UDC:575
DOI: 10.2298/GENSR0903341T
Original scientific paper

DOMINANT LACTIC ACID BACTERIA IN ARTISANAL PIROT CHEESES OF DIFFERENT RIPENING PERIOD

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Terzic-Vidojevic A., J. Lozo, and Lj. Topisirovic (2009):*Dominant lactic acid bacteria in artisanal Pirot cheeses of different ripening period..*– Genetika, Vol. 41, No. 3, 339 - 352.

In this study two raw cow's milk cheeses of a different ripening period were examined. The cheeses were taken from a country household in the region of mountain Stara Planina and manufactured without adding of starter culture. A total 106 lactic acid bacteria (LAB) strains were isolated from both cheeses. They are tested by classical physiological tests as well as by API 50 CH tests. Proteolytic and antimicrobial activities were done too. Identification of LAB isolates was done by repetitive extragenic palindromic-polimerase chain reaction (rep-PCR) with $(GTG)_5$ primer. The

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LAB isolates from cheese BGPT9 (four days old) belonged to the eight species of LAB (*Lactobacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus delbrueckii*, *Lactobacillus brevis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans* and *Leuconostoc garlicum*), while in the BGPT10 cheese (eight months old) only two species were present (*Lactobacillus plantarum* and *Enterococcus faecium*). Proteolytic activity showed 30 LAB from BGPT9 cheese, mainly enterococci. From BGPT10 cheese only one isolate (which belonged to the *Lactobacillus plantarum* species) possessed partial ability to hydrolyze β-casein. Seven enterococci from BGPT9 cheese and four enterococci from BGPT10 cheese produced antimicrobial compounds.

Key words: antimicrobial activity, non-starter lactic acid bacteria (NSLAB), Pirot cheese, rep-PCR

INTRODUCTION

The autochthonous way of dairy products manufacturing, especially cheese from raw milk, is present in Serbia. Those products are generally designated as "artisanal", since such dairy products are manufactured following unique traditional techniques without the addition of starter cultures routinely used in industrial cheese production.

Different steps during the manufacturing of artisanal cheeses, such as renneting of the milk, acidification, heating, whey drainage, salting and ripening, have a great influence on the final characteristics of the cheese, and play the major role in the microbial composition (RANDAZZO *et al*., 2009). Besides that, the specific heterogeneous autochthonous non-starter lactic acid bacteria population of dairy products is highly affected by the fact that those products are being made in the farm households situated in different geographic and climate localities of the authentic flora and fauna (POZNANSKI *et al.,* 2004).

The NSLAB composing natural milk microbiota belong to the mesophilic and thermophilic bacterial groups (BERESFORD *et al*., 2001). The activity of those bacteria in raw milk during the fermentation, and later during the ripening process, determine a specific texture, aroma and flavor of dairy products (MUSTAFA, 2006; ZAMFIR *et al*., 2006; DEWAN and TAMANG, 2007; DUAN *et al*., 2008). On the other hand, the production of dairy products from raw milk in the farm households is not standardized, and contamination of products, made from raw milk with various microorganisms occurs frequently (DE BAYSER *et al*., 2001). Conventional cultivation methods, prior to the characterization by physiological and biochemical tests, as well as different molecular techniques ensure the quicker, more detail and accurate identification of LAB to the level of species and strains (RANDAZZO *et al*., 2006). The biochemical, genetic and technological characterization of LAB isolated from traditional cheeses has a great importance in identifying the novel strains with

phenotypic and genetic characteristics different from already known (DELGADO and MAYO, 2004).

One of the Serbian artisanal cheeses is white semi-hard cheese, which is produced on the highlands of the mountain Stara Planina, named Pirot cheese. This traditional cheese is manufactured from raw cow's milk without the addition of the starter culture. It is known that the NSLAB composition changes in different ripening periods of cheese (MANNU and PABA, 2002).

Considering the significance of LAB microflora for organoleptic characteristics of the artisanal cheeses, the aim of the study was the identification and phenotypic as well as genotypic characterization of NSLAB isolated from two cheeses of different ripening time. Both cheeses (BGPT9 - four days old, and BGPT10 - eight months old) were manufactured in the same household in the village located on mountain Stara Planina. The isolation of LAB having useful technological properties, from traditional fermented products is one of the ways to provide the strains for eventual industrial starters that can preserve a typical nature of the product.

