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COMPARATIVE ANALYSIS OF ANTIMICROBIAL AND PROTEOLYTIC ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM ZLATAR CHEESE

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Traditional artisan Zlatar cheese belongs to the group of white, semi hard home-made cheeses, which are produced from no pasteurized cow's milk, without addition of any known bacterial starter culture. In total, 253 Gram-positive and catalase negative lactic acid bacteria (LAB) were isolated. Results showed that 70 out of 253 analyzed isolates produced antimicrobial compounds known as bacteriocins. Most isolates from genera *Lactococcus* and *Enterococcus*, and isolates belonging to species *Lactobacillus plantarum* and *Lb. brevis*, do not synthesize extracellular proteinase. In contrast, isolates from subspecies *Lb. paracasei* subsp. *paracasei* showed very good proteolytic activity. It was

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observed that good proteolytic activity of isolates was not in correlation with their good antimicrobial activity in the most of isolates.

Key words: Zlatar cheese, LAB, antimicrobial activity, proteolytic activity

INTRODUCTION

Lactic acid bacteria (LAB) represent a heterogeneous group of Gram–positive and catalase negative micro-organisms. They have an ability to synthesize lactic acid from lactose and they are used for production of wide range of fermented dairy, meat and vegetable products. Owing to some of its metabolic properties, LAB contributes to processes such as flavor development and ripening of fermented products (BERESFORD *et al*., 2001).

LAB also produced a large number of antimicrobial compounds like hydrogen peroxide, diacetyl, bacteriocins, that might be of great importance for food fermentation and preservation, and that is why LAB are used as biopreservative agents giving them great economic significance (DAESCHEL, 1989; BOLM and MORVEDT, 1991; PIARD and DESMAZEAUD, 1992). The most important antimicrobial substances were bacteriocins. Bacteriocins are the group of proteinaceous compounds, which generally show showed antimicrobial activity towards closely related bacteria (NES *et al.,* 1996). However, some LAB is producing bacteriocins with a wider antibacterial spectrum (JACK *et al*., 1995). It was found that such LAB can inhibit not only the growth of Gram-positive pathogenic and spoilage bacteria (ATANASSOVA *et al*., 2003; CINTAS *et al*., 1995), but also the growth of Gram-negative bacteria (CARDI, 2002; STEVENS *et al*., 1991).

The ability to produce extracellular proteinases is a very important feature of LAB. This is because most LAB isolated from fermented dairy products has multiple amino acids auxotrophy and in order to grow in protein rich medium they depend on the expression of a complex proteolytic system for the degradation of casein, the main protein in milk (KOK, 1993; VISSER, 1993). These proteinases catalyze the initial steps in the hydrolysis of milk proteins, providing the LAB with the amino acids that are essential for their growth (KUNJI *et al*., 1996). On the other hand, proteinases have an important role in cheese maturation, because they contribute to the milk protein breakdown and development of flavor during cheese ripening. In addition, proteinases play significant role in formation of cheese texture due to protein degradation (OLSON, 1990).

The microflora of cheese may be divided into two groups: starter lactic acid bacteria (SLAB) and secondary micro-organisms or non starter lactic acid bacteria (NSLAB) (BERESFORD *et al*., 2001). Starter cultures have many roles in fermentation like acid production during manufacture and contribute to the ripening process (BERESFORD *et al*., 2001).

NSLAB is a term used to describe the adventitious bacterial flora capable of growth under the selective conditions of ripening cheese. These bacteria may enter the cheese from the milk and as a consequence of post-pasteurization

contamination from the cheese making equipment or the environment (McSWEENEY *et al*., 1993). These bacteria are mainly present in traditionally home-made cheeses, manufactured at specific ecological niches. They are most probably essential for the development of flavour in artisan cheeses traditionally manufactured in households. It is believed that differences in qualities of such cheeses arise from the presence of NSLAB in the region where the cheese is manufactured.

Large quantities of different cheeses have been produced traditionally for centuries in Serbia. One of them is an artisan white semi-hard Zlatar cheese, manufactured in country household from cow's milk without the addition of any starter culture. Specific diversity of mountain Zlatar must be important for formation of specific microflora found in milk (TERZIC-VIDOJEVIC *et al*., 2007).

In previous work, we have isolated LAB from home-made Zlatar cheese. Phenotypic, biochemical and physiological tests have been used for identification of the microbial flora in Zlatar cheese (VELJOVIC *et al*., 2007). Obtained results showed that characterized isolates belong to the species: *Lactococcus lactis* subsp. *lactis*, *Lactobacillus paracasei* subsp. *paracasei*, *Lb. brevis*, *Lb. plantarum*, *Enterococcus faecalis*, *E. faecium* and *E. avium* (VELJOVIC *et al.,* 2007).

