Arch. Biol. Sci., Belgrade, 63 (1), 11-20, 2011 DOI:10.2298/ABS1101011B

ANALYSIS OF DOMINANT LACTIC ACID BACTERIA FROM ARTISANAL RAW MILK CHEESES PRODUCED ON THE MOUNTAIN STARA PLANINA, SERBIA

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Abstract - Traditional Serbian cheese production has a long history and generates products with rich flavor profiles. To enable the industrial manufacture of these home-made Serbian cheeses, the lactic acid bacteria present in them needs to be characterized. Five fresh white cheeses made from raw cow's milk without commercial starter cultures were collected from households on the mountain Stara Planina, Serbia. According to phenotypical and molecular analysis, 262 isolated LAB were found to belong to *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* or *Enterococcus*. The unique bacterial composition of each cheese indicates that the preservation of household industry is the way to maintain production of distinct cheeses.

Key words: Lactic acid bacteria (LAB), artisanal cheese, fermented products, autochthonous starter cultures. Mt. Stara Planina, Serbia

UDC 577.2:63

INTRODUCTION

The making of home-made fermented products has been conducted in a traditional way for centuries throughout the Western Balkan region, including Serbia. These processes of preparation have been passed down from generation to generation and have not been significantly influenced by food production technology development. The current awareness and appreciation of these artisanal types of cheeses by consumers calls for large scale production. Large quantities of different artisanal cheeses are manufactured in households of this region without using any known starter culture. Since these cheeses are made from non-pasteurized milk, the composition of the "natural starter" depends on the presence of lactic acid bacteria (LAB) in the raw milk. On the other hand, the botanical composition of the pasture affects the flavor of the produced cheeses, particularly when they are manufactured from the raw milk (Alichanidis et al., 2008). Accordingly, one would expect that these cheeses contain LAB specific for the environment where they were originally produced. The composition of such cultures is extremely complex and variable and includes both mesophilic and thermophilic LAB cultures (Cogan et al., 1977, Veljovic et al., 2007).

Many of these LAB possess important features such as the production of antimicrobial compounds, proteinases and/or exopolysaccharides (Veljovic et al., 2006). Although the production of all varieties of semi-hard white cheese generally involves similar protocols, the various steps that are applied give a distinct product with the desired characteristics. These "natural starter" cultures are derived mainly from the previous batch of cheese that is used as the inoculum for the new batch. Furthermore, for the

milk curdling process, most of the farmers still use homemade rennin.

Generally, industrial cheese production involves fully standardized processes, including well-defined bacterial cultures present in the product, leading to the same quality of cheese of a certain type. However, cheese manufacturing in households in Serbia is still a subject of investigation and no definite correlation between a particular type of cheese and the presence of certain LAB species has been established.

The objective of this study was to isolate and characterize LAB strains isolated from traditionally produced semi-hard white cheeses typical for the mountain region of Mt. Stara Planina in Serbia. In addition, the elucidation of the cheese culture composition should enable the development of a defined starter culture for the production of these typical Serbian fresh cheeses on a large scale.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The bacterial strains are listed in Table 1. *Lactobacillus* and *Leuconostoc* strains were cultured in MRS broth (pH 5.7) (Merck, Darmstadt, Germany), whereas *Lactococcus*, *Enterococcus* and *Streptococcus thermophilus* were grown in M17 broth (pH 7.2)

