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ANALYSIS OF THE LACTIC ACID BACTERIA MICROFLORA IN TRADITIONAL CAUCASUS COW'S MILK CHEESES

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Abstract — A total of 157 lactic acid bacteria (LAB) were isolated from three hand-made cheeses taken from different households in the region of the Caucasus Mountains. The cheeses were manufactured from cow's milk without the addition of a starter culture. The isolates of LAB were characterized by subjecting them to phenotypic and genotypic tests. The results of identification of LAB indicate that the examined cheeses contained 10 species, viz., *Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus arizonensis, Lactobacillus farciminis, Lactobacillus brevis, Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc pseudomesenteroides, Enterococcus faecium*, and *Enterococcus faecalis*. The strains within the species *L. plantarum, L. arizonensis, L. paraplantarum, L. farciminis*, and *L. pseudomesenteroides* showed good proteolytic activity.

Key words: Caucasus, cheeses, proteolytic activity, rep-PCR; 16S rDNA

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INTRODUCTION

The population of lactic acid bacteria (LAB) was examined from three cheese samples produced in the region of the Baksan Valley in the Kabardino-Balkaria Republic. The cheeses belong to the group of semi hard cheeses manufactured from raw cow's milk without the addition of a starter culture.

The Caucasus is a vast mountain area consisting of distinct ecological zones. It stretches for 1500 km from the Black Sea to the Caspian, representing the conventional boundary dividing Europe from Asia. The Kabardino-Balkaria Republic is a federal unit of Russia, located on the northern slopes of the central part of the Main Caucasian Ridge and the adjacent plains. This region has one of the world's longest traditions of making fermented milk products. The very distinctive vegetation of the Baksan Valley (birch, ash, and pine forests and meadows rich in flowers) and its specific climate (spring and summer weather is mainly dry, warm, and clear; winter and autumn are cold, dry, and clear) contribute to the formation of a specific epiphyte microflora which from pastures directly enters milk and dairy products (Poznanski et al., 2004). Different types of traditional cheese made from milk possess unique and different microfloras, depending on the production technology as well as ecological characteristics of the localities where they are produced (Centeno et al., 1996; Prodromou et al., 2001; Flórez et al., 2006; Mustafa, 2006; Terzic-Vidojevic et al., 2007; Duan et al., 2008). The local microflora of the milk affects biochemical characteristics of the cheeses (Demarigny et al., 1997; Prodromou et al., 2001; Marino et al., 2003) and contributes to organoleptic characteristics of the final product (Caplice and Fitzgerald, 1999). The biochemical, genetic, and technological characterization of LAB isolated from traditional cheeses is of great importance in identifying novel strains with phenotypic and genetic characteristics different from ones already known (Weerkamp et al., 1996; Corroler et al., 1998; Desmasures et al., 1998; Fitzsimons et al., 1999; Delgado and Mayo, 2004).

The region of the Caucasus is a place where longevity of the native inhabitants is well known. The alimentation of those people is mostly based on dairy products. They are one of the best sources of calcium and of essential nutrients that prevent osteoporosis and minimize the risk of colon cancer (Fuller, 1994). It is also known that fermented dairy products are a source of LAB having certain probiotic qualities (Sanders, 1994). It follows that the significant presence of fermented dairy products in the alimentation of habitants of the Caucasus may be one of the reasons for their longevity. These considerations prompted our interest in examination of LAB isolated from Caucasus cheeses.

In the present study, LAB strains were isolated from three different cheese samples produced in the traditional way in distant households located in the Baksan Valley in villages at different altitudes in the Caucasus region. Isolated LAB were characterized by subjecting them to different phenotypic and genetic tests, with the focus on species that are likeliest to play an important role in the fermentation process of those cheeses. To determine their taxonomic status, rep-PCR and 16S RNA gene sequencing of analyzed isolates was also carried out. To our knowledge, there are no available data about the LAB population in cheeses from the Caucasus region.

MATERIALS AND METHODS

Cheese making and sampling. The cheeses studied in this work were produced in two villages located in the Baksan Valley. The cheese samples designated as BGKAVS2 and BGKAVS3 were taken from two different households in the village of Zayukovo, which is situated in the distal part of the Baksan Valley at an altitude of 645 m. The BGKAVS4 cheese sample was taken from the village of Terskol, situated at an altitude of 2281 m.