The aim of the present study was the analysis of antimicrobial compounds as well as the ability of extracellular proteinses production by LAB isolated from Zlatar cheese. Both these features of LAB are very important, because such isolates could be potential source of new variants of bacteriocins and proteinases. Therefore, they could be used as components of new starter cultures for industrial standardized production of Zlatar cheese or any other kind of cheeses.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. Isolation, physiological and biochemical characterization as well as molecular identification of lactic acid bacteria (LAB) from Zlatar cheese were done as described previously (TERZIC-VIDOJEVIC *et al.,* 2007; VELJOVIC *et al*., 2007). Two hundred and fifty three LAB were isolated from cheeses of different ripening period as well as from milk which was used for the cheese manufacturing. Samples are designated as ZLM- raw milk and as ZLS1, ZLS10, ZLS20, ZLS30, ZLS45 and ZLS60 representing cheeses samples after a ripening period of 1, 10, 20, 30, 45 and 60 days, respectively. *Lactococcus* and *Enterococcus* strains were grown in M17 broth (Merck GmbH Darmstadt, Germany) containing 0.5% glucose (GM17) at 30°C or 37°C for 24-48 h, while *Lactobacillus* strains were grown in MRS broth (Merck) at 30°C or at 37°C for 24-72 h. Agar plates were prepared by adding agar (1.5%, w/v) (Torlak, Belgrade, Serbia) to each broth when used as a solid medium.

Detection of antimicrobial compounds and activity assay. Preliminary production of antimicrobial compounds produced by isolated LAB was screened by the deferred antagonism method using various indicator strains (HARRIS *et al.,* 1989). For the detection of antimicrobial activity, agar–well diffusion assay was

used (TAGG and MCGIVEN, 1971) and used indicator strains are presented in Table 1. Soft GM17 and MRS agar (0.7% wt/vol) containing lactococci or lactobacilli indicator strains, was overlaid onto GM17 and MRS plates, respectively. Wells were made in the lawn of hardened soft agars. Aliquots (50 μl) of overnight cultures (16h) were poured in the wells. To confirm the production of antimicrobial compounds of proteinaceous nature, a crystal of proteolytic enzyme pronase E (Sigma, St. Louis, MO, USA) was placed close to the edge of the potential antimicrobial compound containing well. The plates were incubated overnight at appropriate temperature. A clear zone of inhibition around the well, but not in the vicinity of the pronase E crystal, was taken as an indication of possible antimicrobial compounds of proteinaceous nature production. A sample of overnight culture for which is known that does not produce antimicrobial compound such as strain, *Lactococcus lactis* subsp. c*remoris* NS1, was used as a negative control.

Assay of proteinase activity. Proteolytic activities of lactococcal and lactobacilli strains were performed using the modified procedure of HILL and GASSON (1986), as described previously (KOJIC *et al.,* 1991a). All the strains were grown on milk-citrate agar MCA plates for 48 h at optimal growth conditions prior to cell collection. MCA plates containing 4.4% reconstituted non-fat skim milk (RSM), 0.8% sodium citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar. Collected fresh cells (10 mg; approximate density of 10^{10} cells per ml) were resuspended in 100 mM sodium-phosphate buffer, pH 6.2. The cell suspension was mixed with substrate β-casein (5 mg/ml in 100 mM sodium-phosphate buffer, pH 6.2) (Sigma) and incubated for 3 h at 30°C. The clear supernatant was taken and mixed with solubilisation buffer (125 mM Tris-HCl (pH 6.8), 10 mM disodium EDTA, and 4% sodium dodecyl sulphate (SDS), 25% glycerol, 5% 2-mercaptoethanol, 0.07% bromphoenol blue) at a 1:1 volume ratio. Samples were heated at 80°C for 10 min and analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) for 20 h at 20 mA constant current, stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany) and distained in a mix of methanol (20%) and acetic acid (7%).

RESULTS

*Antimicrobial activity of isolates***.** It was found that among 253 analyzed isolates of lactic acid bacteria (LAB) isolated from Zlatar cheese, 70 of them produced antimicrobial compounds. Test for bacteriocin production with pronase E revealed a proteinaceous nature of antimicrobial compounds, indicating the possibility that they could be a bacteriocin-like substances (BLIS). Isolates exhibited either clear inhibition zone, turbid zone or both kinds of zones against one, two, three or all four indicator strains used in the test. According to the similarity in size and appearance of inhibition zones in the assay of antimicrobial activity and cross immunity tests, all 70 isolates are divided into three groups (Table 1).

