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Research article

SAFETY EVALUATION OF SJENICA CHEESE WITH REGARD TO COAGULASE-POSITIVE STAPHYLOCOCCI AND ANTIBIOTIC RESISTANCE OF LACTIC ACID BACTERIA AND STAPHYLOCOCCI

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Sjenica cheese is an artisanal cheese stored in brine, traditionally produced from raw sheep's milk in the southern part of Serbia - Sjenica Pester plateau.

The aim of this study was to perform the safety evaluation of Sjenica cheese. As one of the safety criteria we considered the number of coagulase positive staphylococci and their enterotoxigenic potential. Antibiotic susceptibility/resistance patterns of autochthonous lactic acid bacteria and coagulase-positive staphylococci isolated from Sjenica cheese was also investigated.

During the monitoring period of the cheese-making process, coagulase positive staphylococci did not reach the value of 10^5 cfu/g. Among coagulase positive staphylococci, 12 (46,15%) isolates showed enterotoxigenic potential and were identified as *Staphylococcus intermedius* (11 isolates) and *Staphylococcus aureus* (1 isolate). Vancomycin resistance was the most prevalent phenotypic resistance profile in coagulase positive staphylococci.

Lactococci present the most dominant population among lactic acid bacteria. The most prevalent resistance phenotype in lactococci was resistance to streptomycin (83.33%), ampicillin and penicillin (70.83%); lactobacilli were characterized by resistance to vancomycin (62.5%) and tetracycline (54.17%), while resistance to streptomycin (82.46%) was the most prevalent phenotypic profile in enterococci.

All coagulase positive staphylococci and lactic acid bacteria isolates that showed resistance to tetracycline on disc diffusion and E-test, were tested for the presence of ribosomal protection proteins, *tet(M)* and *tet(K)* genes. All isolates were positive for ribosomal protection proteins genes; 14 (60.87%) isolates showed *tet(M)* gene presence, while 2 lactobacilli isolates revealed the presence of *tet(K)* gene.

Key words: Sjenica cheese, safety evaluation, coagulase-positive staphylococci, lactic acid bacteria, antibiotic resistance

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INTRODUCTION

Serbia has a strong tradition in regional cheese making. Brined cheeses are the most important family of Serbian artisanal cheeses, and among them, Sjenica cheese received Protected Geographical Indication (PGI) designation on the national level [1]. Sjenica cheese is an artisanal cheese stored in brine, traditionally produced from raw sheep's milk in the southern part of Serbia - Sjenica Pester plateau. It is highly appreciated for its unique flavor and classified as a soft cheese which requires a ripening period of minimum 5 months for developing specific structure and aroma, but also for stabilizing the cheese matrix from the microbiological point of view. The aroma/flavor and textural characteristics of cheese depend strongly on the type of milk, production method, diversity and metabolic activity of lactic acid bacteria (LAB) strains as thermal treatment of raw milk is not done. The specific pedoclimatic microenvironments of Sjenica Pester plateau and selection pressure achieved by traditional technology maintained throughout centuries contribute to the establishment of a unique cheese ecosystem as a fermented microbial society with prevalence of lactic acid bacteria.

On our knowledge very few studies have been conducted on Sjenica cheese mainly regarding the technological characteristics of the traditional cheese making process [2,3] and potential of autochthonous strains of lactic acid bacteria isolated from Sjenica cheese to be used as starter cultures [4]. No literature data exist on safety evaluation of Sjenica cheese.

Traditionally, white-brined cheeses have been manufactured from raw milk and there is a risk that certain pathogens can survive, multiply in restrictive cheese matrix and contaminate the final product [5-7]. Furthermore, traditional cheese-making is open to contamination, so there is a growing need to monitor the process hygiene. *Staphylococcus aureus* is one of the major causes of food-borne intoxication due its' enterotoxigenic potential [8,9]. Sjenica cheese ripens in brine with 6-7% salt, and the temperature profile of curd handling techniques are not restrictive to staphylococcal growth, therefore conditions support the growth and multiplication of mesophilic halotolerant staphylococci, highlighting the risk of enterotoxins synthesis.

