VETERINARSKI ARHIV 88 (4), 521-534, 2018 DOI: 10.24099/vet.arhiv.0007

In vitro evaluation of the antimicrobial potential of *Streptococcus uberis* isolated from a local cheese from Southeastern Serbia

Mirjana Ž. Muruzović[1]*, Katarina G. Mladenović[1], Tanja D. Žugić-Petrović[2], and Ljiljana R. Čomić[1]

[1]*Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia*

[2]*College of Agriculture and Food Technology, Prokuplje, Serbia*

MURUZOVIĆ, M. Ž., K. G. MLADENOVIĆ, T. D. ŽUGIĆ-PETROVIĆ, LJ. R. ČOMIĆ: *In vitro* evaluation of the antimicrobial potential of *Streptococcus uberis* isolated from a local cheese from Southeastern Serbia. Vet. arhiv 88, 521-534, 2018.

ABSTRACT

Streptococcus uberis is an environmental bacterium responsible for bovine mastitis. It is occasionally described as a human pathogen. In our study, the isolation was undertaken of lactic acid bacteria from a local cheese from Southeastern Serbia, produced in a traditional way. *S. uberis* (7 isolates) and *S. agalactiae* (1 isolate) were isolated from the cheese samples taken in the summer. The biochemical and physiological characteristics of the isolates were examined. Using tetracycline, chloramphenicol, novobiocin and rifampicin, the antibiotic susceptibility of the isolates was evaluated. The results demonstrated that all the isolates were susceptible to all the tested antibiotics, with a growth inhibition zone from 36-48 mm. Also, the antagonism was examined of *S. uberis* KGPMF1-7 and *S. agalactiae* KFPMF8 isolates on the growth of *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Klebsiella oxytoca* KGPMF1*, Klebsiella ornithinolytica* KGPMF8 and *Aeromonas hydrophila*, as indicator stains. The results were compared with the activities of chloramphenicol, streptomycin and tetracycline on the tested indicator stains. The strongest antagonism was demonstrated by all *Streptococcus* isolates on the growth of *K. oxytoca* KGPMF1 (growth inhibition zone from 12-20 mm) and the *A. hydrophila* (growth inhibition zone from 13-20 mm). When these results were compared with the results of the sensitivity of tested indicator stains to antibiotics, *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8 isolates showed a moderate antagonistic effect. Due to the specific way cheese is made in from Sokobanja, these isolates probably originate from cows' udders.

Key words: *Streptococcus uberis; Streptococcus agalactiae;* cheese; antagonism; sensitivity to antibiotics

*Corresponding author:

Muruzović Mirjana, University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanović 12, 34000 Kragujevac, Serbia, Phone: +381 69 57 01 555; E-mail: mirkagrujovic@gmail.com

ISSN 0372-5480
Printed in Croatia

Introduction

Traditional agricultural products, especially cheeses, are recognizable by their authentic flavour and texture. The nutrition of dairy cows, the specific attributes of raw milk as well as the basic traditional cheese making process and the natural bacteria responsible for the fermentation process and ripening, have a very significant role in the formation of the flavour and organoleptic properties of raw milk cheeses (PRPIĆ et al., 2003; PAVELJŠEK et al., 2014; MIRECKI et al., 2015).

Streptococcus uberis is known worldwide as an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows, and it is also the predominant organism isolated from mammary glands during the non-lactating period (BRADLEY, 2002; KHAN et al., 2003). TRAJCEV et al. (2013) indicated that most cows manifest one or two cases of clinical mastitis during lactation.

S. agalactiae (Lancefield's serogroup B), *S. dysgalactiae* (Lancefield's serogroup C), as well as streptococci of serogroups D, G, L, O and P are the main agents causing bovine mastitis (KHAN, 2002). *S. uberis* isolates are classified as "non-Lancefield Streptococci" (LANCEFIELD, 1933). CENGIZ et al. (2014), MILANOV et al. (2015) and GALFI et al. (2016) indicated that the most important major mastitis pathogens are *Staphylococcus aureus* and *S. agalactiae,* as contagious pathogens, and *S. uberis*, S. *dysgalactiae*, *Escherichia coli* and *Enterococcus* sp.*,* as environmental pathogens. The presence of minor mastitis pathogens (*Corynebacterium* sp. and coagulase-negative staphylococci), is increasing significantly in udders, probably due to the fact that the prevalence of the major pathogens is decreasing (PYÖRÄLÄ and TAPONEN, 2009; REYHER et al., 2012; IDRISS et al., 2013; GALFI et al., 2016). In their study, MAĆEŠIĆ et al. (2012) indicated a rise in the importance of environmental pathogens in the epidemiology of bovine mastitis (GALFI et al., 2016).

The aims of this study were the evaluation of the biochemical and physiological characteristics of *S. uberis* and *S. agalactiae,* isolated from traditionally made cheese from Sokobanja, and the determination of their susceptibility to antibiotics. Also, the aim was the screening of their possible antagonism against some Gram-negative, food-spoilage bacteria (indicator stains) from their environment.