Table 1. Indicator and bacteriocin producing strains used in this study.

Bacterial strains	Description*	Source or references
Lactococcus lactis subsp. *Cremoris* NS1	Bac^-, Bac^s	KOJIC *et al.,* 1991b
Lactococcus lactis subsp. *Lactis* BGMN1-596	Derivative of strain BGMN1-5 without plasmid, Bac^-, Bac^s	GAJIC *et al*., 1999
Lactococcus lactis subsp. *Lactis* NP45	Nisin producer	Lab. collection
Lactobacillus plantarum A112	Bac^+, Bac^{Im}	VUJCIC and TOPISIROVIC, 1993
Lactobacillus paracasei subsp. *Paracasei* BGBUK2-16	Bac^+, Bac^{Im}	LOZO *et al*., 2004
Lactobacillus paracasei subsp. *Paracasei* BGBUK2-16/K4	Derivative of strain BGBUK2-16 without plasmid, Bac^-, Bac^s	LOZO *et al*., 2004

*Bac^+ - bacteriocin producer; Bac^{Im} - immunity to bacteriocin Bac^S - sensitivity to bacteriocin, Bac^- - no bacteriocin activity.

First group (Group I) consists of 13 bacteriocin producing LAB isolated from milk and one, 10 and 20 days old cheeses (Table 1). All these isolates are identified as *Lactococcus lactis* subsp. *lactis*, which are showing zones of inhibition against all four indicator strains. However, clear zones of 5 mm in size were observed when lactococcal indicator strains BGMN1-596 and NS1 were used in the test. In contrast, when lactobacillus indicator strains BGBUK2-16/K4 and A112 were used 3mm in size turbid zones were detected. Isolate BGZLM1-24 is used as a representative of Group I (Figure 1). Cross immunity bacteriocin test with the nisin producing strain *Lc. lactis* subsp. *lactis* NP45, showed that the isolate BGZLM1-24 most probably produced a nisin. Results of PCR analysis by using primers specific for the detection of the *nis* gene, confirmed that isolate BGZLM1-24 is nisin producer (VELJOVIC *et al*., 2007).

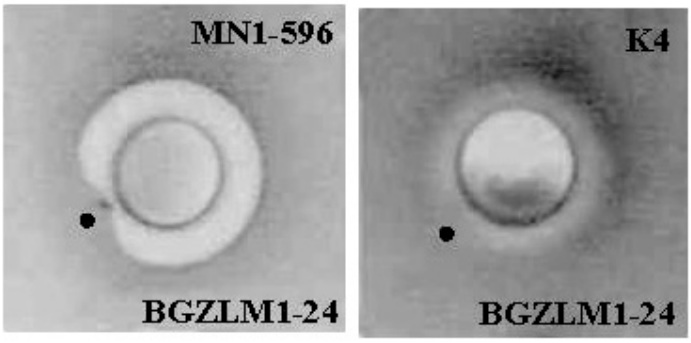

Figure 1. Inhibitory effect of isolate *Lc. lactis* subsp. *lactis* BGZLM1-24 on indicator strains: *Lc. lactis* subsp. *lactis* MN1-596 and *Lb. paracasei* subsp. *paracasei* BGBUK2-16/K4, ● - crystal of pronase E.