Table 1. The list of strains used in this study

Bacterial strains	Source or reference
Lactococcus lactis subsp. *lactis* BGMN1-596[a]	Gajic et al., 1999
Lactobacillus paracasei subsp. *paracasei* BGBUK2-16/K4[a]	Lozo et al., 2004
Lactobacillus plantarum A112[b]	Vujcic et al., 1990
Lactobacillus plantarum ATCC14917[b]	ATCC
Lactobacillus paraplantarum LMG9208[b]	LMG
Lactobacillus paracasei subsp. *paracasei* LMG13552 and LMG10774[b]	LMG
Lactobacillus delbrueckii subsp. *lactis* BGAZEJ1-49[b]	Laboratory collection
Lactobacillus delbrueckii subsp. *bulgaricus* BGAZEJ2-89[b]	Laboratory collection
Lactobacillus brevis LMG11984[b] and LMG7761[b]	LMG
Lactococcus lactis subsp. *lactis* BGSM1-19[b]	Laboratory collection
Lactococcus lactis subsp. *cremoris* NS1[b]	Laboratory collection
Streptococcus thermophilus BGAZEJ3-27[b]	Laboratory collection
Leuconostoc mesenteroides subsp. *mesenteroides* B-4370[b]	NRRL
Enterococcus durans BGZLS20-35b[b]	Laboratory collection
Enterococcus faecium BGGJ8-3[b]	Laboratory collection
Enterococcus faecalis BGZLS60-26a[b]	Laboratory collection

[a] used for antimicrobial activity detection; [b] used for $(GTG)_5$-PCR; [c] Strains identified by molecular methods in the Laboratorium voor Microbiologie, Universitet Gent, Gent, Belgium.
ATCC – American Type Culture Collection, Rockville, Md, USA
LMG – Bacteria Collection, Laboratorium voor Microbiologie - Universiteit Gent, Gent, Belgium
NRRL – Agricultural Research Service Culture Collection, Peoria, Il, USA

(Merck) supplemented with glucose (0.5%, w/v; GM17 broth). To each medium, agar (2% w/v; Torlak, Belgrade, Serbia) was added when used as a solid medium. The plates were incubated overnight at appropriate temperatures, 30°C or 42°C, depending on the strain.

Manufacturing and sampling of cheeses

White semi-hard cheese samples were collected from villages on the mountain Stara Planina where they were originally manufactured. The cheeses were manufactured in a traditional process, without the addition of any commercial starter cultures. The households were located in villages situated at different altitudes: 400 m (Veliko Selo, PT1, PT2), (Dojkinci, PT3), 800 m (Visočka Ržana, PT4) and 1400 m (Jelovica, PT5) above the sea level. The cheeses were manufactured from raw bovine milk immediately after milking, without any additional treatment of the milk. The rennet was added to 10 l of milk and the formation of a curd took approximately one hour. Afterwards, the curd was transferred into a linen strainer, which was tied into a knot over the curd. A stone of approximately 4 kg was put at the top of the prepared curd and kept as long as the whey was leaking out. The curd was then cut into the rectangular pieces, salted and arranged in layers into a barrel. The barrel filled with curd was completely overlaid with brine containing 20 g l^{-1} NaCl. The cheeses were matured between 15°C and 18°C in household chambers with constant temperatures used for this purpose. The cheese ripening usually takes one to two months. For the production of 1 kg of cheese between 5 to 7 l of milk were required. The collected cheese samples were taken into a sterile plastic container and transported to the laboratory in a hand refrigerator. The age of the cheese at the moment of the sampling was as follows: one day (PT2, PT3, PT5), two days (PT4) and three days (PT1). The cheese analysis was performed within the following 24 h.

Microbiological analysis

20 g of each sample was taken from the cheese interior, homogenized with pastille in a sterile mortar and transferred into 180 ml sterile 8 mM trisodium citrate solution. Serial dilutions (10^{-2} to 10^{-7}) of cheese samples were prepared in sterile 0.15M sodium chloride. One milliliter of these dilutions was pour-plated onto different media for the isolation of the lactic acid bacteria (LAB): MRS agar for the presumptive lactobacilli, and GM17 agar for the presumptive lactococci.

The plates were inoculated in triplicate with the bacteria from 10^{-4} to 10^{-7} dilutions when incubated at 30°C, and from 10^{-2} to 10^{-5} dilutions when incubated at 45°C for 3-5 days under aerobic and anaerobic conditions [anaerobic jars with Anaerocult A (Merck)]. The total counts of bacteria were enumerated and the results were expressed as colony forming units (CFU) per gram of cheese.