Materials and methods

Cheese-making, manufacture and sampling. The tested cheese was produced in countryside households around Sokobanja, Southeastern Serbia, in the traditional way. Raw, uncooked cow's milk was filtered after morning and evening milking, and then heated to a temperature of 30-40 ºC. ″SIRELA″ (Cacak, Serbia), a liquid rennet of microbiological origin based on chymosin obtained from the fungi *Rhizomucor miehei* and *Mucor miehei* (85% chymosin - 15% pepsin) was used for milk coagulation. The entire production of the cheese was carried out in wooden vessels. The cheese was salted

to 6-8% based on the total weight of the cheese. Cheese samples (300 g of each), taken in summer, from three different producers (households), were used for analysis. Each cheese sample was three days old, so the samples were in the same phase of ripening. The same procedure of cheese sampling was done in the autumn, from the same three households, in the same phase of ripening. Samples were transported aseptically to the microbiology laboratory at the Department of Biology and Ecology, University of Kragujevac. The samples were stored at 4 °C in a refrigerator.

Streptococcus sp. isolation and identification. The working cheese sample (10 g) was homogenized in 90 mL of 2% sodium citrate solution (pH 7.5) (Alkaloid, Skoplje, Macedonia), previously heated to 45°C and thoroughly mixed in a vortex until complete homogenization was reached. This was done for each sample, separately. Then, successively, 10-fold dilutions (up to 10^{-7}) were prepared with 2% sodium citrate, and 1 mL from 10^{-6} and 10^{-7} were added to M17 agar (Sigma- Aldrich, Sent Luis, USA), and incubated at 37 °C for 48 hours. A single colony resembling *Streptococcus* sp., was subcultured onto another M17 agar to obtain purification of isolates for further identification (ŠKRINJAR, 1994). For long-term preservation, the purified strains were stored at -20 °C in cryotubes containing M17 broth (80%) supplemented with 20% of glycerol (Zorka Sabac, Serbia). Then isolates were subjected to microscopic observation.

Furthermore, Gram-positive and catalase-negative isolates were identified to genus level using tests as follows: arginine, esculin and hippurate hydrolysis, fermentation of melibiose, sorbitol, inulin, lactose, arabitol, ribose, production of CO_2 from glucose, growth at 15 and 45 °C, tolerance to 4, 6.5 and 8% of sodium chloride, citrate utilization and hemolysis in blood agar.

Arginine and esculine hydrolysis, the production of CO_2 from glucose, and growth in the presence of different concentrations of sodium chloride, were performed according to ISENBERG (1992). The hippurate hydrolysis test was performed according to COWAN (1993)

The ability of citrate utilization was evaluated on citrate agar. The citrate agar consisted of 10 g skimmed milk powder, 2.5 g casein hydrolyzate (Torlak, Belgrade, Serbia), 5 g glucose, 18 g agar and 1000 mL distilled water. The ingredients were mixed to dissolve, adjusted to pH 6.6 and then sterilized by autoclaving at 121 °C for 15 min. After sterilization, 10 mL each of solution A (10% $K_3Fe(CN)_6$) and solution B (0.025 g/mL Fe-citrate and 0.025 g/mL Na-citrate) was added. The agar was inoculated with tested bacterial culture aseptically and incubated at 37 °C for 24 hours. The dark blue color of the colonies indicated the ability of citrate utilization by the tested bacteria.

The capacity for hemolysis of *S. uberis* and *S. agalactiae* strains was analyzed by inoculation onto Columbia agar with sheep blood (Oxoid, Hampshire, United Kingdom) and incubation at the temperature of 37 °C for 24 h. The plates were examined for the

existence of light zones, whereby α-hemolysis (green-hued zones around colonies), β-hemolysis (clear zones around) and γ-hemolysis (without aureole around colonies) indicated hemolytic activity, *i.e*. the pathogenesis of the examined strains.

Commercial kit identification - Microgen Strept ID. Isolates that gave equivocal results with the conventional tests were further analyzed using the Microgen Strept ID test, following the manufacturer's instructions. The dehydrated substrates (n = 20) were rehydrated by adding the bacterial suspension. The inoculum was prepared with freshly grown bacteria at 37 °C overnight on Columbia agar with sheep blood plus (Oxoid, Hampshire, United Kingdom), adjusted with 2 mL sterilized distilled water to at least McFarland No.2 turbidity standard. Following the manufacturer's recommendations, the test strip was inoculated and incubated. The color changes after incubation were read directly or after the addition of reagents. Isolates were identified by referring to the identification tables provided by the manufacturer.

Determination of the antibiotic susceptibility of tested isolates. In this study, *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8 isolates were tested for susceptibility to some antibiotics: tetracycline (Biolab zrt, Budapest, Hungary) 30 μL; chloramphenicol (Torlak, Belgrade, Serbia) 30 μL; novobiocin (Bioanalyse, Ankara, Türkiye) 30 μL; rifampicin (Torlak, Belgrade, Serbia) 5 μL. The bacterial reference strain, used as a control, was *Staphylococcus aureus* ATCC 29213. The ATCC strain was provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial strains were kept in glycerol stock at -80 °C until use.