All three cheese samples were manufactured from raw cow's milk, without the addition of starter cultures. Rennet produced in a household by drying a ruminant's stomach was added to milk warmed at a temperature of 30-40°C. A piece of dried stomach was immerged in brine 2-3 days before making the cheese and then kept in a refrigerator. One glass of rennet (about 250 ml) prepared in this way was added to 10 l of milk. The curdling lasted for 5-10 min (for the cheese BGKAVS4) and 30 min for the cheeses BGKAVS2 and BGKAVS3. The created curd was drained in a strainer for 10-12 h (for the cheeses BGKAVS2 and BGKAVS3) or drained by occasional twisting of the curd on a plate over which brine was poured (for the cheese BGKAVS4). The cheese was then salted with dry salt in an amount constituting 3-5% of the quantity of gained cheese. The obtained cheese was kept in a cool room (at about 10-15°C) and covered with clean cloth. It can be eaten the following day or covered for a few more days in a cool room.

The cheese samples selected for microbiological analyses were sampled in sterile conditions and put in a refrigerator until they were delivered to the laboratory. The cheeses BGKAVS2, BGKAVS3, and BGKAVS4 were 24, 5, and 17 days old, respectively.

Bacterial strains, media, and growth conditions. The list of reference strains used in this study is shown in Table 1. *Lactobacillus* and *Leuconostoc* strains were grown in MRS broth (pH 5.7) (Merck GmbH, Darmstadt, Germany), while *Lactococcus* and *Enterococcus* strains were grown in M17 broth (pH 7.2) (Merck GmbH) supplemented with 0.5% (w/v) glucose (GM17 broth). A solid medium was obtained by adding 2% (w/v) agar (Torlak, Belgrade, Serbia) to broth. The plates were incubated 24-48 h under appropriate incubation conditions that varied depending on the strain. Purified strains of LAB were stored at -80°C in appropriate media containing 15% glycerol (w/v).

Isolation and phenotypic characterization of lactic acid bacteria. Isolation of LAB was done from 20 g of each cheese sample taken from the interior of the cheese, homogenized with a pestle in a sterile mortar, and transferred to 180 ml of sterile 2% (w/v) tri-sodium citrate solution. Decimal dilutions (10^{-1}-10^{-7}) were prepared with sterile 0.85% (w/v) sodium chloride. One milliliter of these dilutions was pour-plated on the respective media. For isolation of lactococci and lactobacilli, GM17 and MRS agar plates were used, respectively. Incubation of agar plates was performed under aerobic as well as anaerobic

Table 1. Reference strains used in this study for rep-PCR with (GTG)5 primer. ATCC - American Type Culture Collection, Rockville, MD, USA. LMG - Bacteria Collection, Laboratorium voor Microbiologie - Universitet Gent, Gent, Belgium. NRRL - Agricultural Research Service Culture Collection, Peoria, IL, USA. (a) These strains were identified by molecular methods, AFLP, SDS-PAGE, and rep-PCR with (GTG)5 primer in the Laboratorium voor Microbiologie, Universitet Gent, Gent, Belgium.

Reference strain	Source or reference
Lactobacillus plantarum LMG9206	LMG
Lactobacillus plantarum LMG9208	LMG
Lactobacillus plantarum LMG9219	LMG
Lactobacillus plantarum LMG18024	LMG
Lactobacillus plantarum LMG11475	LMG
Lactobacillus brevis ATCC14869	ATCC
Lactobacillus brevis LMG11438	LMG
Lactobacillus brevis LMG18022	LMG
Lactococcus lactis subsp. *lactis* NP45	Laboratory collection
Enterococcus durans BGZLS20-35b[a]	Laboratory collection[a]
Enterococcus faecium BGGJ8-3[a]	Laboratory collection[a]
Enterococcus faecalis BGZLS60-26a[a]	Laboratory collection[a]
Leuconostoc mesenteroides subsp. *mesenteroides* NRRLB-512	NRRL
Leuconostoc mesenteroides subsp. *mesenteroides* NRRLB-1118	NRRL
Leuconostoc mesenteroides subsp. *mesenteroides* NRRLB-3470	NRRL

conditions at 30 and 45°C for 3-5 days. Anaerocult A (Merck GmbH) was used to obtain anaerobic conditions in anaerobic jars.

Enumeration of total mesophilic and thermophilic bacteria was performed using MRS and GM17 agar plates and incubation at 30 and 45°C for 72 h. Results are expressed as colony-forming units (CFU) per gram of cheese.

