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Artisanal Vlasina Raw Goat's Milk Cheese: Evaluation and Selection of Autochthonous Lactic Acid Bacteria as Starter Cultures

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Summary

The aim of this study is the isolation, characterization and identification of autochthonous lactic acid bacteria (LAB) from artisanal Vlasina raw goat's milk cheese for the selection of potential starter cultures. Soft white Vlasina cheese was manufactured at a household on the Stara Planina Mountain using traditional techniques without starter cultures. One hundred and forty nine LAB isolates were collected from two samples of Vlasina cheese, designated as BGVL2 (5 days old) and BGVL2a (15 days old). The population of LAB in the cheese samples was characterized by phenotype-based assays and presumptively identified using repetitive element palindromic polymerase chain reaction (rep-PCR) with the primer $(GTG)_5$. Results were confirmed by 16S rDNA sequencing. Among the BGVL2 isolates (56), the most numerous LAB species were *Leuconostoc pseudomesenteroides* (27) and *Lactococcus lactis* (26). In 15-day-old BGVL2a (93 isolates), *Lactobacillus plantarum* (33), *Enterococcus durans* (26) and *Pediococcus pentosaceus* (14) were predominant. *Lc. lactis* ssp. *lactis* BGVL2-8 showed good acidification ability and the ability to produce antimicrobial compounds, *Lb. plantarum* BGVL2a-18 had good proteolytic ability and produced exopolysaccharides, while BGVL2-29 and BGVL2-63, which belonged to the species *Ln. pseudomesenteroides*, utilized citrate and produced diacetyl and acetoin. They appeared to be suitable candidates for inclusion in the starter culture. This study contributed to the understanding of the role of autochthonous LAB in the quality of artisanal cheese and the possibility of using the selected LAB as potential starter cultures for cheese making under controlled conditions.

Key words: artisanal Vlasina cheese, lactic acid bacteria, rep-PCR, 16S rDNA sequencing, starter cultures

Introduction

Production and processing of goat's milk can provide a profitable alternative to cow's milk products due to its specific composition, taste, texture, flavour and its natural and healthy aspects when ingested as part of daily diet (*1*,*2*). In addition, Haenlein (*3*) reported that the goat's milk fat and protein are more easily digestible than those of cow's milk and that it contains higher levels of vitamin A, thiamine and niacin.

Goat's milk constitutes about 2.2 % of total milk production. It is mainly produced in Asia (59 % of world production), Africa (21 %) and Europe (16 %) (*4*). After

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the Second World War, goat breeding was prohibited in Yugoslavia, which Serbia was then part of. Goat farming restarted in 1991. No official data on goat population or goat's milk production are available in Serbia, but Žujović *et al*. (*5*) reported 318 000 goats in 2003.

The production of soft and semi-hard white cheeses from raw milk without commercial starter cultures is widely practiced in Serbia. Traditional cheese manufacturing is done in households in the same way as it was centuries ago. The quality of artisanal cheese is closely associated with the specific environment, territory of production and its tradition.

Vlasina cheese belongs to the group of white brined cheeses. This type of cheese represents the most popular cheese consumed in the region. Traditionally, it was mainly made from sheep's and goat's milk according to a specific technology, and ripened and stored in brine until consumption (*6*). As the milk fat of sheep's and goat's milk does not contain carotenoids, the resulting cheeses are white in colour. They have a salty, slightly acidic taste, and pleasant sensory properties that nowadays have a worldwide acceptance. Freshly made cheeses do not have typical sensory properties. They develop during the ripening process that may last up to 60 days. White brined cheeses have no rind, and no gas holes or other openings should be present in the cheese mass except, sometimes, small mechanical openings. The texture of white brined cheeses is smooth, soft and crumbly. Their shape varies, depending on the shape of the container.

The indigenous flora of milk is the main factor affecting the specific consistency, aroma and flavour of raw milk cheeses (*7*,*8*). Furthermore, the wild type of LAB represents a natural reservoir of microbial cultures that contains diverse genetic information. Isolation and screening of LAB from natural processes have always been the most powerful means for obtaining useful cultures for commercial purposes. There are a few studies about Serbian raw milk cheeses (*9–13*), which report about the biodiversity of LAB and their most differentiating traits, such as acidification and proteolytic activity, synthesis of bacteriocins and production of exopolysaccharides. The use of commercial LAB cultures and pasteurized milk for industrial cheese production has led to the loss of flavour and a reduction in the diversity of dairy microflora. Sensorial differences between raw and pasteurized milk cheeses could be minimized by using LAB strains isolated from raw milk cheeses (*14*,*15*).

We have found a household with five goats of an autochthonous breed in the village Mlečiške Mehane situated at an altitude of 1307 m near Lake Vlasina, Serbia. The household members mainly manufacture cheese from raw goat's milk. The domestic white breed Sana goats were fed on meadow grass and once a week with wheat bran, while water came from the local supply. No studies on Vlasina cheese LAB microflora are available in literature. Considering the significance of autochthonous LAB for the sensorial characteristics of artisanal cheeses, the aim of the first part of this study is to isolate, identify and characterise the LAB from Vlasina raw goat's milk cheese. For this purpose, 5-day-old (BGVL2) and 15-day-old (BGVL2a) cheeses were analysed at the physicochemical and microbiological levels. One hundred and forty-nine LAB were isolated from both samples and their technological characteristics were determined. All of them were identified by rep-PCR with $(GTG)_5$ primer and the most interesting isolates were also analysed by 16S rDNA sequencing. The LAB isolates showing the best technological characteristics were selected as potential candidates for use in starter culture construction.