MATERIALS AND METHODS

Cheese manufacturing and sampling. – LAB was isolated from white semi-hard Pirot cheese, which was manufactured from raw cow's milk. The cheese designated as BGPT9 was four days old and made in April. The cheese named as BGPT10 was eight months old and produced in September. The cheeses were taken from the household settled in village Slavinja at 730 m of altitude on the mountain Stara Planina. The Pirot cheese is made by adding the commercial rennet called „Sirnik" (Kraljevo, Serbia) into raw non-pasteurized milk. Two tablespoons of rennet (the strength 1:5000) are added in 8 l of tepid milk. The coagulation takes 60-90 min at temperature about 28°C.

The obtained curd is passed in layers with the plate from the container in which the milk was curdled into the linen strainer beneath which is a metal cup with a cone-shaped hole on the center of it. The draining is done by twisting the linen strainer by hands during 1h. After that, particular drained curd is pulled out from the linen strainer and placed between two boards on which 4 kg stone are put. This additional draining under pressure lasts for 40 min.

The obtained cheese base was 20 x 5 cm in size. It is cut into pieces of 10 cm x 10 cm x 5 cm. The cheese pieces are left for 24 h. afterwards, the cheese pieces are transferred in the plastic vessel, which bottom is covered with dry sea salt. The surface of cheese is salted, too. The procedure is repeated until the vessel is full. During the whole process of filling the vessel, the board on which stone is putted presses the cheese. Thus, cheese is for all time in its own whey. The cheese is kept in the special room at temperature of 15-18ºC. In those conditions, the cheese can be kept for 8 months. From 8 l of milk, 1300 g of cheese is made.

The cheese samples were taken under sterile conditions and transported to the laboratory under refrigeration for microbiological analysis.

Bacterial strains and growth conditions. – Reference strains used in this study are listed 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). Solid medium was obtained by adding 2% (w/v) agar (Torlak, Belgrade, Serbia) into broth. The plates were incubated for 24-48 h at corresponding incubation conditions depending on the strain. The Anaerocult A (Merck GmbH) was used to obtain the anaerobic growth conditions in anaerobic jars. Purified strains of LAB were stored at –80ºC in appropriate broth supplemented with 15% glycerol (w/v).

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

Bacterial strains	Source of reference
Lactobacillus plantarum LMG9206[b]	BCCM/LMG
Lactobacillus plantarum LMG9219[b]	BCCM/LMG
Lactobacillus paracasei subsp. *paracasei* BGBUK2-16/K4[a]	LOZO *et al.*, 2004
Lactobacillus paracasei subsp. *paracasei* LMG4560[b]	BCCM/LMG
Lactobacillus delbrueckii subsp. *bulgaricus* BGAZEJ2-96[b]	Laboratory collection
Lactobacillus brevis LMG7761[b]	BCCM/LMG
Leuconostoc garlicum BGGJ1-13[b]	Laboratory collection
Lactococcus lactis subsp. *lactis* BGMN1-596[a]	GAJIC *et al.*, 1999
Streptococcus thermophilus BGAZEJ2-2[b]	Laboratory collection
Enterococcus durans BGZLS20-35b[b]	Laboratory collection[c]
Enterococcus durans BGGJ4-2[b]	Laboratory collection[c]
Enterococcus faecium BGGJ8-3[b]	Laboratory collection[c]
Enterococcus faecalis BGZLS60-26a[b]	Laboratory collection[c]

[a] Used for BLIS-activity detection; [b] Used for rep-PCR.

[c] This strain was identified by molecular methods, AFLP, SDS-PAGE and rep-PCR with $(GTG)_5$ primer in the Laboratorium voor Microbiologie, Universitet Gent, Gent, Belgium.

BCCM/LMG - Bacteria Collection, Laboratorium voor Microbiologie - Universiteit Gent, Gent, Belgium.

Isolation of microorganisms and physiological characterization. – LAB isolation was done from 20 g of each cheese sample taken from the inside of cheese, homogenized with pestle in sterile mortar and transferred into 180 m 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 ml of these dilutions was poured in MRS agar plates for lactobacilli and in GM17 agar plates for lactococci. Incubation of agar plates was performed under aerobic conditions as well as anaerobic at 30ºC and 45ºC for 3-5 days. Fifty to seventy colonies per sample were randomly picked from both MRS and GM17 agar plates (30ºC and 45ºC) corresponding to the highest dilution at which growth occurred.