Fifty to eighty colonies per sample were randomly picked from both MRS and GM17 agar plates (30 and 45°C) corresponding to the highest dilution at which growth occurred. The colonies were purified twice to three times by streaking on respective fresh agar plates. After the catalase test, Gram staining, and microscopic observations (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany), a total of 157 Gram-positive and catalase-negative isolates were chosen for further analyses.

The following physiological tests were used for phenotypic characterization of all 157 LAB isolates: growth in GM17 and MRS broth at different temperatures (15, 30, and 45°C); and the ability to grow in GM17 and MRS broth with different concentrations of NaCl (2, 4, 6.5, and 8%). The tests were repeated three times. Also performed was testing for: gas production from glucose by sub-culturing the isolates in modified MRS broth (De Man et al., 1960) in tubes containing inverted Durham bells; hydrolysis of L-arginine; hydrolysis of esculin; citrate utilization; production of acetoin from glucose (the Voges-Proskauer or V.P. test) (Zourari et al., 1991); growth and production of slime from sucrose on MSE agar plates (Mayeux et al., 1962); formation of

a black zone on bile-esculin agar (BEA) (Himedia, Mumbai, India) – only for cocci-like LAB; diacetyl production – only for LAB which coagulated casein; and activity in milk and in litmus milk (Prescott et al., 1996).

Identification of LAB isolates based on their phenotypic characteristics was performed according to the methods and criteria of Sharpe (1979), Hardie (1986), Kandler and Weiss (1986), Mundt (1986a, 1986b), and Sneath et al. (1986).

Proteolytic activity. The ability of the chosen LAB isolates to degrade β-casein was established according to the procedure described by Kojic et al. (1991). Fresh cells were collected (10 mg with an approximate density of 10^{10} cells/ml) and resuspended in 0.1 mol/l sodium-phosphate buffer, pH 6.5. The cell suspension was mixed with β-casein (5 mg/ml in 0.1 mol/l sodium-phosphate buffer, pH 6.5) (Sigma, St. Louis, MO, USA) and incubated for 3 h at 30°C. After incubation, the cells were pelleted by centrifugation (5 min at 13000 rpm), and the clear supernatant was taken and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out on 12.5% polyacrylamide gel. Gels were stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany) and destained in a mixture of methanol (20%) and acetic acid (7%).

Rep-PCR analysis and 16S rDNA sequencing of LAB isolates. The method of total DNA isolation and purification described by Hopwood et al. (1985) was applied in this study.

Identification of all 157 LAB isolates was done by rep-PCR analysis. For rep-PCR analysis, total DNA from isolates of LAB was used as a template for PCR amplifications with the $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) oligonucleotide primer, with its optimal PCR program (Versalovic et al., 1994). The PCR reaction mixtures (50 µl) consisted of 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 3 mmol/l $MgCl_2$, 50 mmol/l each of the four deoxynucleotide triphosphates (dNTP), 1 U of *Taq* polymerase, and 5 pmol/l of primer (Fermentas UAB, Vilnius, Lithuania). Template DNA (1 µg) was added to the reaction. The samples were amplified in GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) programmed as follows: initial denaturation of DNA for 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and extension of incomplete products for 7 min at 72°C. The obtained PCR products were quantified by electrophoresis on 1% agarose gel containing ethidium bromide and visualized by a Biometra BDR2/5/6 CCD camera (Bio Doc Analyze GmbH, Göttingen, Germany).

For sequencing of 16S rDNA, total DNA from the chosen LAB isolate, was utilized as a template for PCR amplifications with the U968 (5′-AACGCGAAGAACCTTAC-3′) and L1401 (5′-GCGTGTGTACAAGACCC-3′) primers (Zoetendal et al., 1998; Randazzo et al., 2002), using the same PCR reaction mixture (50 µl) as described previously (Terzic-Vidojevic et al., 2007). The obtained PCR product was purified with the aid of the QIAquick PCR Purification Kit/250 (Qiagen GmbH, Hilden, Germany) and sequenced by the Macrogen Sequencing Service (Seoul, Korea). The BLAST algorithm was used to determine the most closely related sequence relatives in the NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS AND DISCUSSION

The indigenous microfloras of milk play a fundamental role in the fermentation process during cheese manufacturing and exert significant influence on the biochemical and organoleptic characteristics of the cheese. Cheeses made from raw milk following traditional procedures are generally designated as "artisanal". Although many studies of the microfloras of artisanal cheese have been published, exploration of cheeses produced in inaccessible areas is still of great interest. Dairy products manufactured in such localities could eventually be a source of LAB bearing new properties different from those already used in the dairy industry as starter cultures. In this study, the LAB population of artisanal cheeses made in the traditional way in households in the region of the Caucasus Mountains has been examined. Microbiological characteristics of those cheeses were hitherto unknown.