Materials and Methods

Cheese making and sampling

The autochthonous BGVL2 (5 days old) and BVGL2a (15 days old) Vlasina raw goat's milk cheeses were made from two different batches of milk without any starter cultures. Warm (30 °C) raw milk was filtered through a gauze into an enamelled pot immediately after the morning or evening milking. One tablespoon of commercial rennet, strength 1:3000 (Radan-Leskovac, Leskovac, Serbia) was added to every 10 L of milk. The curdling took place after 30-60 min (as indicated by the separation of light green whey). The curd and whey were then stirred with a spoon and transferred into another pot covered with a gauze. The edges of the gauze were moved up and down for about 10 min to drain off the whey. The gauze was then tied at the top and turned upside down onto a metal pan. Another pot containing 3-4 kg of water was placed on top of the curd-filled gauze. After 20 min, the curd was removed from the gauze and placed in a plastic container, layer by layer, each of which was salted with 10 g of dry salt. When the container was full of cheese, the unsalted whey was poured over it to cover it completely. A cotton cloth was placed on top, which was then covered with a wooden board, and finally with a 1-kg marble stone. The container with the cheese was kept in a cellar at 15 °C. Six litres of milk were needed for the production of 1 kg of cheese. Generally, the cheese can be consumed fresh, *i.e.* immediately after production, or during 60 days of ripening.

The cheese samples, BGVL2 (5 days old) and BGVL2a (15 days old), were collected under sterile conditions and transported to the laboratory in a portable refrigerator for microbiological and chemical analysis.

Physicochemical analysis

Dry matter content was determined by heating the samples at (102±1) °C to a constant mass (*16*). Fat content was determined by the Van Gulik method (*17*) and expressed as fat percentage in dry matter. Total nitrogen was evaluated by the Kjeldahl method (*18*), while salt content was determined by the titrimetric Volhard method (*19*). A pH meter (C931; Consort, Turnhout, Belgium) was used for pH measurement. Titratable acidity was determined as described previously (*20*). All analyses were performed in triplicate. Moisture in the non-fat cheese was calculated based on the content of moisture and fat in the cheese.

Microbiological analyses

Enumeration, isolation and physiological characterization of LAB

Twenty grams of sample were taken from the interior of each BGVL2 and BGVL2a cheese and homogenized with a pestle in a sterile mortar with 180 mL of sterile 2 % (by mass per volume) trisodium citrate solution (Astrachem Zemun, Belgrade, Serbia). Cheese suspensions were serially diluted (10^{-2} to 10^{-7}) in sterile physiological solution (0.85 % by mass per volume sodium chloride). Using the pour plate technique, sample aliquots were inoculated on GM17 agar plates (for isolation of cocci) and on MRS agar plates (for isolation of rods). Agar plates from both media were incubated under aerobic and anaerobic conditions for 3 to 5 days at 30 and 45 °C, respectively. The results were expressed as average number of colony forming units (CFU) per gram of cheese obtained from three independent experiments.

After counting, one hundred colonies from each cheese sample were randomly taken from both MRS and GM17 (30 and 45 °C) agar plates under aerobic and anaerobic conditions corresponding to the highest dilution at which the growth occurred. Colonies were purified by streaking on the same isolation medium. Pure cultures were grown in appropriate liquid media and stored at –80 °C after adding 15 % (by mass per volume) glycerol (POCh, Gliwice, Poland).

Cell morphology was determined by microscopy (Olympus BX51 microscope; Olympus Europe Holding, GmbH, Hamburg, Germany). Isolates were also Gram-stained and tested for catalase production. Preliminary identification and grouping was based on cell morphology and phenotypic properties, such as growth at 15, 30 and 45 °C and in 2, 4, 6.5 and 8 % NaCl broth, hydrolysis of L-arginine and esculin, citrate utilization, production of CO_2 from glucose in reconstituted MRS broth with inverted Durham tubes, production of acetoin from glucose (Voges-Proskauer or VP test), time required for curd formation and the litmus milk test. Bile esculin agar (BEA; HiMedia, Mumbai, India) was used as selective medium for detecting members of the genus *Enterococcus* (*Ec. faecium*, *Ec. durans* and *Ec. faecalis*) and fecal streptococci (group D). It tests the ability of organisms to hydrolyze esculin to esculetin in the presence of bile. The esculetin reacts with the ferric citrate (in the medium), forming a dark brown or black phenolic iron complex. Brownish-black colonies surrounded by a black zone are positive. Diacetyl production was tested for LAB strains that were able to coagulate milk and it was assessed qualitatively as follows: LAB strains were inoculated in reconstituted skimmed milk for 16 h. To 1 mL of coagulated milk, 0.1 g of creatinine and 0.1 mL of 30 % NaOH (by mass per volume) were added. Diacetyl generation was indicated by the formation of a red ring at the top of the tubes after 2 h. Exopolysaccharide (EPS) production was visually detected as long strands when colonies were extended with an inoculation loop.