Pre-inoculation was also done from first dilution in MRS and in GM17 broths, as in skimmed milk. Inoculated broths and skimmed milk were anaerobically incubated at 30ºC and 45ºC for 24 h. Bacteria from incubated MRS and GM17 broths and from skimmed milk were streaked on MRS and GM17 agar plates after 24 h. Plates were incubated anaerobically at 30ºC and 45ºC for 3-5 days. The colonies were purified two to three times by streaking on fresh separate agar plates. After catalase test, Gram staining and microscopic observations (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany), LAB isolates are taken for further analysis.

For characterization of chosen Gram-positive and catalase-negative isolates, the same physiological tests were used as described previously (TERZIC-VIDOJEVIC *et al.*, 2009). Chosen LAB isolates were also tested using the API 50 CH tests (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Identification of LAB isolates to genus level was carried out following schemes recommended by several authors (SHARPE, 1979; GARVIE *et al.*, 1986; KANDLER and WEISS, 1986; MUNDT, 1986a, b; SNEATH *et al.*, 1986).

Antimicrobial activity. – For the detection of antimicrobial activity, agar-well diffusion assay was used (TAGG and MCGIVEN, 1971). Soft GM17 or MRS agar (0.7%, w/v), containing lactococci (BGMN1-596) or lactobacilli (BGBUK2-16/K4) indicator strains were overlaid onto the GM17 and MRS plates, respectively. To confirm the production of antimicrobial compounds of proteinaceous nature, a crystal protease TYPE XIV (Sigma Chemie GmbH, Deisenhofen, Germany) was placed close to the edge of the antimicrobial compound containing well. The plates were incubated overnight (16 h) at 30ºC. A clear zone of inhibition around the well, but not near the protease crystal, was taken as an indication of possible antimicrobial compounds production.

Degradation of β-casein. – Proteolytic activities of the isolates were assayed as previously described (KOJIC *et al.*, 1991). Collected fresh cells (10 mg approximate density 10^{10} cells per ml) were resuspended in ammonium acetate buffer (100 mM, pH 6.8). The cell suspension was mixed with β-casein (5 mg per ml in 100 mM ammonium acetate buffer, pH 6.8) (Sigma Chemie GmbH). The mixtures were incubated for 3 h at 30°C or 37°C depending on growth temperature of the tested strain. After centrifugation of the mixtures (5 min at 12,000 rpm) to remove the cells, the degradation of β-casein in the samples was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was

carried out on 12.5% polyacrylamide gel. Gels were stained with Coomassie brilliant blue R250 (SERVA, Heidelberg, Germany) and distained in a mix of methanol (20%) and acetic acid (7%).

DNA isolation and rep-PCR analysis. – The total DNA from LAB isolates was isolated and purified by the method given by HOPWOOD *et al*. (1985). For repetitive extragenic palindromic-polymerase chain reaction, (rep-PCR) analysis total DNA from different isolates of LAB was used as a template for PCR amplifications with $(GTG)_5$ (5´-GTGGTGGTGGTGGTG-3´) oligonucleotide primer, with its optimal PCR program (VERSALOVIC *et al*., 1994), using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). Reactions were carried out in a thermal cycler Gene AmpR PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR amplifications were performed starting with the initial denaturation of DNA at 95°C for 7 min, followed by 33 successive cycles of melting DNA at 94°C for 1 min, annealing at 40°C for 1 min, and elongation at 65°C for 8 min, and the last elongation at 65°C for 16 min. The PCR products were separated by electrophoresis on 1.5% agarose gel (15 x 20 cm) for 16 h in 1 x TAE buffer on 55 V at 4°C (VERSALOVIC *et al*., 1994). Electrophoresis was performed using an Electrophoresis Power Supply EPS 301 (Amersham Biosciences, Piscataway, NJ, USA). The $(GTG)_5$-PCR fingerprints were visualized under ultraviolet light, followed by digital image capturing using a CCD camera Biometra BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany).