Table 2. Total number of viable bacteria in cheese samples from the Caucasus region grown on MRS and GM17 agar plates. Abbreviations: ND - not detected. (a) Average values of three independent experiments.

Cheese samples	CFU/g per sample[a]			
	Total count of bacteria		Total count of molds	
	GM17	MRS	GM17	MRS
BGKAVS2	$2.56x10^8$	$2.13x10^8$	$3.2x10^1$	$1.8x10^2$
BGKAVS3	$2.22x10^8$	$3.47x10^7$	$2.1x10^1$	$1.3x10^2$
BGKAVS4	$1.73x10^8$	$2.96x10^7$	ND	$4.0x10^2$

The counts of viable microorganisms in three different cheese samples from the Caucasus are shown in Table 2. The total count of bacteria in cheese samples was in the range of 10^8 CFU/g on GM17 agar plates and between 10^7 and 10^8 CFU/g on MRS agar plates. These values correspond to those reported by Fitzsimons et al. (2001), Veljovic et al. (2007), and Kagkli et al. (2007). On MRS and GM17 media, besides the growth of bacteria, growth of molds was noticed too. The total number of molds was 10^1 CFU/g on GM17 agar plates and 10^2 CFU/g on MRS agar.

Classical and molecular approaches were used to investigate 157 isolates belonging to different species of LAB. Physiological tests showed that in three different cheeses from the Caucasus region, five types of bacteria were identified: *L. plantarum*, *L. brevis*, *Leuconostoc* spp., *L. lactis*, and *Enterococcus* spp. All five types of bacteria were detected in BGKAVS3 cheese, whereas *L. lactis* was not detected in BGKAVS2 cheese. In addition, *L. brevis* and *Enterococcus* spp. were not identified in BGKAVS4 cheese (Table 3).

Among various microorganisms involved in cheese making, lactobacilli play the major role in primary cultures, their function being to produce lactic acid from lactose (Bernardeau et al., 2008). Many species of mesophilic lactobacilli have been isolated from cheeses, but the ones most frequently encountered were *Lactobacillus casei*, *Lactobacillus paracasei*, *L. plantarum, Lactobacillus rhamnosus*, and *Lactobacillus curvatus* (Beresford et al., 2001). The results of microbiological analysis show that in all three Caucasus cheese samples tested, *L. plantarum* is more present than the other species of this genus. Dominant presence of *L. plantarum* has been seen in the microfloras of cheeses originating from Egypt (Ayad et al., 2004), Morocco (Ouadghiri et al.,

Table 3. Number of LAB isolated from each Caucasus cheese sample identified by physiological tests.

Species	Number of isolates in cheese samples		
	BGKAVS2 (24 days old)	BGKAVS3 (5 days old)	BGKAVS4 (17 days old)
L. plantarum	55	17	32
L. brevis	6	1	0
Leuconostoc spp.	6	7	4
L. lactis	0	16	9
Enterococcus spp.	3	1	0
Total number of isolates	70	42	45

Table 4. Physiological characteristics of isolated LAB from three different Caucasus cheese samples. Abbreviations: NT - not tested. (a) More than 90% of the strains. (b) Between 10 and 90% of the strains. A - acid production, C - curd formation, R - litmus reduction, P - purple (unchanged reaction).

Tests	Lactic acid bacteria				
	L. plantarum	*L. brevis*	*Leuconostoc* spp.	*L. lactis*	*Enterococcus* spp.
Morphology	Rods	Rods	Coccoid	Cocci	Cocci
Growth at 15°C	+[a]	+	+	+	+
Growth at 30°C	+	+	+	+	+
Growth at 45°C	-[a]	-[a]	-	-	+
Growth in 2% NaCl	+	+	+	+	+
Growth in 4% NaCl	+	+	+	+	+
Growth in 6.5% NaCl	+	+[b]	-[a]	-	+
Growth in 8% NaCl	+[a]	-	-	-	±[a]
Hydrolysis of arginine	-	+	±	+[a]	+
Utilization of citrate	+[b]	-	-	-	-
Hydrolysis of esculin	+[b]	-[a]	+[a]	±[a]	+
Production of CO_2	-	+	+	-	-
Production of diacetyl	-	-	-	-[a]	-
V.P. test	+	-	-	-[a]	+
Black zone on BEA	NT	NT	NT	-	+
Slime on MSE agar	-	-	-[a]	-	-[a]
Activity in milk (h)	24[a]	No activity	No activity	24[a]	24
Litmus milk	ACR	P	P	ACR	ACR
Number of strains	104	7	17	25	4