S. uberis KGPMF1-7 and *S. agalactiae* KGPMF8 isolates were cultured on Columbia agar with sheep blood plus (Oxoid, Hampshire, United Kingdom) at 37 °C/24 h. *S. aureus* ATCC 25923 was cultured on nutrient agar at 37 °C, overnight. Bacterial suspensions were prepared by the direct colony method (ANDREWS, 2005). The turbidity of the initial suspension was adjusted using a McFarland densitometer (Biosan, Latvia). Bacterial suspensions contained about 10^8 colony-forming units (CFU)/mL, prepared in sterile 0.85% saline.

The bacterial suspension was inoculated onto Mueller-Hinton agar (Torlak, Belgrade, Serbia) supplemented with 5% defibrinated sheep blood, using a sterile cotton swab. The excess moisture was allowed to be absorbed for five min before applying the antibacterial disks. The disks were placed on the agar plates and the plates were incubated at 37°C for 16 to 18 h and the diameter of the inhibition zones was recorded. Zones of inhibition for the tested antibiotics were determined according to the Kirby-Bauer agar disk diffusion susceptibility test (BAUER et al., 1966).

Screening of S. uberis KGPMF1-7 and S. agalactiae KGPMF8 antimicrobial potential. The antimicrobial potential of the isolated *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8 was screened by the agar-well diffusion method (TAGG and McGIVEN, 1971),

using *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Klebsiella oxytoca* KGPMF1*, Klebsiella ornithinolytica* KGPMF8 and *Aeromonas hydrophila* as indicator strains. The ATCC strains were provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The Gram-negative isolates used in this test were also isolated from cheese from Sokobanja (MLADENOVIĆ et al., 2018), in order to test and compare the antagonistic potential of *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8. The bacterial strains were kept in glycerol stock at -80 °C until use.

Soft Nutrient agar (0.7%, w/v), containing indicator strains, was overlaid onto M17 plates. Wells were made in the lawn of the hardened soft agars. Overnight cultures (18 h) were centrifuged (Hettich Mikro 120 centrifuge, Sigma-Aldrich, Sent Luis, USA) at 10000 rpm/30 min/4 °C. Aliquots (100 μL) of the supernatant of the overnight cultures were filtered, neutralized to pH 6.2, and placed in the wells. The plates were incubated overnight at 37 °C. The clear zone of inhibition around the well was measured.

For comparison, indicator stains were tested for susceptibility to the antibiotics chloramphenicol (Torlak, Belgrade, Serbia) (30 μg), streptomycin (10 μg) and tetracycline (Biolab zrt, Budapest, Hungary) (30 μg), using the Kirby-Bauer agar disk diffusion susceptibility test (BAUER et al., 1966).

Statistical analysis. One sample *t*-test was used to compare the inhibitory effects of antibiotics and isolated LAB against the indicator strains. Data were analyzed using SPSS version 20 software (SPSS Inc., Chicago, IL, USA).

Results

Physiological and biochemical characteristics of isolated S. uberis KGPMF1-7 and S. agalactiae KGPMF8. In this article, the presence, biochemical, and physiological characteristics were investigated of streptococci isolated from cheeses produced in Southeastern Serbia (Sokobanja). The results obtained by testing their physiological and biochemical abilities demonstrated that 7 isolates belonged to *S. uberis* and 1 isolate to *S. agalactiae* (Table 1, 2).

Table 1. Physiological and biochemical characteristics of *S. uberis* and *S. agalactiae* isolates

Species	HIP	ESC	ARG	MEL	SOR	INU	LAC	ARA	RIB	CO_2
S. uberis KGPMF1	+	+	+	+	+	-	+	-	+	+
S. uberis KGPMF2	+	+	+	+	+	-	+	-	+	-
S. uberis KGPMF3	+	+	+	+	+	-	+	-	+	+
S. uberis KGPMF4	-	+	+	+	+	-	+	-	+	-
S. uberis KGPMF5	+	+	+	+	+	-	+	-	+	-
S. uberis KGPMF6	+	+	+	+	+	-	+	-	+	-
S. uberis KGPMF7	+	+	+	+	+	-	+	-	+	-
S. agalactiae KGPMF8	+	-	+	+	-	-	+	-	+	+

HIP-hippurate; ESC-esculin; ARG-arginine; MEL-melibiose; SOR-sorbitol; INU-inulin; LAC-lactose; ARA-arabitol; RIB-ribose; CO_{2}- production of CO_2 from glucose; + positive; - negative

All *S. uberis* and *S. agalactiae* isolates were isolated from summer samples of cheese from Sokobanja. From the first sample of cheese, all *S. uberis* isolates were isolated, while from the second sample, *S. agalactiae* KGPMF8 was isolated. The third sample of cheese, as well as all three samples from the autumn, did not contain *S. uberis*. All the tested isolates, except *S. uberis* KGPMF4 demonstrated the ability to hydrolyze hippurate. All the tested isolates, except *S. agalactiae* KGPMF8, demonstrated the ability to hydrolyze esculin, while all isolates demonstrated the ability to hydrolyze arginine. All the tested isolates showed the ability to ferment melibiose, lactose and ribose, but they showed no ability to ferment inulin and arabitol. All the isolates, except *S. agalactiae* KGPMF8, showed the ability to ferment sorbitol. *S. uberis* KGPMF1, *S. uberis* KGPMF3 and S. *agalactiae* KGPMF8 showed the ability to produce CO_2 from glucose (Table 1).

