermentation of carbohydrates using Hiss culture media (Himedia, India). The cultures were inoculated to the half-liquid media using the method of inoculation loop. For the determination of biochemical properties, an amount of 2% of the following carbohydrates was added to the Hiss media: arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, sucrose, sorbitol, trehalose and xylose.

For accurate identification of LAB of "wild" microbiota of the traditional Carpathian cheese, which was studied for the first time, we used polymerase chain reaction (PCR). The isolation of genome DNA was made using a Genomic Mini (A&A Biotechnology) set in accordance with the manual. The DNA samples were analyzed qualitatively and quantitatively, using a NanoDrop 2000 (Thermo Scientific, USA) spectrophotometer. The DNA samples were maintained at the temperature of 20 °C for further use.

The DNA fragments, which included the 16S pPHK gene, were amplified using the following universal primers:

EGE1 (5-AGAGTTTTGATCCTGGCTCAG-3),
1492R: 5'-TACGGYTACCTTGTTACGACTT-3'.

Every reaction mixture for PCR contained 2 µl (50 ng) of DNA, 1 µl 10 µm of dNTP mixture, 1 µl 10 µm of every primer, 5 µl of 10xPCR-buffer (Fermentas, Lithuania) and 1.25 U Taq DNA-polymerase (Fermentas) with addition of up to 50 µl of sterile Milli-Q water.

The DNA amplification programme consisted of primary denaturalization at the temperature of +96 °C for 5 min, 35 cycles (96 °C for 30 s, 52 °C for 45 s, 72 °C over 1.5 min). The final stage of polymerization was carried out at the temperature of 72 °C during 10 min.

Disintegration into fragments was carried out using restrictive endonucleases, according to the manual. For the disintegration into restrictive fragments, we used the following ferments: HinfI, RsaI (Roche, Switzerland), Sau3A (Fermentas) i HhaI (Takara); they were used in separate reactions. All disintegrations were carried over a period of 2 hours at 37 °C. The fragments were divided in 2 agarose gel.

RAPD-PCR was carried out in a volume of 25 µl, which included 1 µl (~ 25 ng) of each DNA, 1 µl 10 µm of dNTP, 1 µl 10 µm of primer 1254 (5'-CCGCAGCCAA-3'), 2.5 µl of 10xPCR (Fermentas), 1.5 µl 50мM of $MgCl_2$ and 0.75 U of Taq DNA polymerase (Fermentas). The PCR programme consisted of 4 cycles (5 min at the temperature of 96 °C, 5 min at 36 °C and 5 min at 72 °C), then 30 cycles (1 min at 96 °C, 1 min at 36 °C, and 2 min at 72 °C). The final 10-minute extension was conducted at 72 °C. After the amplification 20 µl of PCR product was divided in 2 agarose gel.

The 16S r RNA area was amplified using polymerase chain reaction (PCR) as described previously. The amplified fragments were cleaned using Gel Purification GPB Mini Kit (GenoPlast, Poland) according to the manufacturer's instruction and sequenced in a Genomed (Poland) commercial service. The determination of sequencing was made in both directions with the same primers, which were used for amplifying 16S r DNA. The sequencing of 16S rRNA was compared with the sequencing of the fragments from the NCBI base (www.ncbi.nlm.nih.gov). The search for similarities between the sequenced fragments was conducted using the BLAST algorithms, available at the www.ncbi.nlm.nih.gov/blast.

The statistical analysis was made using the Statistica 6 (StatSoft Inc., USA) software. The difference between the data was considered significant at $P < 0.05$ (ANOVA).

Results

We established that the total number of LAB (Table 2), isolated from the sample of bryndza (A), was 1.5–1.9 times less than for the samples of budz (B and C). Such difference is explained by the effect on the

survivability of the LAB caused by the table salt, which composed 5.5% of the bryndza. The samples B and C were unsalted budz cheese.

Table 2
The number of lactate bacteria which
were isolated from samples (CFU/g, x ± SE, n = 3)

Bryndza (A)	Budz (B)	Budz (C)
$4.4 \pm 0.12 \cdot 10^5$	$6.7 \pm 0.13 \cdot 10^5$	$8.5 \pm 0.12 \cdot 10^5$

Lactate bacilli were grown on a MRS dense growth media, forming white or grey colonies of 1 to 5 mm diameter, sometimes of lenticular or starlike shape. The surface of the colonies was mostly smooth and shiny (S-shape), though in some cases there were rugged colonies (R-shape). The incubation was made at the temperature of 37 °C. The lactic acid cocci were observed to have a distinctive growth on a dense media M_{17} in the form of rounded and cymbiform colonies. The rounded colonies with distinct edges were formed on the surface of the growth media, whereas the cymbiform colonies grew slightly into the agar. The bacteria were incubated at 25 and 42 °C.