Inoculation was also done from the first dilution in the MRS and GM17 broths, as well as in skimmed milk, and they were incubated anaerobically at 30°C or 45°C for 24 h. Subsequently, bacteria from the overnight cultures were streaked on MRS and GM17 agar plates and incubated anaerobically at the appropriate temperature for 3 to 5 days.

50 to 126 colonies per five cheese samples were randomly taken from all the plates with a countable number of colonies (50-200 CFU per plate) regardless of the dilution and temperature of incubation. A total set of 530 different colonies was selected for further analysis.

The cell morphology of all the LAB strains was determined by microscopy after staining with Zichl-Neelsen carbol fuchsin (Olympus U-RFL-T, BX51, GmbH, 20097 Hamburg, Germany). After microscopic observations and catalase activity testing, the overall 406 Gram-positive and catalase-negative isolates were chosen for further analysis.

Phenotypic characterization

In order to identify the 406 isolates to the genus level the following tests were performed: (a) colony morphology and pigmentation, (b) growth at 15°C,

Table 2. Total number of viable bacteria in cheese samples from the Stara Mt. Planina region grown on MRS and GM17 agar plates

Cheese samples	CFU g^{-1} per sample[a]							
	GM17 at 30°C		GM17 at 45°C		MRS at 30°C		MRS at 45°C	
	A	AN	A	AN	A	AN	A	AN
PT1	1.99×10^8	6.87×10^8	8.18×10^6	5.49×10^6	1.76×10^8	2.68×10^8	2.78×10^5	6.75×10^6
PT2	3.62×10^8	9.55×10^8	4.26×10^7	1.23×10^7	3.35×10^7	6.78×10^7	4.41×10^6	6.76×10^6
PT3	5.03×10^7	5.64×10^7	4.55×10^6	3.41×10^6	3.78×10^7	$2. \times 10^7$	3.15×10^5	6.79×10^5
PT4	3.42×10^8	1.77×10^8	6.10×10^6	6.28×10^6	1.38×10^8	2.06×10^8	1.52×10^6	1.23×10^6
PT5	3.28×10^7	7.52×10^7	2.74×10^6	4.94×10^6	1.49×10^6	5.14×10^6	3.40×10^5	5.99×10^5

[a] Average values of independent three experiments.
A - aerobic conditions of cultivation; AN - anaerobic conditions of cultivations.

30°C and 45°C in MRS and GM17 broth, (c) growth in different concentrations of NaCl (2.0% to 8.0%) in MRS and GM17 broth, (d) production of carbon dioxide from glucose by sub-culturing the isolates in MRS broth tubes containing Durham bells, (e) L-arginine and esculin hydrolysis, (f) citrate-utilization (g) survival after heating at 63.5°C for 30 min (h) production of acetoin from glucose, determined by using the Voges-Proskauer (V.P.) test (Zourari et al., 1991), (i) growth and production of slime on Mayeux, Sandine, Elliker (MSE) agar plates (Mayeux et al., 1962), (j) black zone formation on bile-esculin agar (Himedia, Mumbai, India) performed only for cocci-like LAB (k) diacetyl production - only for LAB which coagulated casein, (l) activity in milk, (m) test in litmus milk. Identification of the isolates was performed according to established methods and criteria (Hardie,1986; Kandler et al., 1986; Mundt, 1986; Sharpe, 1979; Sneath, 1986). The tests (b) and (c) were repeated three times. Preliminary identification of selected isolates was performed by using API 50 CH tests (BioMérieux, F-69280 Marcy l'Etoile, France).