Table 2. Growth of *S. uberis* and *S. agalactiae* isolates in different conditions

Species	αHE	βHE	Growth in °C		NaCl (%)			Citrate Utilization
			15 °C	45 °C	4%	6.5%	8%	
S. uberis KGPMF1	+	-	+	+-	+	+	+-	+-
S. uberis KGPMF2	+	-	+	+	+	+	+-	+
S. uberis KGPMF3	+	-	+	+-	+	+	+-	+
S. uberis KGPMF4	+	-	+	+-	+	+	+-	-
S. uberis KGPMF5	+	-	+	+-	+	+-	-	-
S. uberis KGPMF6	+	-	+	+-	+	+-	-	-
S. uberis KGPMF7	+	-	+	+-	+	+-	-	-
S. agalactiae KGPMF8	-	-	+	+-	+	+	+-	+

αHE-α-haemolysis; βHE-β-haemolysis; + positive; - negative; +- partially positive

All *S. uberis* isolates showed α-hemolysis on blood agar. S. *agalactiae* KGPMF8 showed no hemolysis (γ-hemolysis). All the tested isolates demonstrated the ability to grow at 15 °C, but at 45 °C, the growth was reduced. All isolates demonstrated the ability to grow in broth with 4% and 6.5% sodium chloride, but in broth with 8% sodium chloride, the growth was reduced. *S. uberis* KGPMF1, *S. uberis* KGPMF2, *S. uberis* KGPMF3 and S. *agalactiae* KGPMF8 showed the ability to utilize citrate (Table 2).

Using the disc-diffusion method, the susceptibility was tested of seven *S. uberis* isolates and one *S. agalactiae* isolate to tetracycline, chloramphenicol, rifampicin and novobiocin. The results are shown in Table 3.

Table 3. Antibiotic sensitivity of *S. uberis* and *S. agalactiae* isolates

Species	Tetracycline		Chloramphenicol		Rifampicin		Novobiocin	
	GI*	S*	GI*	S*	GI*	S*	GI*	S*
S. uberis KGPMF1	40	(S)	42	(S)	44	(S)	38	(S)
S. uberis KGPMF2	44	(S)	46	(S)	38	(S)	42	(S)
S. uberis KGPMF3	40	(S)	40	(S)	40	(S)	44	(S)
S. uberis KGPMF4	48	(S)	40	(S)	44	(S)	40	(S)
S. uberis KGPMF5	42	(S)	38	(S)	40	(S)	36	(S)
S. uberis KGPMF6	40	(S)	46	(S)	44	(S)	46	(S)
S. uberis KGPMF7	42	(S)	44	(S)	42	(S)	40	(S)
S. agalactiae KGPMF8	40	(S)	40	(S)	40	(S)	38	(S)
Staphylococcus aureus ATCC 25923	22	(S)	28	(S)	30	(S)	32	(S)

GI* - growth inhibition in mm (millimeter); S* - sensitivity (S - susceptible; I- intermediate; R-resistance)

All the tested bacteria were susceptible to all the tested antibiotics. The inhibition zone diameter for tetracycline was 40-48 mm, for chloramphenicol 38-46 mm, for rifanpicin 38-44 mm and for novobiocin 36-46 mm. *S. aureus* ATCC 25923 was also susceptible to all the tested antibiotics, with an inhibition zone diameter of 22 mm, 28 mm, 30 mm and 32 mm for tetracycline, chloramphenicol, rifampicin and novobiocin, respectively.

Table 4. Antimicrobial potential of *S. uberis* and *S. agalactiae* isolates

Species	*E. coli* ATCC 25922	*P. mirabilis* ATCC 12453	*K. oxytoca* KGPMF1	*K. ornithinolytica* KGPMF8	*A. hydrophila*
S. uberis KGPMF1	19*	14	20	9	18
S. uberis KGPMF2	10	14	20	10	20
S. uberis KGPMF3	/	12	20	15	18
S. uberis KGPMF4	16	18	14	12	15
S. uberis KGPMF5	16	18	16	14	16
S. uberis KGPMF6	19	/	12	12	13
S. uberis KGPMF7	20	/	15	/	15
S. agalactiae KGPMF8	/	16	18	16	16

* values are given in mm (millimeter); / - no zone of inhibition

Antimicrobial potential of S. uberis KGPMF1-7 and S. agalactiae KGPMF8. In this work, using the agar-well diffusion method, the potential was tested of the isolated *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8 species to inhibit the growth of *E. coli* ATCC 25922 and *P. mirabilis* ATCC12453, as well as three isolates, *K. oxytoca* KGPMF1, *K. ornithinolytica* KGPMF8 and *A. hydrophila.* The results are shown in Table 4. Also, these indicator stains were tested for antibiotic resistance (Table 5). The antagonistic potential of the isolated streptococci compared to the effect of the chosen antibiotics was rated.