For studying the cheese samples, we selected 106 isolates of lactic acid bacteria. All isolated cultures were gram-positive and catalase-negative. The bacterial cultures were identified at the level of genus on the basis of their cell morphology, fermentation of carbohydrates, the ability to release CO_2 when cultivated in the media with glucose, hydrolysis of arginine, growth at the temperature of 10, 15 and 45 °C, and also capability of growth in the presence of 2.0, 4.0, 6,5% NaCl according to Wood & Holzapfel (1995). We found that the isolated cultures belong to four genera (Table 3).

Table 3
Tinctorial, cultural and physical-biochemical features
of lactate cultures isolated from traditional sheep cheese

Indicators	Genus			
	Lactobacillus	*Lactococcus*	*Leuconostoc*	*Enterococcus*
The number of isolated cultures	31	26	24	25
Morphology	bacilli	cocci	cocci	cocci
Staining	gram-positive	gram-positive	gram-positive	gram-positive
Catalase activity	absent	absent	absent	absent
Release of CO_2 while growing in a media with glucose, number of cultures / %	0	0	24 / 100	25 / 100
Arginine hydrolysis, number of cultures / %	0	26 / 100	0	25 / 100
Fermentation of carbohydrates, number of cultures / %				
arabinose	24 / 77	0 / 0	16 / 67	12 / 48
fructose	31 / 100	26 / 100	24 / 100	25 / 100
galactose	31 / 100	26 / 100	24 / 100	25 / 100
glucose	31 / 100	26 / 100	24 / 100	25 / 100
lactose	31 / 100	26 / 100	24 / 100	25 / 100
maltose	31 / 100	17 / 65	24 / 100	25 / 100
mannitol	31 / 100	0 / 0	0 / 0	23 / 92
mannose	31 / 100	26 / 100	24 / 100	25 / 100
raffinose	31 / 100	0 / 0	24 / 100	6 / 24
sucrose	31 / 100	4 / 15	24 / 100	25 / 100
sorbitol	26 / 84	0 / 0	0 / 0	0 / 0
trehalose	31 / 100	26 / 100	24 / 100	25 / 100
xylose	8 / 26	0 / 0	12 / 50	0 / 0

According to a complex of tinctorial, cultural and physical-biochemical features, we found that the cultures which grew at the temperature of 10 and 15 °C in the presence of 6.5% NaCl, but not at 45 °C, with no gas release when grown in the media with glucose and capability of arginine hydrolysis were lactate acid bacilli. Cocci-like bacteria, but with prolonged body shape, which often occur in pairs or as short chains, grew at the temperature of 10 °C, but not at 45 °C and had no capability of arginine hydrolysis; they released CO_2 while grown in the media with glucose, which indicates heterofermentative fermentation. These bacterial cultures were identified as *Leuconostoc*. All other cocci hydrolyzed agrinine, not releasing CO_2 when grown in a medium with glucose and grew at the temperature of 10 °C and in the presence of 4% NaCl. Some of them were able to grow at the temperature of 45 °C, and in the presence of 6.5% NaCl, therefore were classified as *Enterococcus*, whereas the isolates, which did not grow at the temperature of 45 °C, and in the presence of 6.5% NaCl were classified as lactate cocci.

From the bryndza sample, we isolated 68 cultures, 31 of them belong to the following genera: *Lactobacillus* (46%), 5 – *Lactococcus* (7%), 24 – *Leuconostoc* (35%), 8 – *Enterococcus* (12%). From the sample of budz prepared in the highlands, we isolated 10 cultures of the *Lactococcus* genus, which were 56% of the total and 8 cultures of *Enterococcus* genus (44%). The LAB of the budz sample prepared in pre-mountain area also belong to two genera: *Lactococcus* (11 cultures, 55%) and *Enterococcus* (9 cultures, 45%). In the sample of bryndza, we found representatives of four genera, whereas in two other samples, we found only two. It should be noted that all samples of cheese had a high content of *Enterococcus* bacteria.