There is a growing scientific evidence that commensal microbiota in food could become a reservoir of antibiotic resistance genes disseminated via the food chain by ubiquitously demonstrated mechanism of lateral gene transfer [10-12]. The most frequent antibiotic resistance genes documented to widely occur in lactic acid bacteria are *tet* genes [13] - mostly due to their localization on mobile genetic elements such as plasmids and transposons and extensive use of tetracycline in human and animal therapy. Also, tetracycline resistance (TCr) is one of the most prevalent resistance phenotypes among staphylococci isolated from various food substrates due to the fact that the genetic base of TCr is encoded on transmissible plasmids and conjugative transposons.

The aims of the present work were therefore to perform the safety evaluation of Sjenica cheese regarding the risk of coagulase positive staphylococci presence; also to determine the current susceptibility/resistance patterns of autochthonous LAB and staphylococci originated from Sjenica cheese using phenotypic methods. The genetic base of tetracycline resistance in resistant staphylococcal and LAB population was revealed by applying molecular techniques.

MATERIALS AND METHODS

Material

The material for investigation consisted of 5 samples of raw sheep milk, 5 samples of curd before salting and 9 samples of Sjenica cheese from 1 to 90 days of ripening, collected from three households in the southern part of Serbia – Sjenica Pester plateau.

Microbiological examination

Microbiological examination was carried out in order to estimate the safety of the product and characterize the main microbial groups of lactic acid bacteria. As one of the safety criteria, coagulase-positive staphylococci (CoPS) were enumerated. Furthermore, the microbiota of LAB was studied in order to estimate their succession during the ripening process.

Plating and enumeration

About 25 ml (g) of the sample were aseptically homogenized in 225 ml of a 2% (w/v) sodium citrate solution prewarmed at 45°C using a stomacher. Sample homogenates were tenfold diluted in buffered peptone water and the dilutions were plated in duplicate on selective culture media. The isolation and enumeration procedure of coagulase positive staphylococci followed the ISO standard [14]. Also, for enumeration of the main group of LAB, serial dilutions were plated on specific agar media: thermophilic lactobacilli on MRS Agar (Merck) at 37°C for 48h, mesophilic lactococci on M17 Agar (Merck) at 30°C for 48h; enterococci on Kanamycin Aesculin Azide agar (Oxoid) at 37°C for 24h.

Determination of enterotoxigenic potential of coagulase-positive staphylococci and their identification

VIDAS® Staph enterotoxin II (SET2; bioMeri  ux, REF 30 705, 2004) Enzyme Linked Fluorescent Assay (ELFA) technique was used to determine the enterotoxigenic potential of CoPS isolates. API Staph biochemical test kit (bioMeri  ux REF 20 500) identification system was used for the identification of enterotoxigenic coagulase positive staphylococci.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was accomplished with the commercial test BBL Sensi-Disc Antimicrobial Susceptibility Test Discs performing the standard agar disc diffusion method in accordance with the Clinical Laboratory Standards Institute guidelines [15].

The staphylococcal isolates were tested for resistance to 5 antibiotics in the following concentrations: tetracycline (TE 30 µg), penicillin (P 10 IU), oxacillin (OX1 µg), ampicillin (AM 10 µg), and vancomycin (Va 30 µg). The disks containing 1 µg oxacillin (zone diameter ≥ 13 mm sensitive, ≤ 10 mm resistant) were used for resistance to methicillin [15].

The lactococci were subjected to disc diffusion procedure using antibiotic discs in the following concentrations: erythromycin (E 15 µg), gentamicin (GM 10µg), tetracycline (TE 30 µg), penicillin (P 10 IU), vancomycin (Va 30 µg), ampicillin (AM 10µg) and streptomycin (S 10µg).