E. coli ATCC 25922 showed resistance to *S. uberis* KGPMF3 and *S. agalacticae* KGPMF8, while for the other *S. uberis* isolates the inhibition zone diameter was 10-20 mm. *P. mirabilis* ATCC 12453 showed resistance to *S. uberis* KGPMF6 and *S. uberis* KGPMF7, while for the other *S. uberis* isolates the inhibition zone diameter was 12-18 mm, and the *S. agalactiae* inhibition zone diameter was 16 mm. All the tested isolates inhibited the growth of *K. oxytoca* KGPMF1, the inhibition zone diameter was 12-20 mm. *K. ornithinolytica* KGPMF8 showed resistance to *S. uberis* KGPMF7, while for the other tested isolates the inhibition zone diameter was 9-16 mm. All the tested isolates inhibited the growth of *A. hydrophila*, where the inhibition zone diameter was 13-20 mm.

Table 5. Antibiotic sensitivity of the tested indicator stains

Species	Tetracycline		Streptomycin		Chloramphenicol	
	ZI*	S*	ZI*	S*	ZI*	S*
E. coli ATCC 25922	22	(S)	17	(S)	31	(S)
P. mirabilis ATCC 12453	10	(R)	22	(S)	45	(S)
K. oxytoca KGPMF1	15	(I)	17	(S)	24	(S)
K. ornithinolytica KGPMF8	22	(S)	20	(S)	28	(S)
A. hydrophila	15	(I)	20	(S)	32	(S)

ZI* - growth inhibition in mm (millimeter); S* - sensitivity (S- susceptible; I- intermediate; R-resistance)

All the tested indicator stains demonstrated sensitivity to streptomycin and chloramphenicol. *P. mirabilis* ATCC 12453 showed resistance to tetracycline (10 mm). Tetracycline (15 mm) had an intermediate effect on *K. oxytoca* KGPMF1 and *A. hydrophila*.

All the tested isolates, except *S. uberis* KGPMF6, showed a better effect on *A. hydrophila* than tetracycline (>15 mm). Chloramphenicol showed a better effect on the indicator stains than all the tested streptococci ($P<0.05$). Streptomycin showed a better effect on the indicator stains than *S. uberis* KGPMF4 and *S. uberis* KGPMF5 ($P<0.05$). According to the results, it may be concluded that the S. *uberis* KGPMF7-1-7 isolates and the S. *agalactiae* KGPMF8 isolate showed moderate antagonistic activity on the growth of the tested indicator stains.

Discussion

S. uberis is commonly described as an environmental pathogen, due to the fact that it possesses a great ability to survive and multiply in extreme sites. In our research, for the first time, the presence of *Streptococcus* sp. in cheese from Sokobanja, and their biochemical and physiological characteristics were investigated. *S. uberis* has also been isolated from the straw bedding of housed cattle (BRAMLEY, 1982). WILESMITH et al. (1986) determined the incidence of clinical mastitis and the associated bacteria in dairy herds. *S. uberis,* followed by *S. agalactiae,* were the most frequent pathogens isolated from clinical cases (WARD and SCHULTZ, 1974). FRANCIS et al. (1986) demonstrated the similar epidemiological features of clinical mastitis in dry cows in a three year study. It was reported that *S. uberis* was the predominant pathogen associated with dry period clinical cases, and that the risk of clinical cases in the dry period was higher than during lactation. Clinical mastitis increased during the winter housing period (October to March), with a peak occurring at the end of this period, while a secondary peak in incidence was recorded in August (FRANCIS et al., 1986). To the best of the author's knowledge, there are practically no data about the presence of this species in milk or cheese, or any other milk products in Serbia. In cheeses from Sokobanja, S. *uberis* KGPMF1-7 and S. *agalactiae* KGPMF8 were only isolated from summer samples of cheese. Due to the specific form of production of cheese from Sokobanja, these isolates, probably, originated from the cows' udders, and not as a cause of clinical mastitis.

MOREA et al. (1999) examined the dominant bacterial population in traditional mozzarella cheese. Their techniques allowed the identification of potential pathogens in a non-ripened cheese produced from raw milk. They isolated *S. uberis* with other *Streptococcus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, *Carnobacterium* and *Leuconostoc* species. The identification of the bacterial population was performed from their physiological properties. In our study, S. *uberis* KGPMF1-7 and S. *agalactiae* KGPMF8 were isolated as a part of the bacterial population which may be found in the traditionally made cheese from Sokobanja.

Antibiotic therapy is very important for controlling mastitis infections. Understanding the prevalence of the resistance and antibiotic susceptibility patterns of the microorganisms isolated from the milk of cows with the infection can improve the treatment of mastitis. Antibiotic intramammary infection therapy is initiated prior to microbiological culturing (GUTERBOCK et al., 1993; MILNER et al., 1997; WONGKATTIYA, 2008). ERBAS et al. (2016) examined the susceptibility of *Enterococcus* spp. isolated from mastitis milk to some antibiotics, and most isolates were resistant to tetracycline. ZDOLEC et al. (2016) indicated that the high prevalence of the development of resistance in enterococci isolated from milk samples of healthy cows, could be the result of animal cohabitation and cross-contamination. In our research, *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8 isolates shown tetracycline susceptibility. The reason may be found in fact that the bacteria were

isolated from cheese made from milk samples from healthy cows, not from bovine mastitis, so they may be treated as environmental. Several antibiotic susceptibilities of *S. uberis* isolated from bovine mastitis have been reported (BROWN and SCASSERRA, 1990; SALMON et al., 1998; ROSSITTO et al., 2002; McDOUGALL et al., 2014). However, there are no recent data available in the literature describing the susceptibility to any antibacterial agents of *S. uberis* isolated from milk and cheeses made in Serbia. In our research, all the tested isolates showed susceptibility to all tested antibiotics.