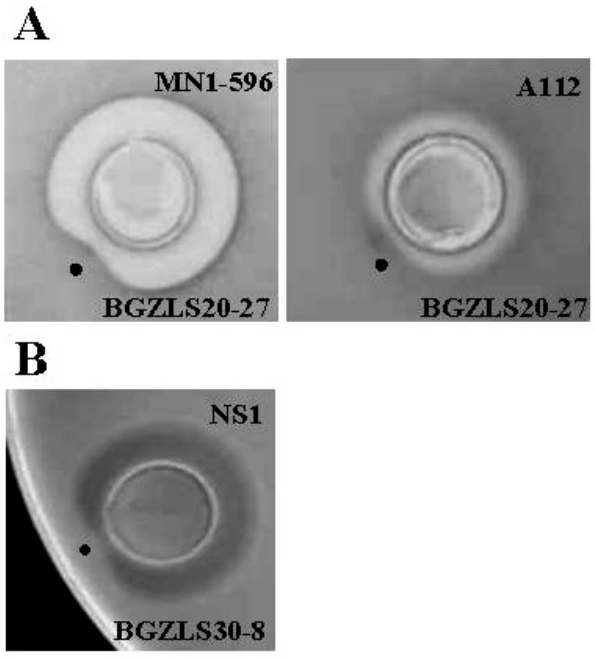

Figure 2. Inhibitory effect of isolates *E. faecalis* BGZLS20-27 (A) and *E. faecalis* BGZLS30-8 (B) on indicator strains: *Lc. lactis* subsp. *lactis* MN1-596, *Lc. lactis* subsp. *cremoris* NS1 and *Lb. plantarum* A112. ● - crystal of pronase E.

The second group (Group II) contains 20 *E. faecalis* isolates (Table 1). On the basis of similarity in size and appearance of inhibition zones on all indicator strains, this group is divided into two subgroups. Fifteen isolates forming Subgroup I, which were isolated from one, 10 and 20 days old cheeses, gave 5 mm clear zone on BGMN1-596 and NS1 indicator strains, and 1.5 mm turbid zones on indicator strain A112 and BGBUK2-16/K4. As the representative of Subgroup I the isolate BGZLS20-27 was chosen (Figure 2A).

Five isolates belonging to Subgroup II, all of which are isolated from 30 days old cheese, gave 4 mm clear zone only on lactococcal indicator strains NS1 and BGMN1-596, but there is no an inhibitory effect on the growth of lactobacillus indicator strains used in the test. The isolate BGZLS30-8 is a representative of this Subgroup (Figure 2B).

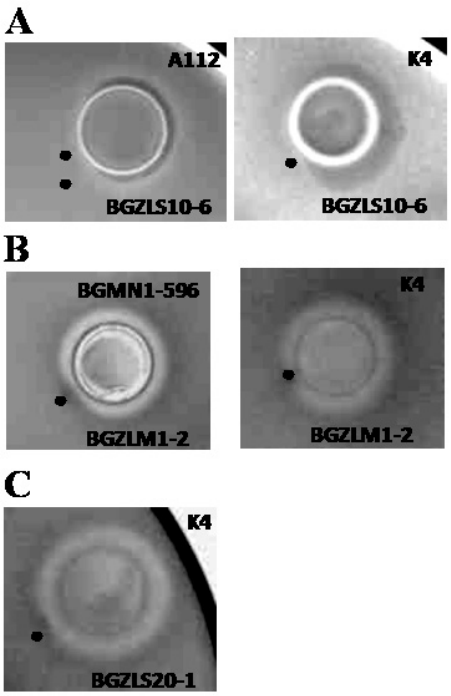

Figure 3. Inhibitory effect of isolates *Lb. paracasei* subsp. *paracasei* BGZLS10-6 (A), *Lb. paracasei* subsp. *paracasei* BGZLM1-2 (B) and *Lb. paracasei* subsp. *paracasei* BGZLS20-1 (C) on indicator strains: *Lc. lactis* subsp. *lactis* MN1-596, *Lb. plantarum* A112 and *Lb. paracasei* subsp. *paracasei* BGBUK2-16/K4, ● - crystal of pronase E.

The third group (Group III) comprises 37 isolates that belongs to subspecies *Lb. paracasei* subsp. *paracasei* (Table 1). This group is divided into three subgroups. Eight isolates of Subgroup I isolated from one and 10 days old cheeses, gave 1.5 mm and 5 mm clear zones only on lactobacillus indicator strains A112 and BGBUK2-16/K4, respectively. Isolate BGZLS10-6 is a representative of this Subgroup (Figure 3A).

Two isolates of Subgroup II, isolated from milk, showed 2 mm turbid zone on BGMN1-596, BGBUK2-16/K4 and A112 indicator strains. Representative of this Subgroup is isolate BGZLM1-2 (Figure 3B). Remaining 27 isolated lactobacilli were classified into Subgroup III. They exposed 3.5 mm turbid zone only on BGBUK2-16/K4 indicator strain and all of them are isolated from 20 days old cheese. The representative of this Subgroup is isolate BGZLS20-1 (Figure 3C).

*Proteolytic activity of isolates from Zlatar cheese***.** The ability of 43 randomly selected LAB isolates to hydrolyse β–casein fraction were tested. For that purpose, cells were grown on MCA plates and their proteolytic activities were determined by following the β–casein hydrolysis.