According to the results of set of bacteriological methods, the studied LAB cultures were classified as follows: *L. lactis* ssp. *lactis* (13 cultures) and *L. garvieae* (13 cultures); *L. plantarum* (31 cultures); *E. faecium* (25 cultures); *L. mesenteroides* ssp. *mesenteroides* (24 cultures).

It was found that about 90% and 93% strains of *L. plantarum* grew at the temperature of 10 °C and 15 °C, whereas no growth was observed at 45 °C. Similar growth was observed in *L. lactis* ssp. *lactis* − 83% and 87% at the temperature of 10 and 15 °C respectively; in *L. garvieae* − 81% and 89% and in *L. mesenteroides* – 85% and 92%. It should be noted that the highest activity of growth was observed among *E. faecium* strains (87% and 90% at the temperature of 10 and 15 °C, and also 90% at 45 °C). The results of the tests are given in Table 4.

Most strains of *L. lactis* ssp. *lactis* (over 90%) were active acidifiers and coagulated milk in 3–9 hours, whereas *L. garvieae* and *E. faecium* were less active, most of them coagulated milk in more than 24 hours (Table 5).

Table 4
Growth of lactate acid bacteria in relation
to the temperature and concentration of NaCl (number of strains in %)

Species of lactate bacteria	Growth at the temperature			Growth at the concentration of NaCl, %		
	10 °C	15 °C	45 °C	2.0	4.0	6.5
L. plantarum (n = 31)	90.3	93.4	0.0	97.6	86.6	58.8
L. lactis ssp. *lactis* (n = 13)	82.8	87.5	0.0	95.9	83.7	0.0
L. garvieae (n = 13)	80.6	89.4	0.0	96.4	84.6	0.0
E. faecium (n = 25)	87.5	89.7	98.6	100.0	98.5	93.3
L. mesenteroides (n = 24)	85.1	92.3	0.0	97.3	89.2	43.5

Table 5
Milk-coagulating and acidifying activity of strains of lactate bacteria

Species of lactate bacteria	Milk-coagulating activity		Acidifying activity		
	the speed of bunch formation, hours	number of strains, %	titrated acidity, °T	active acidity, IU pH	number of strains, %
L. plantarum (n = 31)	3	5.5	100–120	4.8	19.4
	6	30.0	90–100	5.0	41.9
	9	42.9	80–90	5.3	35.5
	>24	22.6	60–80	5.5	3.2
L. lactis ssp. *lactis* (n = 13)	3	15.2	100–120	4.8	15.3
	6	25.5	90–100	5.0	46.2
	9	51.8	80–90	5.3	30.8
	>24	7.5	60–80	5.5	7.7
L. garvieae (n = 13)	3	0.0	100–120	4.8	7.6
	6	7.7	90–100	5.0	23.1
	9	23.1	80–90	5.3	38.5
	>24	69.2	60–80	5.5	30.8
E. faecium (n = 25)	3	0.0	100–120	4.8	8.0
	6	20.4	90–100	5.0	19.8
	9	26.5	80–90	5.3	35.2
	>24	53.1	60–80	5.5	37.0
L. mesenteroides (n = 24)	3	0.0	100–120	4.8	8.3
	6	12.5	90–100	5.0	12.5
	9	41.7	80–90	5.3	25.0
	>24	45.8	60–80	5.5	54.2

However, it is important that all isolated cultures manifested a milk-coagulating property. The bordering acidity of *L. lactis* ssp. *lactis* and

L. plantarum was 120 °T, over 61% of strains of these species caused the acidity of milk up to 90–120 °T. Only 30%, 28% and 21% of *L. garvieae*, *E. faecium* and *L. mesenteroides* strains relatively brought the acidity of milk to the mentioned values. However, the composition of these cultures is capable of providing milk acidity sufficient for formation of a bunch during the cheese making.

Figure 1 provides an electrophoretogram of disintegration of amplified DNA fragments by restrictive enzymes. The analysis of profiles of fingerprints of the studied cultures' DNA fragments amplified by the HinfI, RsaI, Sau3A and HhaI enzymes showed the presence of five species: *L. plantarum*, *L. lactis*, *L. garvieae*, *L. mesenteroides*, *E. faecium*. Every enzyme proved the presence of these species. The results of RAPD typing coincide with the results received using bacteriological methods.

Conducting the polymerase chain reaction using the 1254 primer allowed isolation of five primary clusters (I–V) with the level of similarity of 51–85%. The results of RAPD typing found significant intraspecific heterogeneity of the studied LAB, which indicates the necessity of further studies in identification of LAB at the level of strains. The RAPD-PCR results are provided in Figure 2.