2005), Azerbaijan (Terzic-Vidojevic et al., unpublished data), and Tibet (Duan et al., 2008). On the other hand, in cheeses originating from Northern Europe (Fitzsimons et al., 2001; Østlie et al., 2004), Spain (Arizcun et al., 1997; Pérez-Elortondo et al., 1998; Ortigosa et al., 2006), Italy (Marino et al., 2003), and the Balkans (Terzic-Vidojevic et al., 2007; Nikolic et al., 2008), the most common species is *L. paracasei.*

The principal physiological characteristics of LAB isolates investigated in this work are summarized in Table 4. According to data given by Kandler and Weiss (1986), Centeno et al. (1996), and Badis et al. (2004), the species *L. plantarum* is not supposed to survive the presence of 6.5% NaCl in the growth media. Interestingly, our results showed that most of the tested *L. plantarum* strains isolated from Caucasus cheeses showed good growth in the presence of high NaCl concentrations (6.5 and 8%). The activity in milk of almost all LAB isolates was very weak. Only four isolates that curdled skimmed milk within 8 h of incubation could be distinguished from the BGKAVS3 cheese sample (one of *L. lactis* and three of *L. plantarum*). Arizcun et al. (1997) reported previously that the majority of lactococci strains are slow acid producers. It was determined that three isolates from the cheese BGKAVS3

Table 5. Identification of isolated LAB by rep-PCR with (GTG)5 primer and 16S rDNA sequencing. (a) Demonstrated identity with 16S rDNA sequences of relevant species deposited in GenBank database (NCBI).

Isolates	Identification by rep-PCR	Identification by 16S rDNA sequencing	(% identity)[a]
BGKAVS2-1	*Enterococcus* spp.	*E. faecalis*	(99%)
BGKAVS2-13	*L. plantarum*	*L. plantarum/L. pentosus*	(87%)
BGKAVS2-20	*L. plantarum*	*L. paraplantarum*	(99%)
BGKAVS2-41	*L. brevis*	*L. brevis*	(94%)
BGKAVS2-43	*L. plantarum*	*L. pentosus/L. plantarum*	(90%)
BGKAVS2-46	*L. plantarum*	*L. plantarum*	(94%)
BGKAVS2-48	Unidentified	*L. farciminis*	(98%)
BGKAVS2-50	*L. brevis*	*L. brevis*	(99%)
BGKAVS2-58	*L.. brevis*	*L. brevis*	(99%)
BGKAVS2-70	*L. plantarum*	*L. pentosus/L. plantarum*	(99%)
BGKAVS3-2	*L. lactis*	*L. lactis* subsp. *lactis*	(99%)
BGKAVS3-4	*L. lactis*	*L. lactis* subsp. *lactis*	(99%)
BGKAVS3-18	*Lactococcus* spp.	*L. lactis* subsp. *lactis*	(99%)
BGKAVS3-23	*Leuconostoc* spp.	*L. pseudomesenteroides*	(100%)
BGKAVS3-31	Unidentified	*L. arizonensis*	(99%)
BGKAVS3-35	Unidentified	*L. brevis*	(99%)
BGKAVS3-37	*Leuconostoc* spp.	*L. mesenteroides* subsp. *mesenteroides*	(99%)
BGKAVS3-38	*L. plantarum*	*L. plantarum/L. pentosus*	(87%)
BGKAVS3-54	Unidentified	*E. faecium*	(94%)
BGKAVS3-67	*L. plantarum*	*L. plantarum/L. pentosus*	(99%)
BGKAVS4-1	*L. lactis*	*L. lactis* subsp. *lactis*	(99%)
BGKAVS4-8	*Leuconostoc* spp.	*L. mesenteroides* subsp. *mesenteroides*	(99%)
BGKAVS4-9	*L. plantarum*	*L. plantarum*	(99%)
BGKAVS4-12	Unidentified	*L. plantarum/L. pentosus*	(99%)
BGKAVS4-23	*Leuconostoc* spp.	*L. mesenteroides*	(99%)
BGKAVS4-25	*L. brevis*	*L. brevis*	(99%)
BGKAVS4-58	*L. plantarum*	*L. arizonensis*	(98%)