Classification of LAB isolates based on phenotypic characteristics was done according to the criteria of Bergey's Manual of Determinative Bacteriology (*21*). The carbohydrate fermentation patterns of selected strains (potential components of starter cultures) were determined using the API 50CH system (bioMérieux, Marcy l'Etoile, Lyon, France) according to the manufacturer's instructions.

LAB strains and cultivation conditions

Lactobacillus and *Leuconostoc* were cultivated on MRS medium (pH=5.7) (Merck GmbH, Darmstadt, Germany), while lactococci, enterococci and pediococci were cultivated on M17 medium (pH=7.2) (Merck GmbH) supplemented with 0.5 % (by mass per volume) glucose (GM17). The incubation was carried out at appropriate growth temperatures depending on the LAB strain (30 or 37 °C) during 24-48 h under anaerobic conditions in jars using the Anaerocult A (Merck GmbH). Indicator and reference LAB strains used in this study for detection of antimicrobial activity and rep-PCR analyses are listed in Table 1.

Table 1. The list of reference strains used in this study

Bacterial strain	Source
Lactobacillus plantarum A112[a] (bac+)	Lab. collection
Lactobacillus plantarum BGAZES2-87[b]	Lab. collection
Lactobacillus paraplantarum BGKAVS2-20[b]	Lab. collection
Lactobacillus paracasei ssp. *paracasei* BGSJ2-8[b]	Lab. collection
Lactobacillus paracasei ssp. *paracasei* BGBUK2-16[a] (bac+)	Lab. collection
Lactobacillus casei NRRLB-441[b]	NRRL[d]
Lactobacillus pentosus NRRLB-227[b]	Lab. collection
Lactococcus lactis ssp. *lactis* BGMN1-5[a] (bac+) and BGMN1-596 [a] (bac–)	Lab. collection
Lactococcus lactis ssp. *lactis* BGZLM1-24[a,b] (nisine+)	Lab. collection
Lactococcus lactis ssp. *cremoris* NS1[a] (bac+)	Lab. collection
Enterococcus durans BGZLS20-35b[b]	Lab. collection[c]
Enterococcus faecium BGGJ8-3[b]	Lab. collection[c]
Enterococcus faecalis BGZLS60-26a[b]	Lab. collection[c]
Enterococcus faecalis BG221[a](bac+)	Lab. collection
Leuconostoc mesenteroides ssp. *mesenteroides* BGKAVS4-8[b]	Lab. collection
Leuconostoc pseudomesenteroides BGKAVS3-23[b]	Lab. collection
Leuconostoc citreum NRRLB-742[b]	NRRL[d]
Pediococcus pentosaceus NRRLB-14009[b]	NRRL[d]

[a]used for bacteriocin-like inhibitory substance (BLIS) activity detection
[b]used for rep-PCR
[c]these strains were identified by amplified fragment length polymorphism (AFLP), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and rep-PCR with $(GTG)_5$ primer in the Microbiology Laboratory, University of Ghent, Ghent, Belgium
[d]NRRL, Agricultural Research Service Culture Collection, Peoria, IL, USA

rep-PCR analysis and 16S rDNA sequencing of LAB isolates

Total DNA from all 149 LAB isolates was extracted as described by Hopwood *et al.* (*22*). For rep-PCR, the oligonucleotide primer $(GTG)_5$ (5'-GTGGTGGTGGTGG-TG-3') was used with its optimal PCR programme (*23*).

PCR amplification with $(GTG)_5$ primer and electrophoresis were performed as previously described in detail by Terzic-Vidojevic *et al.* (*24*).

A statistical matrix was formed with the isolated and reference strains as variables (columns of input matrix), and the position of bands as statistical cases (rows of the matrix). In the statistical matrix, only two characteristics of the bands were used: 0 (no band present) and 1 (band present). Clustering was carried out in STATISTICA v. 7.0 for Windows (*25*) using the algorithm 'unweigthed pair-group with average linkage'. Distances between the clusters were estimated using 'percent disagreement'.

For 16S rDNA sequencing, total DNA from the chosen LAB isolate was utilized as a template for PCR amplifications with primers U968 (5'-AACGCGAAGAACCTTAC-3') and L1401 (5'-GCGTGTGTACAAGACCC-3') (*26*). The complete procedure was previously described (*9*). The obtained PCR amplicons were purified by QIAquick PCR Purification Kit/250 (Qiagen GmbH, Hilden, Germany), and sequenced by the Macrogen sequencing service (Seoul, Korea). The basic local alignment search tool (BLAST) algorithm was used to determine the most related sequence relatives in the National Center for Biotechnology Information (NCBI) nucleotide sequence database (*27*). 16S rDNA sequences were submitted to the European Nucleotide Archive (ENA; *28*).