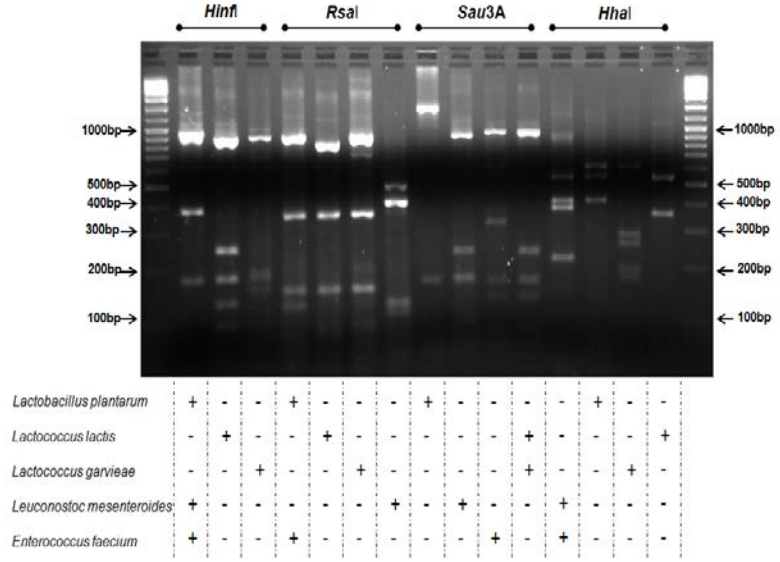

Fig. 1. Electrophoretogram of disintegration of amplified DNA fragments by Hinf1, Rsa1, Sau3A, Hhal restrictive enzymes: the first and the last tracks are markers of molecular mass of the DNA fragments, every track divides the mixture of DNA; "+" indicates the presence of the species

Discussion

Bryndza and budz cheese, which have been made from sheep milk in the Carpathians for centuries, have a specific taste and aroma, which are formed due to fermentative activity of the bacteria involved in the cheese making. Milk with natural content of LAB is highly rated in the production of different kinds of cheese made out of either raw or pasteurized milk. Raw sheep milk is highly polluted bacterially, which is related to the conditions in which it was obtained. Apart from the LAB content, it usually contains undesirable bacteria. For safety of sheep cheese, the milk should be pasteurized; however this causes death also to the LAB bacteria, which could have valuable technological and probiotic properties. Rendering the LAB composition which is typical for original Carpathian cheese in bacterial preparations for industrial production, is a subject of interest and an important issue.

Out of three samples of Carpathian cheese made in the highlands and in pre-mountain zone, 106 LAB cultures were isolated. The total number of LAB was the lowest in bryndza, which included 5.5% of salt, whereas the two other samples did not contain salt. However, the sample of bryndza is distinctive for the highest bacterial diversity, including four genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*), which were represented by five species (*L. lactis* ssp. *lactis*, *L. garvieae*, *L. plantarum*, *E. faecium*, *L. mesenteroides* ssp. *mesenteroides*). LAB isolated from the samples of non-salted budz cheese were represented by only three species (*L. lactis* ssp. *lactis*, *L. garvieae*, *E. faecium*) and two genera respectively (*Lactococcus* and *Enterococcus*). We did not isolate cultures of other two species (*L. plantarum* and *L. mesenteroides* ssp. *mesenteroides*). This, in our opinion, is related to the high salt-resistance. Low salt content and insignificant representation in fresh cheese allowed the bacteria to survive and successfully compete with less salt-resistant species during salting, ripening and maintaining of bryndza in 18% brine, which is part of the production technology. The impact of salt was also proven by the lower overall of LAB in bryndza. It should be noted that the two samples of budz have similar representation: in two samples, we isolated 55–56% cultures of *Lactococcus* genus and 44–45% of *Enterococcus*. This leads us to think that there are no significant differences in the LAB content of raw sheep milk between the highland and pre-mountain areas of the Carpathians. However, the study in this direction should be continued due to reports on the differences in the content of "wild" microbiota in relation to the geography of the making of traditional fermented products, though on a significantly larger scale regarding both the products and location – China, Mongolia, and Russia (Zhong et al., 2016).

LAB isolated from all samples of cheese belong to mezophilic and thermophilic cultures, which coincides with the reports by Poznanski et al. (2004), who studied the bacterial composition of the traditional national cheese made in the conditions of alpine tundra.