The examination of their proteolytic activity revealed that isolates belonging to *Lactococcus* and *Enterococcus* species exhibited weak proteolytic activity. Isolates BGZLM1-5, identified as *E. faecium* and BGZLS1-11 as well as BGZLS10-33, identified as *Lc. lactis* subsp. *lactis,* degraded ß-casein with low efficiency (Figure 4). Other lactococcal and enterococcal isolates do not degrade β-casein (data not shown).

Among 15 isolates identified as *Lb. paracasei* subsp. *paracasei* it was found that 8 of them exhibited pretty good proteolytic activity, while 7 of them showed poor degradation of β-casein. Representatives of isolated strains, identified as *Lb. plantarum* and *Lb. brevis*, degraded ß-casein poorly or not at all (Figure 5, 6 and 7).

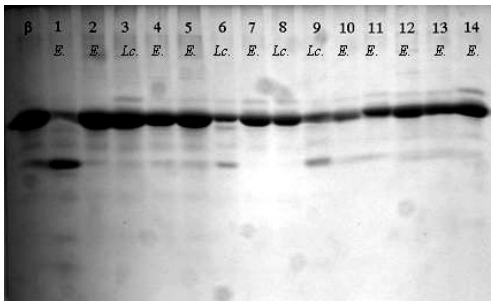

Figure 4. Proteolytic activity of *Lactococcus* (*Lc.*) and *Enterococcus* (*E.*) isolates.

ß – substrate ß-casein, Lane 1, BGZLM1-5, Lane 2, BGZLM1-20, Lane 3, BGZLM1-24, Lane 4, BGZLM1-35, Lane 5, BGZLS1-4, Lane 6, BGZLS1-11, Lane 7, BGZLS10-27, Lane 8, BGLZS10-35, Lane 9, BGZLS10-33, Lane 10, BGZLS20-27, Lane 11, BGZLS20-35, Lane 12, BGZLS30-8, Lane 13, BGZLS30-9, and Lane 14, BGZLS30-19.

Figure 5. Proteolytic activity of *Lactobacillus paracasei* subsp. *paracasei* isolates.
ß – substrate ß-casein, Lane 1, BGZLM1-4, Lane 2, BGZLS1-1, Lane 3, BGZLS10-6, Lane 4, BGZLS10-8, Lane 5, BGZLS20-8, and Lane 6, BGZLS30-23.

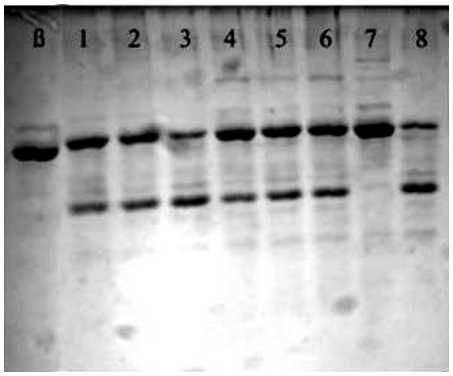

Figure 6. Proteolytic activity of *Lactobacillus* isolates.
ß – substrate ß-casein, Lanes 1, 2, 3, 5 and 8, *Lactobacillus paracasei* subsp. *paracasei* BGZLM1-2, BGZLS10-1, BGZLS20-5, BGZLS20-42 and BGZLS45-25, respectively.
Lanes 4 and 7, *Lactobacillus plantarum* BGZLS20-20 and BGZLS30-41.
Lane 6, *Lactobacillus brevis* BGZLS10-5

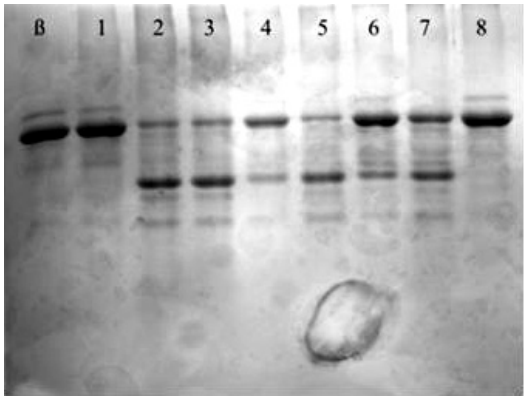

Figure 7. Proteolytic activity of *Lactobacillus* isolates.
ß – substrate ß-casein, Lanes 2, 3, 4, and 5, *Lactobacillus paracasei* subsp. *paracasei* BGZLS45-46, BGZLS45-50, BGZLS60-32, BGZLS60-38, respectively.
Lanes 7 and 8, *Lactobacillus plantarum* BGZLS60-43 and BGZLS60-58.
Lane 1 and 6, *Lactobacillus brevis* BGZLS45-36 and BGZLS30-2.