Detection of bacteriocin-like inhibitory substance

An agar well diffusion assay was used to detect bacteriocin activity for all 149 LAB isolates (*29*). Soft GM17 and MRS agars (0.7 % by mass per volume) containing *Lactococcus*, *Enterococcus* or *Lactobacillus* indicator strains (Table 1) were overlaid onto GM17 and MRS plates, respectively. To confirm the production of antimicrobial compounds of proteinaceous nature, a pronase E crystal (Sigma Chemie GmbH, Deisenhofen, Germany) was picked with a sterile toothpick and placed close to the edge of the well containing the antimicrobial compound. Plates were incubated overnight at appropriate temperatures depending on the LAB strain. A clear zone of inhibition around the well, but not in the vicinity of the protease crystal, was taken as a positive signal for possible bacteriocin-like inhibitory substance (BLIS) production.

Proteolytic activity

The proteolytic activity of all 149 LAB isolates was assayed as previously described (*30*). Cell suspensions were mixed with a 5 mg/mL of β-casein solution (Sigma-Aldrich, St. Louis, MO, USA) dissolved in the same buffer (100 mmol/L of sodium phosphate, pH=6.8) at a 1:1 volume ratio. Mixtures were incubated for 3 h at 30 °C. In addition, the LAB isolates chosen as potential starter cultures were tested for degradation of α_{s1}- and κ-casein.

Resistance to antibiotics and biogenic amine production

The minimum inhibitory concentration (MIC) in μg/mL of seven antibiotics (ampicillin, tetracycline, chloramphenicol, gentamicin, streptomycin, erythromycin and vancomycin) was determined for 16 LAB strains that showed the best technological characteristics according to NCCLS (*31*). In addition, their ability to produce biogenic amines was qualitatively determined on an improved screening medium as described by Bover-Cid and Holzapfel (*32*) using four precursor amino acids: histidine, lysine, ornithine and tyrosine.

Results and Discussion

Physicochemical properties of Vlasina cheese

The results of the physicochemical analysis of BGVL2 and BGVL2a cheeses are listed in Table 2. According to the point values of moisture (55.77 %) and fat percentage in dry matter (FDM; 45.35 %) given by Abd El-Salam and Alichanidis (*6*), the moisture and FDM values of BGVL2 and BGVL2a cheeses were within the range acceptable for white cheeses. According to the Codex General Standard for Cheese (*33*), based on the content of FDM, the examined cheeses are full-fat cheeses. Based on the moisture on a fat-free basis, they can be classified as soft cheeses.

Table 2. Physicochemical characteristics of Vlasina BGVL2 and BGVL2a cheeses

Physicochemical parameters	Cheese	
	BGVL2	BGVL2a
Dry matter/%	47.15±0.06	42.52±0.01
MNFC/%	67.76±0.08	74.17±0.01
Fat/%	22.0±0.0	22.5±0.0
FDM/%	46.66±0.06	52.92±0.01
TN/%	2.887±0.03	2.415±0.03
Protein/%	18.4±0.2	15.4±0.2
Salt/%	3.46±0.05	3.60±0.04
Titratable acidity/°SH	33.6±0.5	92.25±0.07
pH	5.1±0.2	5.2±0.3

Mean values and standard deviations of three independent measurements on different samples
MNFC=moisture in the non-fat cheese, FDM=fat in dry matter, TN=total nitrogen

Because of a higher moisture content in the cheese sample BGVL2a, more intensive biochemical transformations were expected during ripening. In the course of these transformations larger amount of acid components with higher buffer capacity was formed. This assumption is confirmed by titratable acidity as the measure of buffer capacity. Furthermore, there is no correlation between titratable acidity and pH value. Therefore, differences in titratable acidity in the examined samples (33.62 and 92.25 °SH) do not have to result in significant differences in pH values, which is confirmed in our investigations (samples had close pH values: 5.13 and 5.18). Appropriate explanation and conclusion about differences in physicochemical composition between these two cheeses is difficult to give, since it can be caused by different factors, such as autochthonous microflora, which does not have the same composition in both cheeses, different ripening time, chemical composition and traditional technology.

Enumeration and identification of lactic acid bacteria from Vlasina cheese

Mesophilic bacterial counts in both cheeses were 10^8 CFU/g. Total bacterial counts at 45 °C ranged from 10^2 CFU/g in BGVL2 to 10^4 CFU/g in BGVL2a cheese on MRS, and reached 10^5 CFU/g on GM17 in both cheeses (Table 3). The number of viable bacteria grown on GM17 agar plates at 45 °C was higher than the number of bacteria on MRS agar plates also incubated at 45 °C. This is probably because enterococci and pediococci can grow better on GM17 agar plates at both 30 and 45 °C under aerobic and anaerobic conditions than on MRS agar plates. On the other hand, lactobacilli and lactococci were mainly isolated after incubation on MRS and GM17 agar plates, respectively, at 30 °C under aerobic and anaerobic conditions. The results related to total number of bacteria in Vlasina cheeses are comparable to those reported for other Serbian raw milk cheeses (*10*,*34*).