Assays of proteolytic activity

The detection of caseinolytic activity after the induction by growth of strains on MCA plates was performed as described previously (Kojic et al., 1991). A β-casein solution (5 g l^{-1}) was prepared in 10 mM ammonium acetate buffer (pH 6.8) (Sigma-Aldrich Chemie GmbH, 82024 Taufkirchen, Germany). The degradation of β-casein in the samples was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Test for antimicrobial activity

For the detection of bacteriocin activity, an agar-well diffusion assay was used (Tagg et al.,). Soft GM17 or MRS agar (7 g l^{-1}), containing indicator strains, was overlaid onto GM17 and MRS plates, respectively. The indicator strains used were *Lactococcus lactis* subsp. *lactis* BGMN1-596 and *Lactobacillus paracasei* subsp. *paracasei* BGBUK2-16/K4. Wells were made in the lawn of hardened soft agars. Aliquots (50 μl) of the supernatant of the overnight cultures (16 h) were poured into the wells. The plates were incubated overnight at 30°C. A clear zone of inhibition around the well was taken as a positive signal of antimicrobial activity.

DNA and polymerase chain reaction analysis

The total DNA from the LAB isolates was purified by the method given by Hopwood et al. (1985). For the

Table 3. Physiological characteristics of LAB isolated from homemade PT cheeses

Test	Cheese samples				
	PT1	PT2	PT3	PT4	PT5
Total number of tested isolatesa	37	30	32	34	33
Growth at 15°C	32	19	25	34	32
Growth at 30°C	36	30	32	34	33
Growth at 45°C	14	1	9	12	24
Growth in 2% NaCl	1	11	2	0	1
Growth in 4% NaCl	16	15	10	14	7
Growth in 6% or 6.5% NaCl	18	4	17	18	4
Growth in 8% NaCl	0	0	3	2	21
Arginin hydrolysis	32	19	13	17	22
Production of CO_2 from glucose	3	0	12	12	5
Citrate utilization	13	16	25	29	24
Diacetyl production	10	1	5	1	0
Curd formation in milk after 6 h	0	0	0	3	0
Curd formation in milk after 10 h	6	0	2	5	0
Curd formation in milk after 24 h	6	0	3	5	0
EPS producers[b]	3	0	0	9	0

[a] Values in Table 3. indicate the number of isolates showing positive reaction results.
[b] Formation of slime colonies during the growth on MSE agar plates.

repetitive extragenic palindromic-polymerase chain reaction (rep-PCR), $(GTG)_5$ primer was used as previously described (Versalovic et al., 1994). The PCR products were separated by electrophoresis on 1.5% agarose gel for 16 h in 1xTAE buffer on 55 V at 4°C. The $(GTG)_5$-PCR fingerprints were visualized under ultraviolet light, followed by digital image capturing using a CCD camera Biometra BDR2/5/6 (Biometra, D-37079 Göttingen, Germany).

Table 4. Proteolytic and antimicrobial activity of LAB isolated from homemade PT cheeses[a]

Test	Cheese samples				
	PT1	PT2	PT3	PT4	PT5
Total number of tested isolates	52	51	52	52	55
Proteolytical activity	43	31	38	38	38
Antimicrobial activity	15	3	7	3	0

[a] Values in Table 4. indicate the number of isolates with positive reaction results

RESULTS

Total count of lactic acid bacteria

The total viable counts of bacteria in five cheeses samples on GM17 and MRS agar plates under aerobic and anaerobic conditions of cultivation varied among the tested cheeses and were in the range of 10^5 and 10^8 CFU g^{-1}. The lowest total counts of bacteria in the PT5 cheese (3.4 x 10^5 CFU g^{-1} on MRS agar plates at 45°C in aerobic condition and 7.5 x 10^7 CFU g^{-1} on GM17 agar plates at 30°C in anaerobic condition) (Table 2).