DISCUSSION

Production of cheeses and other milk products has a long tradition in Balkan region. These products could be a valuable source of lactic acid bacteria (LAB) that inhabits different ecological niches. This bacteria represent the specific microflora, which most probably contribute to the differences in flavour, texture and taste between manufactured cheeses (TOPISIROVIC *et al*., 2006) Recent studies have shown that artisan cheeses have a distinct and typical microbial population dynamics related to the local production technology and geographic area of origin (GOBBETTI *et al.,* 2002; MANNU *et al.,* 2002). Isolation of LAB from autochthonous milk products is a base for selection of new strains that had desirable properties for starter cultures. Criteria for strains selection are good activity in milk, proteolytic activity and ability to synthesize diacetyl and antimicrobial compounds.

Due to increased public interest in food safety, including demands for less artificial additives, attention in the research of LAB has been focused on the use of naturally occurring metabolites produced by food-grade bacteria. Bacteriocin produced by LAB may be a very promising source of biological food preservatives (BOLM and MORVEDT, 1991; PIARD and DESMAZEAUD, 1992). Capability of LAB to produce bacteriocins enables them an advantage in competition during the growth in mixed population.

A large number of LAB isolated from the Zlatar cheese produces antimicrobial compounds. The first group of LAB is lactococci. The most of them

produced nisin. Bearing in mind a wide spread use of nisin in fermentation industry, nisin producing LAB isolates from Zlatar cheese could be used as one of component in mixed starter cultures for the industrial manufacturing of cheese and other fermented milk products.

The second group of bacteriocin producers consists of enterococcal isolates. On the basis of similarity of size and appearance of zones of inhibition on all indicator strains, it could be speculated that all of them produced enterocins. However, in order to confirm such presumption, further analysis is required. Enterocins are of considerable importance because they have wide antimicrobial range against gram-positive bacteria including *Listeria monocitogenes* (RODRIGUEZ *et al*., 2002). The third group of bacteriocin producers found in Zlatar cheese is *Lb. paracasei* subsp. *paracasei* isolates.

Dairy LAB has manifolding auxotrophy and in order to grow in protein rich medium such as milk they depend on the expression of a complex proteolytic system (KUNJI *et al*., 1996). LAB isolated from Zlatar cheese showed a different ability in β-casein degradation. Generally, lactococci and enterococci isolated from Zlatar cheese showed very low proteolytic activity, if any. Similar results were obtained in testing the proteolytic activity of *Lb. plantarum* and *Lb. brevis* isolated from the same cheese. In contrast, *Lb. paracasei* subsp. *paracasei,* exhibited pretty good proteolytic activity and almost completely degraded ß-casein. The results of the examination showed that isolates with good proteolytic activity mainly have not good antimicrobial activity. Taking into account that LAB showing only Prt^+ or only Bac^+ phenotype are isolated from the same cheese sample, it could be inferred that their co-existence is feasible. Therefore, such LAB could be considered as a good source for construction of new starter cultures. Moreover, lactobacilli that contemporary showed good proteolytic and antimicrobial activity, for instance isolate BGZLS10-6, detected in Zlatar cheese support this conclusion.

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KOMPARATIVNA ANALIZA ANTIMIKROBNE I PROTEOLITIČKE AKTIVNOSTI BAKTERIJA MLEČNE KISELINE ISOLOVANIH IZ ZLATARSKOG SIRA

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I z v o d

Tradicionalni zlatarski sir pripada grupi belih, polutvrdih sireva proizvedenih u domaćinstvu. Sir se proizvodi od nepasterizovanog kravljeg mleka bez dodavanja bilo kakvih poznatih starter kultura. Ukupno je izolovano 253 Gram pozitivnih i katalaza negativnih bakterija mlečne kiseline (BMK). Rezultati su pokazali da 70 od 253 analiziranih izolata proizvodi antimikrobna jedinjenja poznatih kao bakteriocini. Većina izolata koji pripadaju rodovima *Lactococcus* i *Enterococcus*, kao i izolati vrsta *Lactobacillus plantarum* i *Lb. brevis* ne sintetišu ekstracelularne proteinaze. Nasuprot njima, izolati prodvrste *Lb. paracasei* subsp. *paracasei* pokazuju veoma dobru proteolitičku aktivnoist. Pokazano je da ne postoji korelacija izmedju dobre proteolitičke i antimikrobne aktivnosti u većini izolata.

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