Table 3. Viable bacterial count (CFU/g) in Vlasina BGVL2 and BGVL2a cheese samples grown on MRS and GM17 agar plates

Medium (growth conditions)	Counts (CFU/g)* in cheese sample	
	BGVL2	BGVL2a
GM17 (30 °C, A)	$(5.6\pm0.1)\cdot10^8$	$(4.1\pm0.3)\cdot10^8$
GM17 (30 °C, AN)	$(4.5\pm0.1)\cdot10^8$	$(2.4\pm0.2)\cdot10^8$
MRS (30 °C, A)	$(2.1\pm0.1)\cdot10^8$	$(7.6\pm0.2)\cdot10^8$
MRS (30 °C, AN)	$(5.9\pm0.2)\cdot10^8$	$(4.1\pm0.2)\cdot10^8$
GM17 (45 °C, A)	$(4.0\pm0.5)\cdot10^5$	$(1.0\pm0.1)\cdot10^5$
GM17 (45 °C, AN)	$(3.3\pm0.2)\cdot10^5$	$(2.7\pm0.3)\cdot10^5$
MRS (45 °C, A)	$(2.3\pm0.3)\cdot10^2$	$(7.0\pm0.1)\cdot10^4$
MRS (45 °C, AN)	$(0.9\pm0.2)\cdot10^2$	$(5.1\pm0.3)\cdot10^4$

*mean values and standard deviations of three independent experiments
A=under aerobic conditions, AN=under anaerobic conditions

Preliminary identification of LAB based on cell morphology and phenotypic characteristics (Table 4) showed significant differences in microflora composition between cheeses of different ripening time. The most numerous LAB groups in BGVL2 cheese were *Leuconostoc* and *Lactococcus* spp., whereas in the older BGVL2a cheese, besides *Lactobacillus* and *Enterococcus*, which were dominant populations, *Pediococcus*, *Leuconostoc* and *Lactococcus* species were also present. rep-PCR was used for more precise identification of all 149 LAB isolates by comparison of the band patterns with those of appropriate reference strains (Table 4). It was found that *Ln. pseudomesenteroides* and *Lc. lactis* species were predominant in BGVL2 cheese, while *Ec. faecium* and *Ln. citreum* were also present in lower numbers. On the other hand, more different species were obtained from the older BGVL2a cheese than from the younger BGVL2 cheese. Identification of LAB using rep-PCR analysis, confirmed by sequencing, showed that in BGVL2a cheese *Lb. casei*, *Ec. durans*, *Ec. faecium*, *Ec. faecalis*, *Pd. pentosaceus*, *Ln. mesenteroides* and *Lc. lactis* were present. Examples of PCR product visualization and the dendrograms based on statistical analysis of rep-PCR fingerprints are presented in Fig. 1. Data obtained from rep-PCR analysis revealed differences in the composition of the LAB population between the two cheeses. The distances between the clusters were performed using 'percent disagreement'. High abundance of *Lactococcus* sp. in 1- and 10-day-old artisanal Zlatar cheeses and of *Lactobacillus* and *Enterococcus* sp. in 20-day or older Zlatar cheeses has previously been reported (*9*). *Lactobacillus* and *Enterococcus* populations increase during cheese ripening because they are more resistant to acid conditions and salt concentration than other LAB

Table 4. Grouping of representative strains of LAB isolated from BGVL2 and BGVL2a cheeses

Cheese	Cell shape (Grouped strains)*	Some phenotypic characteristics				Presumptive identification	Reference strain	Presumptive identification by rep-PCR (grouped strains)*
		CO_2 from glucose	Arginine hydrolysis	Growth at 45 °C	Growth in 6.5 % NaCl			
BGVL2	cocci/round (26)	–	+	–	–	*Lactococcus*	BGZLM1-24	*Lc. lactis*
BGVL2a	cocci/round (7)	–	+	–	±			
BGVL2	cocci/round (2)	–	+	+	+	*Enterococcus*	BGGJ8-3	*Ec. faecium*
BGVL2a	cocci/round (28)						BGZLS20-35b	*Ec. durans* (26)
							BGGJ8-3	*Ec. faecium* (1)
							BGZLS60-26a	*Ec. faecalis* (1)
BGVL2	cocci/ovoid (28)					*Leuconostoc*	BGKAVS3-23	*Ln.*
		+	–	–	–		NRRL B-742	*pseudomesenteroides* (27)
								Ln. citreum (1)
BGVL2a	cocci/ovoid (8)						BGKAVS4-8	*Ln. mesenteroides*
BGVL2a	rods (33)	–	–	–	+	homof. lactobacilli	BGAZES2-87	*Lb. plantarum* (33)
	rods (3)	–	–	–	–	homof. lactobacilli	NRRL B-441	*Lb. casei* (3)
	cocci/tetrads (14)	–	+	+	+	*Pediococcus*	NRRLB-14009	*Pc. pentosaceus* (14)