Characterization of lactic acid bacteria

Overall, 530 isolates were collected from five cheese samples and among them 406 isolates were Gram-positive and catalase-negative. According to the cell morphology, among Gram-positive and catalase-negative isolates, 97 were rod-like and 309 were cocci-like bacteria. In all the cheeses a significantly larger number of cocci-like bacteria were isolated. Interestingly, cocci-like bacteria were isolated only from PT2 cheese. For further physiological analyses, 262 isolates including all the rod-like bacteria, were chosen according to their cell morphology and the ability to grow on the bile esculin agar plate. All isolates were tested for their proteolytical ability to de-

grade β casein and antimicrobial activity against two indicator strains (Table 4). The results revealed 188 proteolytically active LAB and 28 LAB that produced antimicrobial compounds. Subsequently, LAB exhibiting the same features were divided into groups and the representatives of each group (overall 111 isolates) were identified by $(GTG)_5$ rep-PCR.

Comparison of LAB present in cheeses

Comparison of LAB isolated from PT1 and PT2 cheeses

The comparison was performed in order to find the link between the process of cheese manufacturing and the presence of certain LAB with particular features. Both cheeses were produced in the same geographical location, the milk was obtained from domestic spotted cows fed with hay and clover from the local meadows of village Veliko Selo, and drinking the water from the local water network. The cheese production process was the same in both cases, but the households were different.

Data obtained from $(GTG)_5$-PCR analysis revealed significant differences in the composition of LAB population between these two cheeses (Table 5). While the number of isolated *Lactococcus lactis* was similar for both cheeses, *Streptococcus thermophilus, Enterococcus durans* and *Lactobacillus* species were found only in PT1. Additionally, the LAB population in the PT2 cheese consisted of only two LAB species, *Enterococcus* and *Lactococcus* species.

A significant difference in the growth temperature was observed between these two groups of LAB, since only one isolate from PT2 was able to grow at 45°C (compared to 14 isolates from PT1). Although the tolerance to 4% and 8% salt concentration was similar for both groups, the difference in the number of strains tolerant to 6% and 2% salt concentration was evident.

A significant number of the LAB isolates that efficiently utilize citrate (Cit^+) was discovered in both cheeses. On the other hand, while 10 diacetyl producers were found in cheese PT1, only one strain from PT2 produced diacetyl. Regarding curd formation, these two cheeses significantly differed since none of the isolates in cheese PT2 formed curd even within 24 hours, while six isolates from PT1 curdled the milk after 10 h. Unlike the PT2 cheese, three isolates from the PT1 cheese identified as *L. lactis* ssp. *lactis* produced EPS strains.

The cheese sample PT1 was the most abundant among all the tested cheeses, with 83% of the LAB producing proteinases with the ability to degrade β-casein (Prt^+). On the other hand, in PT2 the lowest number of Prt^+ isolates was detected (Table 4).

Results showed that the highest number of LAB isolates exhibiting antimicrobial activity was detected in the cheese PT1, including two isolates showing concurrent antimicrobial activity against both indicator strains, *L. lactis* BGMN1-596 and *L. paracasei* subsp. *paracasei* BGBUK2-16/K4. In contrast, only three isolates from PT2 exhibited antimicrobial activity against *L. lactis* BGMN1-596. According to the $(GTG)_5$-PCR fingerprinting results, all antimicrobial-compound producers belonged to the *L. lactis* or *Enterococcus* sp. group. Additionally, all antimicrobial-compound producers from the PT2 cheese were also proteolytically active. Among the antimicrobial-compound producers in cheese PT1, one strain, *L. lactis* ssp. *lactis,* was proteolytically inactive.

Comparison of LAB isolated from PT2, PT4 and PT5 cheeses

The aim of this comparison was to determine the specificities of the cheeses produced at the different altitudes of one region. Cheeses PT2, PT4 and PT5 were produced in the same manner in the households situated at 400 m, 800 m and 1300 m, respectively. The cows, all domestic spotted cows, were fed with locally produced hay. The cows whose milk was used in the production of PT2 and PT4 cheeses consumed water from the local water network, while the cow used in the production of PT5 drank water from the river.