RESULTS AND DISCUSSION

Phenotypic characterization of LAB. – The results of preliminary characterization of LAB isolates obtained by testing the physiological abilities are shown in Table 2. From BGPT9 cheese sample, total 40 Gram-positive and catalase-negative bacterial strains were isolated (19 rods and 21 cocci), whereas 66 (35 rods and 31 cocci) Gram-positive and catalase-negative bacterial strains were isolated from BGPT10 cheese sample. Six isolates from the BGPT9 cheese belonged to the heterofermentative LAB strains. All isolates from the BGPT10 cheese samples were homofermentative LAB strains. Generally, the LAB isolates from both cheeses showed weak activity in milk. Two isolates from BGPT9 cheese formed slime on MSE agar. Four isolates from BGPT9 cheese and two isolates from BGPT10 cheese produced acetoin, and no LAB isolate produced diacetyl (data not shown).

API tests were done for 11 LAB strains isolated from the BGPT9 cheese sample and 4 LAB strains isolated from the BGPT10 cheese sample. Those strains were chosen for API 50 CH test according to the results of LAB identification by rep-PCR. According to API tests one isolate from BGPT9 cheese was identified as *Enterococcus durans*, two isolates as *Enterococcu faecium/faecalis*, three isolates as *Lactobacillus paracasei/plantarum*, four isolates as *Lactobacillus plantarum* and one isolate was identified as *Lactobacillus brevis*. Three isolates from BGPT10 cheese were identified as *Lactobacillus plantarum*, and one isolate as *Lactobacillus curvatus*. Results of API test are given in Table 3.

Table 2. Preliminary identification of LAB isolated from BGPT9 and BGPT10 cheeses according to the physiological tests.

The genus of LAB	Cheese	
	BGPT9	BGPT10
Lactobacillus spp.	13	35
Lactococcus spp.	4	–
Leuconostoc/Weissella	6	–
Enterococcus spp.	17	31
Total number of tested isolates	40	66

Note: The following tests used for physiological characterization of isolated LAB:growth at 15ºC, 30ºC and 45ºC; growth in 2%, 4%, 6.5% and 8% NaCl; hydrolysis of argininee and esculin; utilization of citrate; production of CO_2 and acetoin (V.P. test); forming black zone on bile esculin agar; forming slime on MSE agar; activity in milk and litmus milk

Table 3. Identification of LAB by API 50 CH test.

Isolate	Species identified by API test
BGPT9-2P	*Enterococcus durans*
BGPT9-4, BGPT9-5	*Enterococcus faecium/faecalis*
BGPT9-26, BGPT9-37, BGPT9-62	*Lactobacillus paracasei/plantarum*
BGPT9-28, BGPT9-48, BGPT9-59, BGPT9-66	*Lactobacillus plantarum*
BGPT9-53	*Lactobacillus brevis*
BGPT10-46, BGPT10-69, BGPT10-71	*Lactobacillus plantarum*
BGPT10-48	*Lactobacillus curvatus*

Antimicrobial activity. – Analysis of antimicrobial activity showed that seven isolates from the BGPT9 cheese sample (BGPT9-21, BGPT9-22, BGPT9-23, BGPT9-24, BGPT9-25, BGPT9-1P and BGPT9-6PM) were active against BGMN1-596 strain (Figure 1). All of them belong to genus *Enterococcus*. Four enterococci strains out of total 66 isolates from BGPT10 cheese sample (BGPT10-17, BGPT10-18, BGPT10-21 and BGPT10-61) were producers of antimicrobial compounds (data not shown). None of isolates from the BGPT9 and BGPT10 cheese samples was active against BGBUK2-16/K4 strain.

Fig. 1. Terzic-Vidojevic *et al.*

Figure 1. .

Proteolytic activity. – The ability of natural isolates to hydrolyze β-casein was tested in ammonium acetate buffer, pH 6.8, after induction on MCA plates. In this study, the most proteolytic active strains were enterococci and lactobacilli. It was revealed that from 40 analyzed strains from the cheese BGPT9, 30 isolates produced proteinases with the ability to degrade β-casein, while the other tested isolates were proteolytically inactive. Sixteen of them belonged to enterococci, one to lactococci and 13 isolates belonged to rod shaped LAB (data not shown). On the other hand, there were no LAB isolates from BGPT10 cheese sample, which could degrade β-casein. Only BGPT10-71 isolate showed partial proteolytic activity (data not shown).

rep-PCR. – The identification of LAB isolated from both artisanal cheeses, BGPT9 and BGPT10, was also done by rep-PCR with $(GTG)_5$ primer in order to get results that are more precise. Results of rep-PCR analysis are shown in Table 4 and in Figures 2 and 3. Based on those results we conclude that the significantly higher number of LAB species was isolated from the cheese sample BGPT9 (eight species) then from the cheese BGPT10 with two isolated LAB species.