*number of isolates is given in brackets

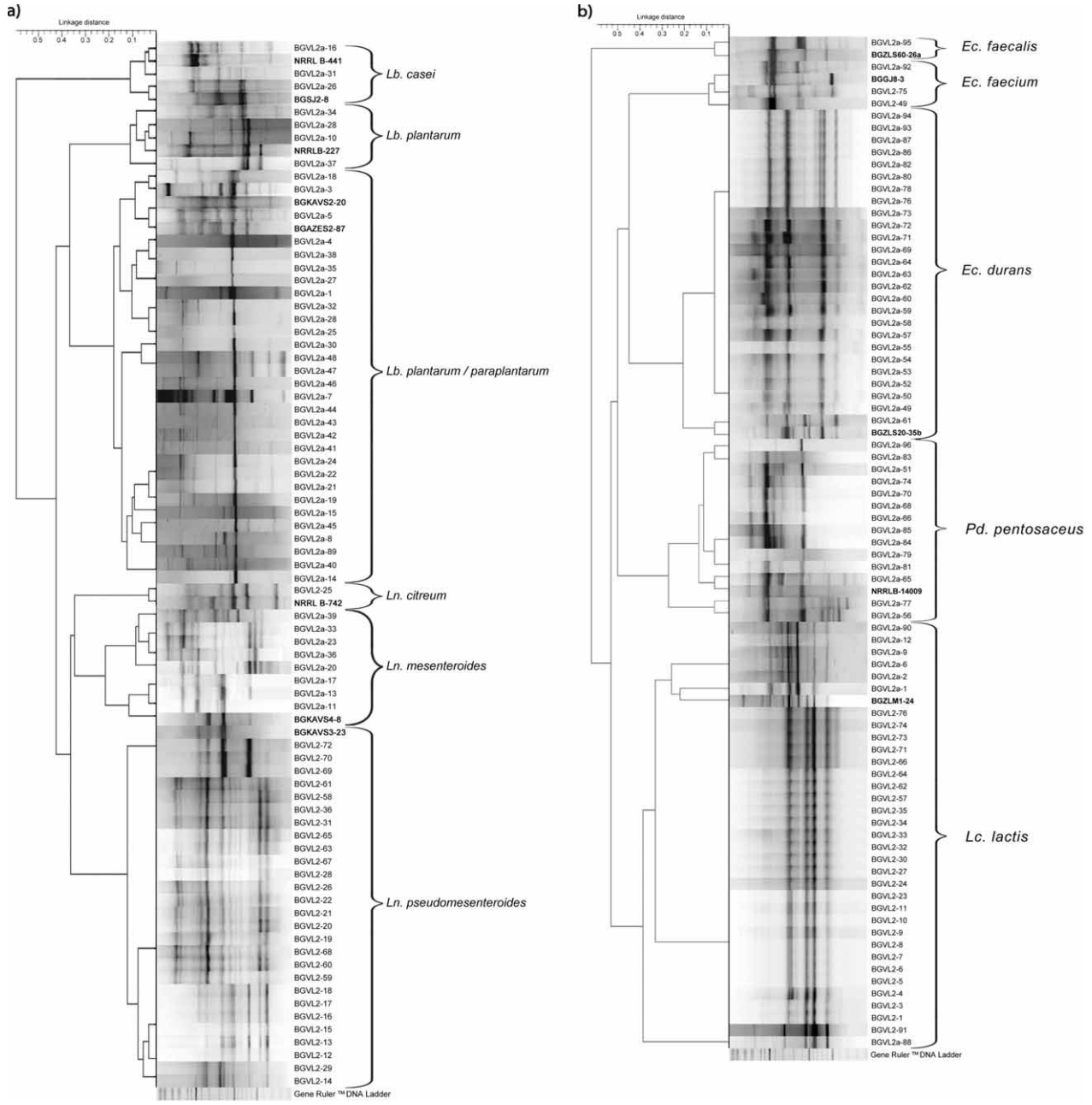

Fig. 1. Dendrograms based on statistical analysis of the $(GTG)_5$-PCR fingerprints of: a) *Lactobacillus* and *Leuconostoc*; b) *Enterococcus, Pediococcus* and *Lactococcus* isolated from autochthonous Vlasina BGVL2 and BGVL2a cheeses. The distances between the clusters were performed using 'percent disagreement'. The algorithm 'unweighted pair-group with average linkage' was used. Reference strains used in the test are given in bold

genera (*35*). This is confirmed in our case, because at (92.25±0.07) °SH the titratable acidity of BGVL2a cheese was significantly greater than that of BGVL2 cheese.

In order to test the validity of the previous identification, the 16S rDNA gene of some LAB isolates was partially sequenced (Table 5). The BLAST algorithm did not give a single hit, and it was not possible to infer from the 16S rDNA sequence if a strain was *Lb. plantarum*, *Lb. paraplantarum* or *Lb. pentosus*. One way to differentiate among them is to perform a multiplex PCR assay with *recA*-derived primers (*36*). The same can be said for *Lb. casei* and *Lb. paracasei* or *Ln. mesenteroides* and *Ln. pseudomesenteroides*.

Characteristics of lactic acid bacteria from Vlasina cheese

Thirty-five out of 36 leuconostocs isolates from BGVL2 and BGVL2a cheeses were able to use citrate (data not shown). This group of LAB is very often present in many raw milk cheeses (*13*,*37*,*38*). Bacteria of *Leuconostoc* genus are very important for cheese quality, as they produce CO_2 and flavour compounds through lactose fermentation and citrate utilization. Produced carbon dioxide is responsible for eye formation in cheese and citrate utilization leads to the production of diacetyl, which is considered the main flavour compound of fermented milk products (*39*).