Table 5. Distribution of detected LAB species amongst homemade PT cheeses

Characterised LAB	Cheese samples				
	PT1	**PT2**	**PT3**	**PT4**	**PT5**
Total of *Lactococcus* sp.	12	13	4	7	2
L. sp.					1
L. lactis	12	13	4	7	1
S. thermophilus	3		3		
Total of *Lactobacillus* sp.	4	0	16	5	2
L. paracasei	2		5	3	1
L. plantarum	1				1
L. paraplantarum			4		
L. brevis	1		1	2	
L. delbrueckii ssp. *bulgaricus*			6		
Total of *Leuconostoc* sp.	0	0	2	2	3
Leuconostoc sp.				2	3
L. mesenteroides			2		
Total of *Enterococcus* sp.	9	3	4	6	10
E. sp.				3	
E. faecium	3	1	3	2	1
E. faecalis	4	2			2
E. durans	2		1	1	7

When analyzing only these three cheeses, it appears that the number of isolated lactococci decreases with altitude. Nevertheless, if we compare cheeses PT3 (400 m, 4 isolates) and PT4 (800 m, 7 isolates), the number of lactococci is higher at the greater altitude. This is why no correlation between the presence of this species and the altitude of the household can be deduced. Regarding *Lactobacillus* sp., *Leuconostoc* sp., and *Enterococcus* sp., the differences between the cheeses were evident and no specific microflora correlated with the altitude of the household. The results of growth at different temperatures revealed that the differences between these three cheeses were in the ability of the isolated LAB to grow at 45°C. The greatest number of LAB able to grow at this temperature was discovered in PT5 (24 isolates or 73%).

The growth test of LAB in the broth containing various salt concentrations showed variations between the tested cheeses. If we compare the results obtained for 8% salt concentration, it appears that at the highest altitude - 1300 m (PT5), the greatest number of LAB isolates (21 or 64%) grew in the broth with a high salt concentration. On the other hand, a significant number of LAB isolates from PT4 (17 or 53% respectively) grew in broth with 6 % salt (mainly *L. plantarum*) or 6.5 % salt (*Enterococcus* sp.).

The highest number of isolates that efficiently utilize citrate (Cit^+) was found in the PT4 cheese (29 isolates or 86%). A low number of diacetyl producers (overall two strains) were found among the LAB isolates from all three cheeses.

Regarding curd formation, only three isolates from the PT4 cheese formed a curd within six hours of incubation. According to $(GTG)_5$-PCR one isolate was identified as *L. lactis* while the other two were identified as *L. paracasei.*

The largest number of EPS producers (9) was discovered in the PT4 cheese where six out of nine belonged to *Leuconostoc* sp., two isolates belonged to *Enterococcus* sp., and one isolate belonged to *L. plantarum*. In the other two cheeses, no EPS-producing LAB were isolated.

In all three cheeses, between 60% and 70% of the isolates were proteolytically active. In cheeses PT2 and PT4, the same number of antimicrobial-compound producers against *L. lactis* BGMN1-596 indicator strain was isolated and they were all proteolytically active. On the other hand, no antimicrobial producers were isolated from the PT5 cheese.

Comparison of PT2 and PT3 cheeses

To determine whether cheeses of the same age are distinguished by the specific microflora, the PT2 and PT3 cheeses were analyzed. Both cheeses were one day old, produced in the same manner, at the same altitude in two villages on the mountain Stara Planina. The bovine milk used for the cheese production came from domestic spotted cows. The cows consumed hay and water from the local water network (PT2) or fresh grass from the local meadows and spring water (PT3).