S. uberis is a lactic acid bacteria (LAB), a group that commonly produces bacteriocins (KLAENHAMMER, 1993; COTTER et al., 2005; PATROVSKÝ et al., 2016). Bacteriocins are proteinaceous antibiotics elaborated by bacteria that typically kill other bacteria of the same or closely related species (TAGG et al., 1976). It has been shown that *S. uberis* produce a novel nisin variant named nisin U. WIRAWAN et al. (2007) reported that, in addition to nisin U, *S. uberis* produce a second bacteriocin that may induce the lysis of metabolically active, susceptible target bacteria, and as a result it has been named uberolysin. In our research, for the first time, the ability of *S. uberis* and *S. agalactiae* isolated from cheese from Sokobanja, to inhibit the growth of some tested Gram-negative bacteria, was investigated and the results were compared with the results of commonly used antibiotics. The authors concluded that S. *uberis* and S. *agalactiae* isolates showed moderate antagonistic activity on the growth of the tested indicator stains.

Conclusion

In conclusion, *S. uberis* and S. *agalactiae* are present on farm premises and can quite easily gain entrance to a cow's udder through environmental contamination. In our research, *S. uberis* KGPMF1-7 and *S. agalactiae* KGPMF8 isolates were isolated from cheese from Sokobanja, which was made from raw milk, so the origin of these bacteria is probably from the cows' udders. All isolates showed susceptibility to all the tested antibiotics. For the first time, the ability was investigated of the isolated streptococci to inhibit the growth of some Gram-negative bacteria, which included potential pathogens. S. *uberis* KGPMF1-7 and S. *agalactiae* KGPMF8 showed moderate inhibitory activity. For these reasons, it is important to understand the way in which bacteria grow on cows' udders, the type of potential mastitis they may cause, and the range of good ways to prevent this from becoming a problem in your herd. Further investigations need to include examination of the potential production of antimicrobial compounds, because they show potential in the growth inhibition of Gram-negative bacteria.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia [41010]

Conflicts of interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

ANDREWS, J. M. (2005): BSAC standardized disc susceptibility testing method (version 4). J. Antimicrob. Chemother. 56, 60-76.
DOI: 10.1093/jac/dki124

BAUER, A.W., W. M. KIRBY, J. C. SHERRIS, M. TURCK (1966): Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45, 493-496.
DOI: 10.1093/ajcp/45.4_ts.493

BRADLEY, A. J. (2002): Bovine Mastitis: An Evolving Disease. Vet. J. 164, 116-128.
DOI: 10.1053/tvjl.2002.0724

BRAMLEY, A. J. (1982): Source of *Streptococcus uberis* in the dairy herd. Isolation from bovine feces and from straw bedding of cattle. J. Dairy Res. 49, 369-373.
DOI: 10.1017/S0022029900022500

BROWN, M. B., A. E. SCASSERRA (1990): Antimicrobial resistance in streptococcal species isolated from bovine mammary glands. Am. J. Vet. Res. 51, 2015-2018.

CENGIZ, S., G. DINÇ, M. Ü. SÖĞÜT (2014): Detection of several virulence properties, antibiotic resistance and phylogenetic relationship in *E. coli* isolates originated from cow mastitis. Acta Vet. Beograd 64, 413-425.
DOI: 10.2478/acve-2014-0039

COTTER, P. D., C. HILL, R. P. ROSS (2005): Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. 3, 777-788.
DOI: 10.1038/nrmicro1273

COWAN, S. T., K. J. STEEL, G. I. BARROW, R. K. A. FELTHAM (1993): Cowan and Steel's Manual for the Identification of Medical Bacteria, Cambridge University Press, p. 332.

ERBAS, G., U. PARIN, S. TURKYILMAZ, N. UCAN, M. OZTURK, O. KAYA (2016): Distribution of antibiotic resistance genes in *Enterococcus* spp. isolated from mastitis bovine milk. Acta Vet. Beograd 66, 336-346.
DOI: 10.1515/acve-2016-0029

FRANCIS, P. G., J. W. WILESMITH, C. D. WILSON (1986): Observations on the incidence of clinical bovine mastitis in non-lactating cows in England and Wales. Vet. Rec. 17, 549-552.
DOI: 10.1136/vr.118.20.549

GALFI, A. L., M. Ž. RADINOVIĆ, S. F. BOBOŠ, M. J. PAJIĆ, S. S. SAVIĆ, D. S. MILANOV (2016): Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings. Vet. arhiv 86, 487-497.

GUTERBOCK, W. M., A. L. VAN EENENNAAM, R. J. ANDERSON, I. A. GARDNER, J. S. CULLOR, C. A. HOLMBERG (1993): Efficacy of lntramammary antibiotic therapy for treatment of clinical mastitis caused by environmental pathogens. J. Dairy Sci. 76, 3437-3444.