Table 5. Identification of isolated LAB by 16S rDNA sequencing

Isolates	Identification by 16S rDNA sequencing	Accession number	Identity* %
BGVL2-1	*Lc. lactis* ssp. *lactis*	HE616195	99
BGVL2-14	*Ln. pseudomesenteroides*	HE616196	99
BGVL2-25	*Ln. citreum*	HE616197	100
BGVL2-49	*Ec. faecium*	HE616198	98
BGVL2a-1	*Lc. lactis* ssp. *lactis*	HE616204	99
BGVL2a-3	*Lb. paraplantarum*	HE616205	97
BGVL2a-5	*Lb. plantarum*	HE616206	97
BGVL2a-11	*Ln. mesenteroides* ssp. *mesenteroides*	HE616208	95
BGVL2a-15	*Lb. plantarum*	HE616209	99
BGVL2a-18	*Lb. plantarum*	HE616210	99
BGVL2a-20	*Ln. mesenteroides* ssp. *mesenteroides*	HE616211	99
BGVL2a-26	*Lb. paracasei* ssp. *paracasei*	HE616212	99
BGVL2a-30	*Lb. plantarum*	HE616213	99
BGVL2a-31	*Lb. casei*	HE616190	99
BGVL2a-34	*Lb. plantarum*	HE616191	99
BGVL2a-39	*Ln. mesenteroides* ssp. *mesenteroides*	HE616214	100
BGVL2a-51	*Pd. pentosaceus*	HE616201	99
BGVL2a-52	*Ec. durans*	HE616194	99
BGVL2a-61	*Ec. durans*	HE616193	100
BGVL2a-65	*Pd. pentosaceus*	HE616192	99
BGVL2a-86	*Ec. durans*	HE616188	99
BGVL2a-92	*Ec. faecium*	HE616202	97
BGVL2a-95	*Ec. faecalis*	HE616203	99
BGVL2a-96	*Pd. pentosaceus*	HE616189	99

16S rDNA sequences were submitted to the European Nucleotide Archive (ENA)
*demonstrated identity with 16S rDNA sequences of relevant species deposited in GenBank database (NCBI)

It is interesting that one group of 33 lactobacilli were highly tolerant to salt. By comparing their rep-PCR fingerprint with the fingerprint of appropriate reference strains (BGAZES2-87), they were identified as *Lb. plantarum* species. *Lb. plantarum* strains isolated from Zlatar raw cow's milk were also able to grow in broth containing 6 % NaCl (*9*).

Concerning technological characteristics in general, the activity of all isolates in milk was weak. Fifteen out of 26 lactococci isolated from BGVL2 cheese, and six out of 28 enterococci from BGVL2a cheese curdled milk within 7.5-8.5 h. Other isolates needed significantly longer time to curd milk or did not curd it at all. This particularly refers to *Leuconostoc* and *Enterococcus* sp. *Lactobacillus* species isolated from the BGVL2a cheese sample generally curdled milk within 16-24 h. Almost all lactobacilli (31 of the 36 isolates) gave a positive VP test, *i.e.* they produced acetoin, while 26 lactobacilli utilized citrate (data not shown).

All lactococci from BGVL2 cheese and almost all the enterococci from BGVL2a cheese were bacteriocin producers, inhibiting the growth of indicator strains BGZLM 1-24 and NS1, and BGMN1-596 and NS1, respectively (data not shown), so one could speculate that more than one agent is produced by those LAB (*40*).

The production of extracellular proteinases is a very important characteristic of LAB for curd formation and flavour development. In relation to the total number of individual groups of LAB, the highest proteolytic activity was shown by lactococci (over 85 %) and lactobacilli (over 60 %), while the other groups of LAB degraded β-casein poorly or not at all (data not shown). Lactococci and lactobacilli often show better proteolytic activity than other groups of LAB.

Numerous lactobacilli from the BGVL2a cheese were EPS producers (52.7 %). EPS-producing LAB are able to modify the adhesion of probiotics and enteropathogens to human intestinal mucous (*41*). These cultures can improve the sensory characteristics of dairy products, since smooth and creamy products have considerable appeal to consumers. Therefore, EPS production is a relevant characteristic to be considered in starter selection (*42*).

Lactic acid bacteria selected as potential starter cultures

For final selection of potential starter cultures, 16 LAB isolates with the most suitable technological characteristics (acidification and proteolytic ability, production of exopolysaccharides, diacetyl and acetoin, utilization of citrate, ability to survive for 30 min at 63.5 °C) were chosen for examination of biogenic amine production and resistance to antibiotics. They included the following strains: 6 *Lc. lactis* ssp. *lactis* (BVL2-1, BGVL2-8, BGVL2a-1, BGVL2a-2, BGVL2a-88 and BGVL2a-91), 2 *Ln. pseudomesenteroides* (BGVL2-29 and BGVL2-63), 7 *Lb. plantarum* (BGVL2a-5, BGVL2a-18, BGVL2a-21, BGVL2a-22, BGVL2a-30, BGVL2a-41 and BGVL2a-89) and 1 *Ec. faecium* (BGVL2a-92). All lactococci coagulated milk within 6–8 h and had the ability to produce bacteriocins. The chosen lactobacilli had the ability to hydrolyse β-casein and produce EPS. All of them, with the exception of BGVL2a-22, utilized citrate, and isolates BGVL2a-18, BGVL 2a-30, BGVL2a-41 and BGVL2a-89 produced acetoin. Both *Leuconostoc* isolates produced EPS, and BGVL2-29 produced acetoin. Enterococcal BGVL2a-92 isolate was bacteriocin and diacetyl producer. All of them could survive for 30 min at 63.5 °C. None of them produced biogenic amines, which is not a desirable property of starter cultures (*43*). Therefore, these Vlasina cheese isolates are suitable starter culture components in this respect. Before using the chosen LAB strains for cheese production, qualitative and quantitative analysis of biogenic amines is necessary.