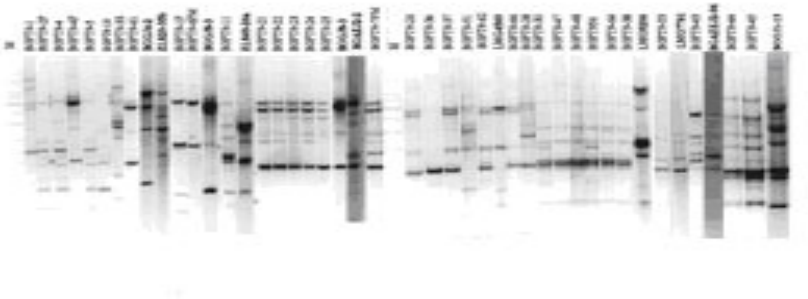

Figure 2. The rep-PCR analysis of LAB isolated from artisanal Pirot BGPT9 cheese. M - Gene Ruler^{TM} DNA Ladder Mix. Reference strains used in the test are given in bold letters.

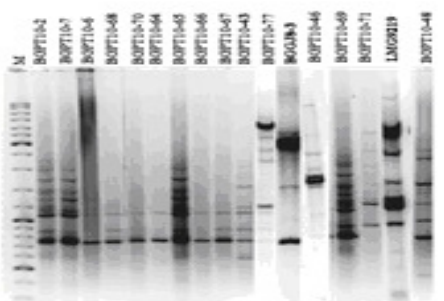

Figure 3. The rep-PCR analysis of LAB isolated from artisanal Pirot BGPT10 cheese. M - Gene Ruler^{TM} DNA Ladder Mix. Reference strains used in the test are given in bold letters.

Table 4. Identification of LAB by rep-PCR.

LAB isolates from BGPT9 cheese	LAB isolates from BGPT10 cheese	Identification by rep-PCR
BGPT9-26, BGPT9-36, BGPT9-37, BGPT9-51, BGPT9-62, BGPT9-66	□	*Lactobacillus paracasei* subsp. *paracasei*
BGPT9-28, BGPT9-30, BGPT9-38, BGPT9-47, BGPT9-48, BGPT9-56, BGPT9-64	BGPT10-46, BGPT10-69, BGPT10-71	*Lactobacillus plantarum*
BGPT9-43	□	*Lactobacillus delbrueckii*
BGPT9-44, BGPT9-45	□	*Leuconostoc garlicum*
BGPT9-53	□	*Lactobacillus brevis*
BGPT9-1, BGPT9-2P, BGPT9-4, BGPT9-4P, BGPT9-5, BGPT9-19, BGPT9-33, BGPT9-61	□	*Enterococcus durans*
BGPT9-1P, BGPT9-6PM, BGPT9-7PM	BGPT10-2, BGPT10-6, BGPT10-7, BGPT10-43, BGPT10-64, BGPT10-65, BGPT10-66, BGPT10-67, BGPT10-68, BGPT10-70, BGPT10-77	*Enterococcus faecium*
BGPT9-11	□	*Enterococcus faecalis*
BGPT9-21, BGPT9-22, BGPT9-23, BGPT9-24, BGPT9-25	□	*Enterococcus* spp./ *Streptococcus* spp.
□	BGPT10-48	Unidentified

DISCUSSION

The production of typical and traditional food represents a great opportunity for the development of rural areas. This is the case of Pirot cheeses, which are produced in the region of mountain Stara Planina. In this research, we made a survey of the LAB microflora present in two cheeses of different ripening period, which were produced at the same location.

The results of the identification of the isolated LAB from four days old BGPT9 cheese by rep-PCR analysis revealed that Lactobacillus plantarum, Lactobacillus paracasei subsp. paracasei, Enterococcus faecium, Enterococcus faecalis, Enterococcus durans, Lactobacillus delbrueckii, Lactobacillus brevis and Leuconostoc garlicum were identified. In total 15% of isolated strains from this cheese belonged to the heterofermentative LAB. Altogether, eight different LAB species were detected in four days old BGPT9 cheese. On the other hand, in eight months old BGPT10 cheese only two species, Lactobacillus plantarum and

Enterococcus faecium, were discovered although more than 50% of LAB isolates were used in determination in this case. ALBENZIO *et al.* (2001) and OUADGHIRI *et al.* (2005) examined LAB microflora in the Canestrato Pugliese cheese and Moroccan soft white cheese (Jben), respectively. They obtained similar results concerning the presence of LAB species in mentioned cheeses. Enterococci comprise a major part of fresh and ripened cheese microflora (SARANTINOPOULOS et al., 2001). This group of bacteria was only coccal-shaped LAB microflora present in 45, 60 and 90 days old Zlatar cheeses (TERZIC-VIDOJEVIC *et al.*, 2007, 2009).