DOI: 10.3168/jds.S0022-0302(93)77682-1

IDRISS, S. H. E., V. FOLTYS, V. TANČIN, K. KIRCHNEROVÁ, K. ZAUJEC (2013): Mastitis pathogens in milk of dairy cows in Slovakia. Slovak J. Anim. Sci. 46, 115-119.

ISENBERG, H. D. (1992): Clinical Microbiology Procedures Handbook, Vol. 1, Part 2. American Society for Microbiology, Washington DC, USA, pp. 1-18.

KHAN, I. U. (2002): Identification and further characterization of *Streptococcus uberis* and *Streptococcus parauberis* isolated from bovine milk samples. PhD thesis, Department of Silk Sciences, Institute of Veterinary food sciences, Justus-Liebig-University, Giessen.

KHAN, I. U., A. A. HASSAN, A. ABDULMAWJOOD, C. LÄMMLER, W. WOLTER, M. ZSCHÖCK (2003): Identification and epidemiological characterization of *Streptococcus uberis* isolated from bovine mastitis using conventional and molecular methods. J. Vet. Sci. 4, 213-224.

KLAENHAMMER, T. R. (1993): Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39-85.

DOI: 10.1111/j.1574-6976.1993.tb00012.x

LANCEFIELD, R. C. (1933): A serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. 57, 571-595.

DOI: 10.1084/jem.57.4.571

MAĆEŠIĆ, N., T. KARADJOLE, G. BAČIĆ, M. BENIĆ, M. KARADJOLE, S. VINCE, M. LIPAR, M. CERGOLJ (2012): A etiology and prevalence of bovine intramammary infection at drying off. Vet. arhiv 82, 125-131.

McDOUGALL, S., H. HUSSEIN, K. PETROVSKI (2014): Antimicrobial resistance in *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae* from dairy cows with mastitis. New Zeal. Vet. J. 62, 68-76.

DOI: 10.1080/00480169.2013.843135

MILANOV, D., B. PRUNIĆ, M. VELHNER, D. TODOROVIĆ, V. POLAČEK (2015): Investigation of biofilm formation and phylogenetic typing of *Escherichia coli* strains isolated from milk of cows with mastitis. Acta Vet. Beograd 65, 202-216.

DOI: 10.1515/acve-2015-0017

MILNER, P., K. L. PAGE, J. E. HILLERTON (1997): The effects of early antibiotic treatment following diagnosis of mastitis detected by a change in the electrical conductivity of milk. J. Dairy Sci. 80, 859-863.

DOI: 10.3168/jds.S0022-0302(97)76008-9

MIRECKI, S., N. POPOVIĆ, N. ANTUNAC, N. MIKULEC, D. PLAVLJANIĆ (2015): Production technology and some quality parameters of Njeguši cheese. Mljekarstvo 65, 280-286.

DOI: 10.15567/mljekarstvo.2015.0408

MLADENOVIĆ, K., M. MURUZOVIĆ, T. ŽUGIĆ-PETROVIĆ, O. STEFANOVIĆ, Lj. ČOMIĆ (2018): Isolation and identification of *Enterobacteriaceae* from traditional Serbian cheese and their physiological characteristics. J. Food Saf. 38, 1-9.

DOI: 10.1111/jfs.12387

MOREA, M., F. BARUZZI, P. S. COCCONCELLI (1999): Molecular and physiological characterization of dominant bacterial population in traditional Mozzarella cheese processing. J. Appl. Microbiol. 87, 574-582.

DOI: 10.1046/j.1365-2672.1999.00855.x

PATROVSKÝ, M., L. KOUŘIMSKÁ, Š. HAVLÍKOVÁ, J. MARKOVÁ, R. PECHAR, R. VOJTĚCH (2016): Utilization of bacteriocin-producing bacteria in dairy products. Mljekarstvo 66, 215-224.

DOI: 10.15567/mljekarstvo.2016.0306

PAVELJŠEK, D., A. TRMČIĆ, B. HACIN, I. ROGELJ (2014): PCR verification of microplate phenotypic system identification for LAB from traditional Western Balkan raw milk cheeses. Mljekarstvo 64, 245-253.

DOI: 10.15567/mljekarstvo.2014.0403

PRPIĆ, Z., S. KALIT, J. LUKAČ-HAVRANEK, M. ŠTIMAC, S. JERKOVIĆ (2003): Krčki cheese. Mljekarstvo 53, 175-194 (in Croatian).

PYÖRÄLÄ, S, S. TAPONEN (2009): Coagulase-negative staphylococci - emerging mastitis pathogens. Vet. Microbiol. 134, 3-8.

DOI: 10.1016/j.vetmic.2008.09.015

REYHER, K. K., I. D. HAINE, R. C. DOHOO, W. REVIE (2012): Examining the effect of intramammary infections with minor mastitis pathogens on the acquisition of new intramammary infections with major mastitis pathogens - a systematic review and metaanalysis. J. Dairy Sci. 95, 6483-6502.