Franciosi *et al.* (*44*) showed that only a small percentage of wild LAB strains from raw cow's milk were sensitive to the six tested antibiotics. The absence of resistance to antibiotics in starter cultures is very important for safety reasons, because bacteria resistant to antibiotics may transfer their resistance to other bacteria. When the previously mentioned 16 LAB isolates were tested for antibiotic resistance, four strains (BGVL2-8, BGVL2a-18, BGVL2-29 and BGVL2-63) were found to be

sensitive to seven different antibiotics regardless of the concentrations used (data not shown). Therefore, they were chosen as a potential starter culture for further examination in the production of different types of cheese.

The physiological and biochemical characteristics of BGVL2-8, BGVL2-29, BGVL2-63 and BGVL2a-18 LAB strains chosen as potential starter cultures are shown in Table 6. It should be noted that *Lc. lactis* ssp. *lactis* BGVL2-8 isolate was selected primarily as acid and BLIS producer, *Lb. plantarum* BGVL2a-18 isolate as an EPS producer with a good proteolytic ability and *Ln. pseudomesenteroides* BGVL2-29 and BGVL2-63 isolates as aroma producers.

Table 6. Physiological characteristics, proteolytic activities and carbohydrate fermentation abilities of the selected lactic acid bacteria

Test	Potential starter cultures			
	BGVL2-8 *Lactococcus lactis* ssp. *lactis*	BGVL2-29 *Leuconostoc pseudomesenteroides*	BGVL2-63 *Leuconostoc pseudomesenteroides*	BGVL2a-18 *Lactobacillus plantarum*
Growth at 45 °C	-	±	-	-
Growth in 8 % NaCl	-	-	±	+
Hydrolysis of arginine	+	±	±	-
CO_2 production	-	+	+	-
Acetoin production	-	+	-	+
Milk curdling time and (pH)	7.5 h (4.66)	28 h (4.76)	30 h (4.71)	24 h (4.52)
Survival at 63.5 °C	±	±	±	±
Black zone on BEA	-	NT	NT	NT
EPS production	-	+	+	+
BLIS production	+	-	-	-
Degradation of α_{s1}-, β- and κ-casein after 3 h of incubation at 30 °C	α_{s1}-casein (–) β-casein (±) κ-casein(–)	α_{s1}-casein (–) β-casein (±) κ-casein (–)	α_{s1}-casein (–) β-casein (–) κ-casein (–)	α_{s1}-casein (+) β-casein (+) κ-casein (+)
Carbohydrate fermentation (API 50 CH)				
D-ribose	+	±	-	-
D-xylose	-	+	+	-
D-galactose	+	±	+	+
D-mannitol	-	-	-	+
N-acetylglucosamine	+	±	-	+
amygdalin	-	-	-	+
arbutin	-	-	-	+
esculin ferric citrate	+	-	-	+
salicin	+	-	-	+
D-celiobiose	+	-	-	+
D-maltose	+	+	±	+
D-melibiose	-	+	+	-
D-saccharose (sucrose)	-	+	+	+
D-melezitose	-	-	-	+
D-raffinose	-	+	+	-
amidon (starch)	±	-	-	-
gentiobiose	±	-	-	±
D-turanose	-	+	+	-

+=positive reaction, -=negative reaction, ±=weak reaction, NT=not tested
BEA=bile esculin agar, BLIS=bacteriocin-like inhibitory substance, EPS=exopolysaccharide
All isolates are sensitive to ampicillin, tetracycline, chloramphenicol, gentamicin, streptomycin, erythromycin and vancomycin
All isolates give positive reaction in terms of growth at 15 and 30 °C in the presence of 6.5 % NaCl as well as in their ability to utilize citrate and hydrolyse esculin and to ferment D-glucose, D-fructose, D-manose, D-lactose and D-trehalose
All isolates give negative reaction to diacetyl production, glycerol, erythritol, D-arabinose, L-arabinose, L -xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate fermentation

Conclusions

In Serbia white cheese is produced traditionally in rural households. Such artisanal cheeses are sold in local open markets and supermarkets. These products are often unsafe. Efforts are being made to control the production of such artisanal cheese, *i.e.* to place it under industrial conditions and to use pasteurized milk, in order to attain a standard of uniform quality and safety. *Lc. lactis* ssp. *lactis* BGVL2-8 and *Lb. plantarum* BGVL2a-18 isolates from autochthonous Vlasina raw goat's milk cheese showed potentially important properties for practical application as mixed starter cultures, while *Ln. pseudomesenteroides* BGVL2-29 and BGVL2-63 isolates acted as adjunct cultures. They were used to prepare four varieties of soft white cheese from pasteurized goat's milk on a laboratory scale, followed by ripening process.

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