Representatives of the *Enterococcus* genus made up a significant part of the total LAB content of all samples of cheese. Currently, the representatives of *Enterococcus* genus are used in the content of fermentation starter preparations for many fermented products (Goh & Philip, 2015), mostly cheese (Neviani et al., 2009; Natarajan et al., 2015), made in South and North Europe out of either raw or pasteurized milk. The presence of *Enterococcus* during the ripening of cheese has a positive effect on their sensory properties (Gatti, 2014), they improve the taste and aroma and prolong the time of maintenance (Franz et al., 2003). The most common species of *Enterococcus* isolated from the cheese were the following species: *E. faecium*, *E. faecalis* and *E. durans* (Belicov et al., 2007; Serio et al., 2007; Veljovic et al., 2007). Some *E. faecium* strains manifest probiotic properties (Saarela et al., 2000; Ammor et al., 2007; Franz et al., 2011; Amaral et al., 2016).

It is worth mentioning that Ukrainian scientists were among the first to determine that *E. faecium* strains manifest probiotic properties. This led to the creation with their participation of Gerolakt, a lactate gerontological nutrition product (Kovalenko et al., 1994). However, the *E. faecium* strains include pathogenic and antibiotic-resistant strains; therefore the study of priobiotic properties should be preceded by study of the safety of separate strains for humans. It should be noted, that there are clear differences in the safety of certain *Enterococcus* strains in relation to the source of isolation, the strains with virulent genes are isolated from ill people, and not from food products (Moreno et al., 2006). Currently, the domestic industry does not use bacterial pre-

parations with representatives of the *Enterococcus* genus, unlike West-European countries, where, as mentioned before, they are included in microbial compositions for cheese production.

According to the results of biochemical fermentation of carbohydrates, all strains of *Enterococcus* fermented galactose. Decreasing the level of galactose is one of modern trends of the dairy industry, for galactose is undesirable in food products for various reasons. Galactose is badly metabolized in the human organism, which could have a negative effect on the well-being of people who suffer lactase deficiency (Novelli & Reichardt, 2000). Accumulation of galactose in dairy products can cause negative effects: darkening of cheese, release of CO_2 by bacteria which are not included in a fermentation starter preparation, development of purulent or pathogenic microflora (Vaillancourt et al., 2004). Therefore, selection of LAB for producing dairy products prove significance of their both technologic and probiotic properties (Goh & Philip, 2015; Natarajan & Parani, 2015; Zhang et al., 2017).