DOI: 10.3168/jds.2012-5594

ROSSITTO, P. V., L. RUIZ, Y. KIKUCHI, K. GLENN, K. LUIZ, J. L. WATTS, J. S. CULLOR (2002): Antibiotic susceptibility patterns for environmental streptococci isolated from bovine mastitis in central California dairies. J. Dairy Sci. 85, 132-138.

DOI: 10.3168/jds.S0022-0302(02)74061-7

SALMON, S. A., J. L. WATTS, F. M. AARESTRUP, J. W. PANKEY, R. J. YANCEY (1998): Minimum inhibitory concentrations for selected antimicrobial agents against organisms isolated from the mammary glands of dairy heifers in New Zealand and Denmark. J. Dairy Sci. 81, 570-578.

DOI: 10.3168/jds.S0022-0302(98)75610-3

ŠKRINJAR, M. (1994): Methods of microbiological control of foodstuffs. Univerzitet u Novom Sadu, Novi Sad, Srbija, p. 16 (In Serbian).

TAGG, J. R, A. S. DAJANI, L. W. WANNAMAKER (1976): Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40, 722-756.

TAGG, J. R., A. R. McGIVEN (1971): Assay system for bacteriocins. Appl. Microbiol. 21, 943.

TRAJCEV, M., D. NAKOV, S. HRISTOV, S. ANDONOV, M. JOKSIMOVIC-TODOROVIC (2013): Clinical mastitis in Macedonian dairy herds. Acta Vet. 63, 63-76.
DOI: 10.2298/AVB1301063T

WARD, G. E., L. H. SCHULTZ (1974): Incidence and control of mastitis during the dry period. J. Dairy Sci. 57, 1341.
DOI: 10.3168/jds.S0022-0302(74)85065-4

WILESMITH, J. W., P. G. FRANCIS, C. S. WILSON (1986): Incidence of clinical mastitis in a cohort of British dairy herds. Vet. Rec. 118, 199-204.
DOI: 10.1136/vr.118.8.199

WIRAWAN, R. E., K. M. SWANSON, T. KLEFFMANN, J. W. RALPH, J. R. TAGG (2007): Uberolysin: a novel cyclic bacteriocin produced by *Streptococcus uberis*. Microbiol. 153, 1619-1630.
DOI: 10.1099/mic.0.2006/005967-0

WONGKATTIYA, N. (2008): Characterization and biological control of bovine of *Streptococcus uberis* strains associated with bovine mastitis. PhD Thesis, School of Applied Sciences, RMIT University, Melbourne, Australia.

ZDOLEC, N., V. DOBRANIĆ, I. BUTKOVIĆ, A. KOTURIĆ, I. FILIPOVIĆ, V. MEDVID (2016): Antimicrobial susceptibility of milk bacteria from healthy and drug-treated cow udder treated cow udder. Vet. arhiv 86, 163-172.

Received: 17 June 2017
Accepted: 17 March 2018

MURUZOVIĆ, M. Ž., K. G. MLADENOVIĆ, T. D. ŽUGIĆ-PETROVIĆ, LJ. R. ČOMIĆ: *In vitro* procjena antimikrobnog potencijala bakterije *Streptococcus uberis* izolirane iz autohtonog sira jugoistočne Srbije. Vet. arhiv 88, 521-534, 2018.

SAŽETAK

Streptococcus uberis jest okolišna bakterija odgovorna za mastitis kod goveda, a povremeno se opisuje kao patogen u ljudi. U ovom su istraživanju izolirane bakterije mliječne kiseline iz autohtonog sira jugoistočne Srbije, proizvedenog na tradicionalni način. *S. uberis* (7 izolata) i *S. agalactiae* (1 izolat) izolirani su iz uzoraka sira prikupljenih tijekom ljeta. Analizirane su biokemijske i fiziološke značajke izolata. Koristeći se tetraciklinom, kloramfenikolom, novobiocinom i rifampicinom, procijenjena je osjetljivost izolata na antibiotike. Rezultati su pokazali da su svi izolati osjetljivi na sve istražene antibiotike, sa zonom inhibicije rasta od 36 do 48 mm. Također, istražen je antagonizam *S. uberis* KGPMF1-7 i *S. agalactiae* KFPMF8 izolata na rast *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Klebsiella oxytoca* KGPMF1, *Klebsiella ornithinolytica* KGPMF8 i *Aeromonas hydrophila* kao indikatorskih sojeva. Rezultati su uspoređeni s aktivnostima kloramfenikola, streptomicina i tetraciklina na testiranim indikatorskim sojevima. Najjači antagonizam pokazali su svi *Streptococcus* izolati na rast *K. oxytoca* KGPMF1 (zona inhibicije rasta od 12 do 20 mm) i *A. hydrophila* (zona inhibicije rasta od 13 do 20 mm). Usporedbom tih rezultata s rezultatima osjetljivosti istraženih indikatorskih sojeva na antibiotike, za *S. uberis* KGPMF1-7 i *S. agalactiae* KGPMF8 izolate utvrđen je umjeren antagonistički učinak. Ti izolati vjerojatno potječu iz vimena krava i u siru iz Sokobanje prisutni su zbog specifičnosti njegove pripreme.

Ključne riječi: *Streptococcus uberis*; *Streptococcus agalactiae*; sir; antagonizam; osjetljivost na antibiotike

---