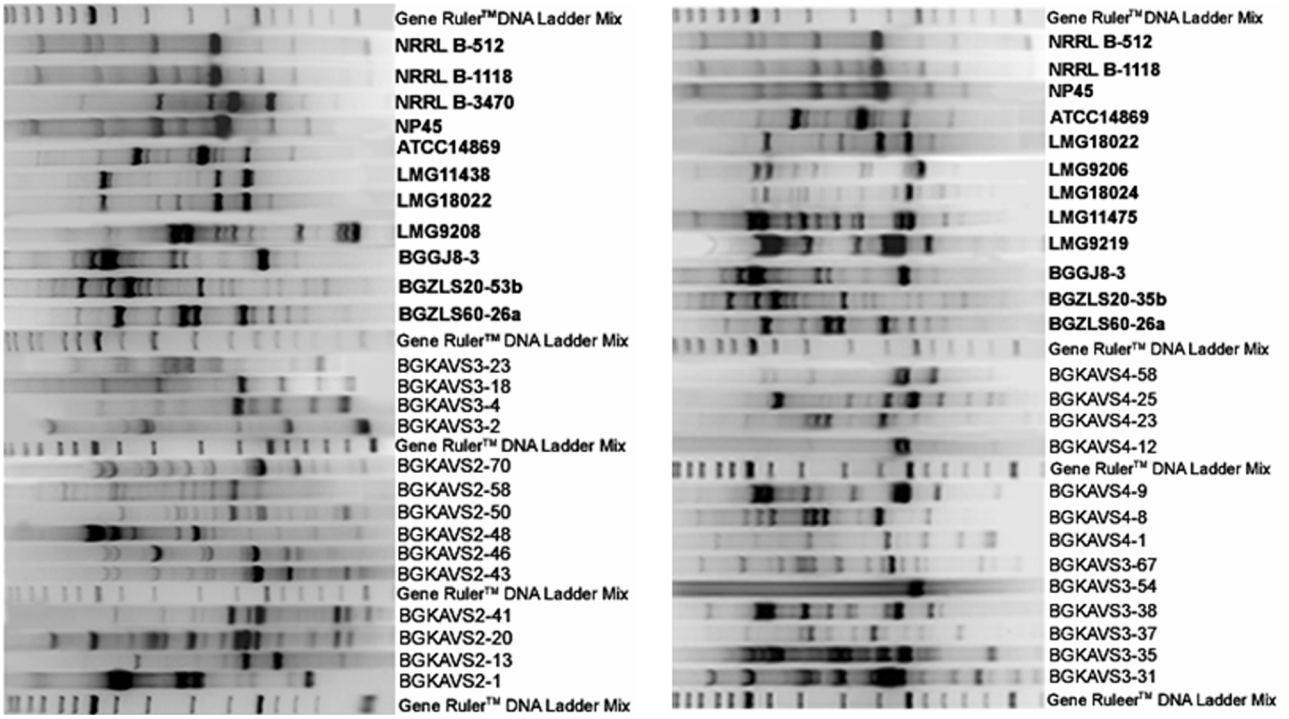

Fig. 1. Analysis by rep-PCR with (GTG)5 primer of chosen lactic acid bacteria isolated from Caucasus cheeses. Reference strains used in the test are given in bold letters.

(*Leuconostoc* spp.) created slime when growing on an MSE agar plate, as well did two lactococci isolates from the cheese BGKAVS4.

Identification of LAB by physiological tests as well as by rep-PCR was done for all 157 isolates. The results showed that rod-shaped LAB belonged mainly to the species *L. plantarum* (Table 5 and Fig. 1). After that, 22 groups of isolates were formed according to their rep-PCR patterns and some physiological characteristics. The rep-PCR patterns of five isolates were quite different and did not match up with those of any referent strain. One representative of each group (22 isolates) as well as five unidentified isolates were therefore subjected to 16S rDNA sequencing. The 16S rDNA analysis of strains revealed the presence of 10 LAB species in the examined cheeses, viz., *L. plantarum*, *L. paraplantarum*, *L. arizonensis*, *L. farciminis*, *L. brevis*, *L. mesenteroides*, *L. pseudomesenteroides*, *L. lactis* subsp. *lactis*, *E. faecalis*, and *E. faecium* (Table 5). One of isolates, BGKAVS2-20, identified as *L. plantarum* on the basis of its rep-PCR pattern, did not grow in broth with 8% NaCl, which distinguished this isolate from the other isolates of *L. plantarum*. Interestingly, 16S rDNA sequencing showed that this isolate is *L. paraplantarum*.