The production of high quality fermented dairy products depends on the proteolytic systems in milk, since the found peptidase and amino acids have a direct effect on the flavor in these products (AYAD *et al.*, 1999). Although the authors AYAD *et al.* (2004) reported that lactobacilli showed a better proteolytic activity than lactococci and enterococci, more than 50% of proteolytic active LAB in our study are enterococci. These results are similar with these obtained by ARIZCUN *et al.* (1997) which highlights the ability of enterococci isolated from Roncal and Idiazabal cheeses to hydrolyze β-casein.

Enterococi have the ability to produce enterocins. It is an important characteristic for their use in food production (GIRAFFA, 2002, 2003; FOULQUIÉ-MORENO *et al.*, 2006). It is known that the enterococci strains, including Enterococcus faecium and Enterococcus faecalis, produce bactericidal peptides, which are called enterocins that generally belong to the II class bacteriocins (FRANZ *et al.,* 2007). Therefore, the subject of future researches will certainly be more detail analysis of antimicrobial components produced by enterococci isolated from Pirot artisanal BGPT9 and BGPT10 cheeses.

In conclusion, present research indicates that enterococci have crucial role in the manufacturing and ripening of the BGPT9 and BGPT10 cheeses. Comparative examination of LAB microflora in the two Pirot cheeses of different ripening time showed that eight various bacteria species are present in younger BGPT9 cheese while only two species were found in older BGPT10 cheese. LAB from the young cheese also has better technological characteristics, especially concerning the proteolytic and antimicrobial activity. Technological characteristics of particular LAB isolates and the possibility to prepare the starter for the cheese production will be analyzed in further experiments.

ACKNOWLEDGMENTS

We greatly thank Hans Brandsma and Wilco Meijer for critically reading the manuscript and for fruitful discussions. The Ministry of Science and Technological Development of the Republic of Serbia (MSTDRS) grant No. 143036 supported this work. This work was also partly financed by CSK food enrichment. The Netherlands.

Received July 16th, 2009.
Accepted December 11th, 2009.

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DOMINANTNE BAKTERIJE MLEČNE KISELINE U PIROTSKIM SIREVIMA RAZLIČITOG PERIODA ZRENJA PROIZVEDENIM U DOMAĆINSTVU

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I z v o d

U ovom radu su ispitivana dva sira od svežeg kravljeg mleka različitog perioda zrenja. Sirevi su uzeti iz seoskog domaćinstva u regionu Stare Planine, a proizvedeni su bez dodatka starter kulture. Iz oba sira je izolovano ukupno 106 sojeva bakterija mlečne kiseline. Sojevi su testirani klasičnim fiziološkim i API 50 CH testovima. Takođe je ispitivana proteolitička i antimikrobna aktivnost. Identifikacija bakterija mlečne kiseline je rađena rep-PCR analizom sa $(GTG)_5$ prajmerom. Osam vrsta bakterije mlečne kiseline je izolavano iz sira BGPT9 starog četiri dana (*Lactobacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus delbrueckii*, *Lactobacillus brevis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans* i *Leuconostoc garlicum*), dok su u siru BGPT10 starom osam meseci bile prisutne samo dve vrste (*Lactobacillus plantarum* i *Enterococcus faecium*). Proteolitičku aktivnost je pokazalo 30 izolata iz sira BGPT9, uglavnom enterokoke. Samo jedan izolat iz sira BGPT10 (koji je pripadao vrsti *Lactobacillus plantarum*) je posedovao delimičnu sposobnost da hidrolizuje β-kazein. Sedam enterokoka iz sira BGPT9 i četiri enterokoke iz sira BGPT10 je proizvodilo antimikrobne substance.

Primljeno 16. VII 2009.
Odobreno 11. XII. 2009.