According to the results presented in Table 5, lactococci were significantly more present in PT2 (13 isolates). On the other hand, from PT3 four different species of lactobacilli, including *L. delbrueckii* ssp. *bulgaricus,* were isolated, as well as *L. mesenteroides,* and it appeared that this cheese contained the most variable LAB population (six different LAB species). The results of the LAB growth at the different temperatures were similar for both strains. The isolates from these cheeses also differed in their ability to grow in the presence of different salt concentrations, citrate utilization and diacetyl production. While no isolates that curdle milk within 24 hours were detected in the PT2 cheese, in PT3 two isolates formed curd in 10 hours and three in 24 hours. No EPS producers were found in either cheese.

DISCUSSION

Although it is hard to set up completely controlled studies of cheese production in domestic households, we selected the manufacturers from Mt. Stara Planina mountain who were known to produce fresh white semi-hard cheese in a similar manner. Overall, five cheese samples were randomly collected from different localities on the mountain. In this work we showed that the specific Serbian fresh white cheeses produced in a traditional way were characterized by heterogeneous non-starter lactic acid bacteria (NSLAB).

The analyzed lactic acid bacteria (LAB) most probably represented two groups of specific microflora. The first was a specific microflora of the milk used for cheese production. Since no starter cultures were used for the cheese production in this region, the initial ripening process was based on the presence of indigenous lactic acid bacteria (LAB) flora in the raw milk. The secondary flora may be composed of microorganisms that accessed the cheese from the environment. During cheese ripening, both groups of microorganisms promoted a complex series of biochemical reactions responsible for the development of specific cheese flavor and texture. The results on the tolerance of the strains to NaCl indicate that salt resistant species play an important role in the ripening of the analyzed cheeses. The highest numbers of isolates that efficiently utilize citrate (Cit^+) were mostly enterococci. In many cheeses, such as Mozzarella and Feta, enterococci comprise a major part of the fresh cheese microflora and contribute to the ripen-

ing and quality of the mature products and in the development of the aroma and flavor due to citrate catabolism (Coppola et al., 1988; Sarantinopoulos et al., 2001; Tzanetakis et al., 1991). Additionally, the proteolytic system of thermophilic lactobacilli is considered to be important for the formation of the flavor and texture of fermented products. The majority of strains analyzed in this study showed a good proteolytical activity. On the other hand, exopolysaccharide (EPS) producing cultures improve different cheese properties including textural, melting and sensory characteristics.

The analysis of the LAB microflora in five PT cheeses revealed all of the main LAB genera including enterococci that were detected in all PT cheeses and are very often present in other types of artisanal cheeses (Psoni et al., 2001). However, the observed differences in the cell counts and composition of the LAB populations among the five analyzed cheeses indicated the potential influence of the cheese-making practice. According to our study, the NSLAB flora in traditionally manufactured cheeses is unique for each household, even in such a small geographical region as the mountain Stara Planina. The differences in LAB composition were evident even when the cheeses were produced in different nearby households. On the other hand, the altitude of the households where the cheeses were manufactured did not play a significant role in the formation of specific LAB cheese composition. Moreover, cheeses of the same age notably differed in LAB composition. Nevertheless, the PT3 cheese contained the greatest number of different LAB groups which maybe indicated the significance of fresh feed in the cows' diet for the development of more diverse cheese microflora. It is most probable that household-to-household variation in the LAB content of milk from which cheeses were made might be a reason for the variation in the LAB populations among cheeses. Additionally, LAB from the microenvironment specific for each household probably contributed to the observed differences. Overall, these data indicate the difficulties in the construction of one LAB starter culture that could be used in the production of these specific artisanal cheeses on a larger scale. Nevertheless, the results point out the importance of the preservation of these small-scale household manufactures. These distinct cheese products could serve as a source of different NSLAB species with the specific features that can subsequently become a part of different autochthonic starter cultures. Moreover, due to the increased interest for bacterial species with potential health-improving properties, our future studies will be focused on these artisanal products as a source of new probiotic LAB.

Acknowledgments - This work was supported by CSK Food Enrichment, Leeuwarden, The Netherlands grant and in part by the Ministry of Science and Technological Development of the Republic of Serbia (MSTDRS) Grant No. 143036.

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