The coccal-shaped LAB isolated from Caucasus cheese samples belong to lactococci and enterococci. Lactococci are ubiquitous in the environment and in food. Most natural isolates of lactococci belong to the *lactis* subspecies (Ayad et al., 2004; Ouadghiri et al., 2005; Veljovic et al., 2007; Nikolic et al., 2008). *Lactococcus* spp. was isolated from cheeses BGKAVS3 (16 isolates) and BGKAVS4 (nine isolates) (Table 3), and they were all identified as *L. lactis* subsp. *lactis*. Lactococci were the most abundant only in 5-day-old BGKAVS3 cheese, which is in agreement with the results reported by Sharpe et al. (1966) and Macedo et al. (2004). They indicated that the coccal-shaped lactic acid bacteria decrease towards the end of a 28-day period of cheese ripening, while the lactobacilli increase, probably due to their ability to grow at low pH and to resist higher salt concentrations than lactococci. In addition, Mangia et al. (2008) reported that the number of *L. lactis* subsp. *lactis* was the highest in curd of Fiore

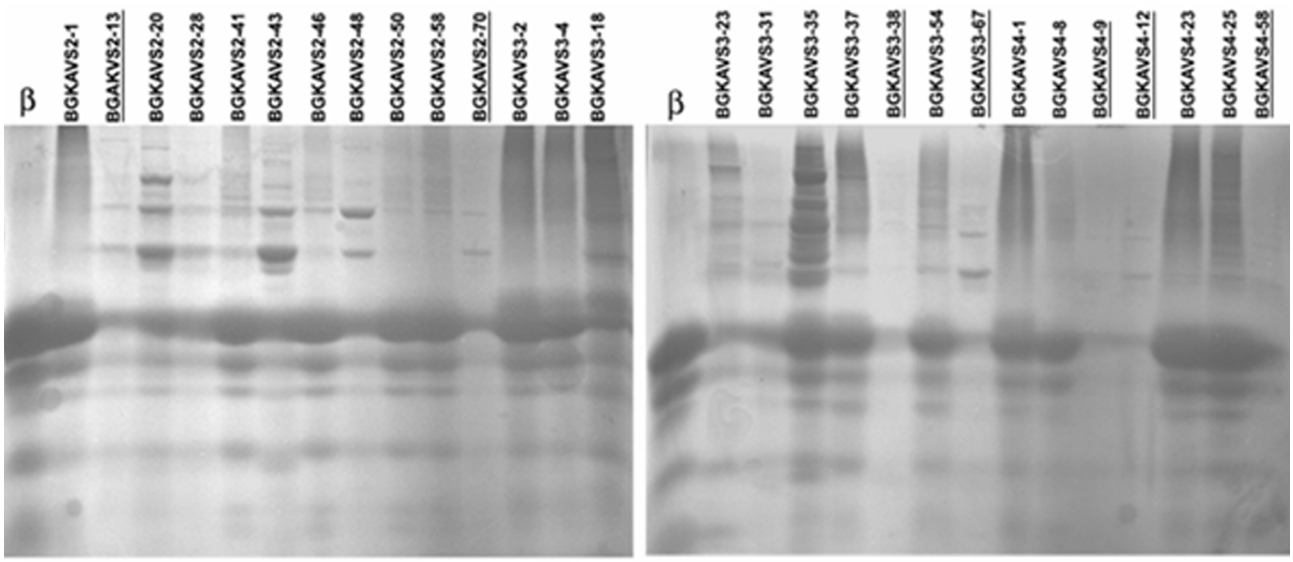

Fig. 2. Proteolytic activity of chosen lactic acid bacteria isolated from Caucasus cheeses. β - substrate β-casein.

Sardo cheese after 5 days of ripening, after which the number of lactococci decreased. Representatives of the genus *Enterococcus* were isolated from cheeses BGKAVS2 and BGKAVS3, but in a smaller number than *L. lactis* species (Table 3). Three enterococcal isolates from cheese sample BGKAVS2 belonged to the species *E. faecalis*, and one isolate from cheese sample BGKAVS3 was identified as the species *E. faecium*.