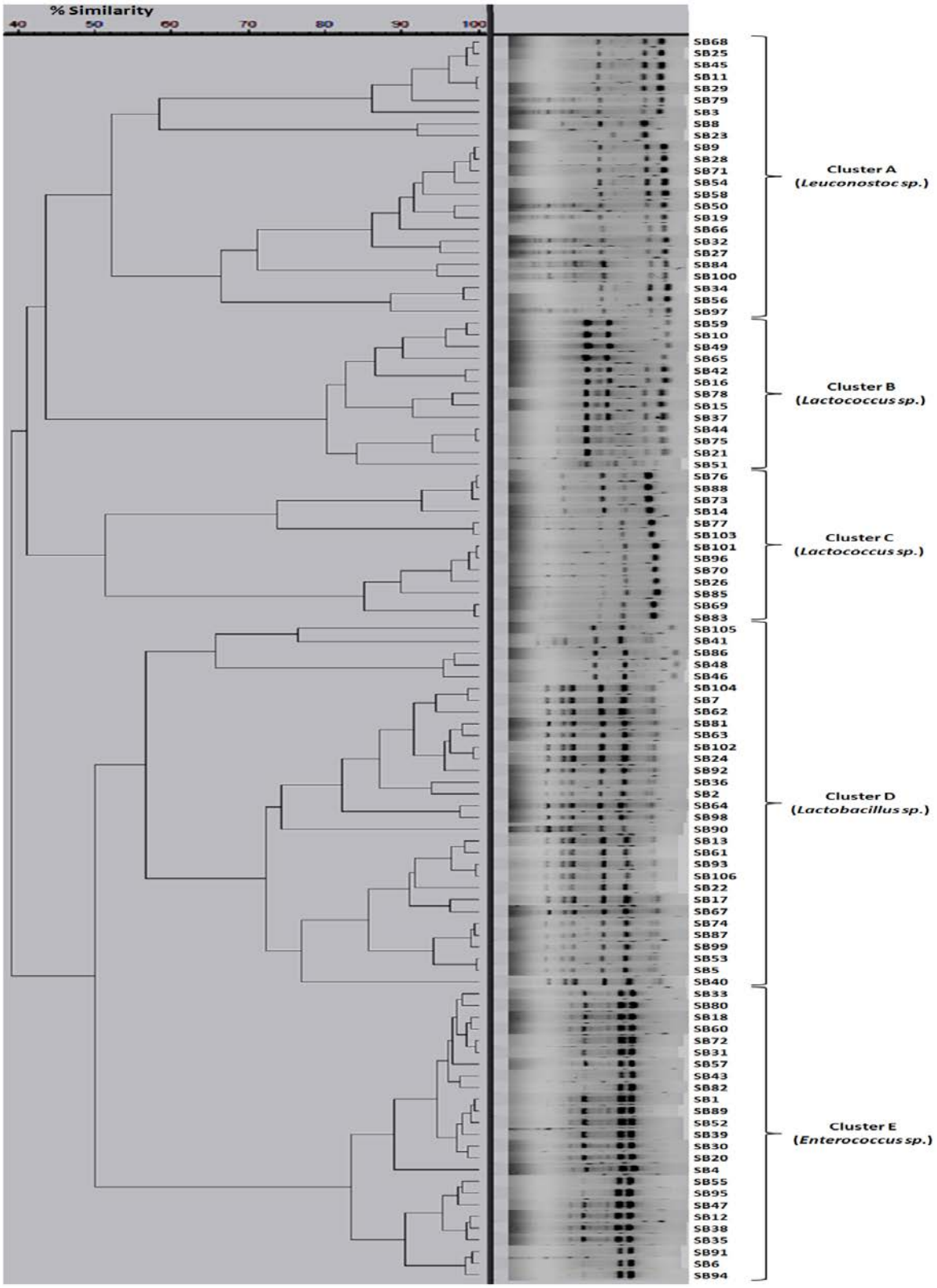

Fig. 2. Electrophoretogram of the products of DNA amplification using 1254 primer

Bacteria of heterofermentative *L. mesenteroides* is typical for home-made cheese of different countries. Their presence is explained by their role in the process of cheese ripening due to proteolytic and lipolytic activity and synthesis of aromatic compounds (Cocolin et al., 2004; Giraffa et al., 2010). The presence of *L. garvieae* can be considered an important part of microbial compound of bryndza, which is related to natural fermentation. The presence of *L. garvieae* is typical for traditional dairy products in different countries, especially Italy (Fortina et al., 2007). *L. garvieae* is a typical culture for production of home-made cheese in this country. Differential characteristic, based only on bacteriological methods, does not always provide the possibility of accurate identification of strains, and sometimes of microorganism species. The ability of some LAB strains to manifest fermentative properties which differ from the properties described for a given species, is a common phenomenon, which complicates the definition of their taxonomic position (Kovalenko & Lashhevskij, 2003; Mayo et al., 2014). The objective of the study was the traditional Carpathian cheeses, previously unstudied , therefore we used molecular-genetic methods, which a number of researchers consider necessary (Yu et al., 2011, 2012), to prove our results of the LAB identification.

To summarize, the microflora of cheese A was determined to be the most diverse in representatives of the LAB species and genera, involved in its making. It should be noted that out of three samples of cheese made from milk of different sheep breeds in different regions with different climatic conditions, we isolated three species of lactate acid bacteria: *L. lactis* ssp. *lactis*, *L. garvieae* and *E. faecium*. Therefore, we consider this microflora compound distinctive for traditional Carpathian bryndza.

Conclusions

We conducted genus and species level identification of LAB isolated from traditional Carpathian cheeses, bryndza and budz, using classic microbiological and molecular-genetic methods. Using a set of tinctorial, cultural and physical-biochemical features, we identified the following LAB bacteria: *L. lactis*, *L. garvieae*, *L. plantarum*, *E. faecium*, *L. mesenteroides*. This was proven by the results of molecular-genetic studies. Using polymerase chain reaction with 1254 primer, we determined significant intraspecific diversity of LAB strains isolated from the samples of cheese. This indicates the necessity of identification of strains and studying their properties.

The sample of bryndza from the Carpathian highlands had the largest species representation. These species can be considered as promising for developing a bacterial preparation for making brined cheese in industrial conditions, for they manifest high resistance to 4% concentration of NaCl and are quite active acidifiers – titrated acidity of milk after their inoculation is 60 to 120ºT.

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