Leuconostocs are very often present in numerous tested varieties of raw milk cheeses. Their role in the formation of aroma and texture is essential in some cheeses (Hemme and Foucaud-Scheunmann, 2004). Our results indicated that *Leuconostoc* spp. are present in all three analyzed Caucasus cheese samples. A significant number of leuconostocs was also detected in Tibetan Qula cheese (Duan et al., 2008).

The ability to produce extracellular proteinases is a very important feature of LAB. These proteinases catalyze the initial steps in the hydrolysis of milk proteins, providing the cell with the amino acids that are essential for growth of LAB (Kunji et al., 1996). This capability is also very important for curd formation, as well as for flavor development. According to the results regarding proteolytic activity of the tested *L. plantarum* strains from Caucasus cheeses, BGKAVS2-13, BGKAVS2-70, BGKAVS3-38, BGKAVS3-67, BGKAVS4-9, and BGKAVS4-12 isolates, as well as the isolate BGKAVS4-58 (which was identified as *L. arizonensis*) showed the best proteolytic activity (Fig. 2, underlined). Significant proteolytic activity of lactobacilli was also described by Parra et al. (1996) in the curd system of goat's milk, in lactobacilli isolated from traditional Pecorino Sardo cheese (Mannu et al., 2000), and in lactobacilli found in goat's milk from different Algerian races (Badis et al., 2004).

It was shown previously that *Leuconostoc* spp. did not exhibit proteolytic activity (Badis et al., 2004). Similar results were obtained in the analysis of most leuconostocs isolated from Caucasus cheeses. However, the isolate BGKAVS3-23, identified as *L. pseudomesenteroides*, showed very good ability to degrade β-casein.

Representatives of *L. brevis*, *L. lactis* subsp. *lactis*, *L. mesenteroides*, *E. faecalis*, and *E. faecium* did not degrade β-casein at all (Fig. 2).

CONCLUSION

In this study, 10 species were isolated from three artisanal cheeses manufactured in the Caucasus region. Analysis of isolated LAB showed that in regard to

the total number of isolates, the presence of the strain *L. plantarum* was very high (66.2%). The next most dominant taxon of LAB (15.9% of total isolates) was *L. lactis* subsp. *lactis*, which belongs to the category of homofermentative cocci. Among heterofermentative LAB, the genus *Leuconostoc* (10.8% of isolates) and *L. brevis* species (4.5% of isolates) were identified. The total number of enterococci isolated from all three Caucasus cheese samples was negligible (2.5% of total isolates). Some strains of the species *L. plantarum*, *L. arizonensis*, and *L. farciminis*, as well as one *L. pseudomesenteroides* isolate (BGKAVS3-23), showed various efficiency in β-casein degradation.

Characterization of LAB from artisanal fermented products to the species level could be used to estimate the microbial diversity present in natural populations. In addition, well characterized strains from the probiotic and technological points of view could eventually be used for construction of new starter cultures for production of new fermented dairy products labeled as functional foods.

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АНАЛИЗА МИКРОФЛОРЕ БАКТЕРИЈА МЛЕЧНЕ КИСЕЛИНЕ У ТРАДИЦИОНАЛНИМ КРАВЉИМ СИРЕВИМА СА КАВКАЗА

АМАРЕЛА ТЕРЗИЋ-ВИДОЈЕВИЋ, МИЛИЦА НИКОЛИЋ, КАТАРИНА ВЕЉОВИЋ, МАЈА ТОЛИНАЧКИ, М. БУСАРЧЕВИЋ И Љ. ТОПИСИРОВИЋ

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Из три ручно прављена сира узета из различитих домаћинстава смештених у региону планине Кавказ, изоловано је укупно 157 бактерија млечне киселине (LAB). Сиреви су прављени од крављег млека без додатка стартер културе. Изолати LAB су окарактерисани фенотипским и генотипским тестовима. Резултати идентификације LAB показују да је у испитиваним сиревима присутно десет врста, као што су: *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus arizonensis*, *Lactobacillus farciminis*, *Lactobacillus brevis*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc pseudomesenteroides*, *Enterococcus faecium* и *Enterococcus faecalis*. Сојеви у оквиру врста *L. plantarum*, *L. arizonensis*, *L. paraplantarum*, *L. farciminis* и *L. pseudomesenteroides* су показивали добру протеолитичку активност.