The lactobacilli population was screened for antibiotic susceptibility/resistance patterns to 10 antibiotics in the following concentrations: erythromycin (E 15 µg), gentamicin (GM 10µg), tetracycline (TE 30 µg), penicillin (P 10 IU), vancomycin (Va 30 µg), clindamycin (CC 2µg), ciprofloxacin (CIP 5µg), chloramphenicol (C 30µg), ampicillin (AM 10µg) and streptomycin (S 10µg). These were the combination of the antimicrobial list of SCAN (Scientific Committee on Animal Nutrition EC) [16] and FEEDAP reports [17]. The isolates were classified as sensitive or resistant following the cut-off points according to the recommendations of the manufacturer.

Testing the antimicrobial susceptibility/resistant patterns, enterococci were exposed to the same antibiotics as lactobacilli, except for ciprofloxacin and additionally gentamicin discs were used in a concentration of 10 and 120 µg.

The tetracycline resistant subpopulation of staphylococci and LAB screened by disc diffusion test was subjected to Minimal Inhibition Concentration (MICs) determination by applying E test strips (AB Biodisk, Sweden) following the manufacturer's recommendations. Evaluation of MIC values for CoPS was done according to manufacturer's instructions; and for LAB according to FEEDAP [17].

Genetic characterization of tetracycline resistance

Isolates displaying tetracycline MICs equal to or higher than the breakpoints were considered resistant and therefore were submitted for PCR-based detection of genes encoding resistance to tetracycline (*tet* genes). In the first PCR assay, *tet* genes encoding tetracycline resistance through ribosomal protection proteins (RPP) were detected using universal primers. If positive for RPP genes, additional PCR test was performed using gene-specific primer for *tet*(K) and *tet*(M). The genomic DNA of tested strains was isolated by using MasterPure Complete DNA and RNA Purification kit (Epicentre, USA) and amplification procedure was performed in a thermocycler

(Applied Biosystems AB2720). The PCR reaction mixture consisted of Amplitaq Gold PCR Master Mix 2x (Invitrogen, USA), 400 nm each of the primers tested and 100 ng of respective DNA. Primer sequences, annealing temperatures and amplicon sizes are listed in Table 1. PCR-based detection of *tet* genes was performed under the conditions given in Table 2. Amplification products were detected by electrophoresis in 1% agarose gel (Invitrogen, USA), stained with ethidium bromide and as a final step were visualized and documented by using UV transilluminator and GelDoc system (Eppendorf, Germany).

Table 1. Primers and PCR conditions for selected antibiotic resistance genes

Resistance gene	Primers	Ta (°C)	Amplicon size (bp)	Sequence	Reference (s)
<i>rpp</i>	rpp-f rpp-r	45	1083	5'-GAYACNCCNGGNCAYRTNGAYTT-3' 5'-GCCCARWANGGRTTNGGNGGNACYTCA-3'	Clermont et al. (1997) [18]
<i>tetM</i>	tetM-f tetM-r	55	1513	5'-GAYACNCCNGGNCAYRTNGAYTT-3' 5'-CACCGAGCAGGGATTTCTCCAC-3'	Clermont et al. (1997) [18]
<i>tetK</i>	tetK-f tetK-r	55	384	5'-TTATGGTGGTTGTAGCTAGAAA-3' 5'-AAAGGGTTAGAACTCTTGAAA-3'	Guillaume et al (2000) [19]

Table 2. Program for multiplex PCR assay (*45°C annealing temperature for RPP encoding gen)

Initial denaturation	95°C	5 min
	95°C	30 s
30 cycles	45*/55°C	60 s
	72°C	120 s
Final elongation	72°C	7 min

RESULTS

Milk, gel, curd and cheese samples in the early phase of Sjenica cheese manufacturing process were subjected to conventional microbiological procedures in order to estimate the number of CoPS (Table 3).

Table 3. Dynamic of coagulase positive staphylococci in the early phase of cheese manufacturing process *log cfu/g mean values (standard deviation)

Cheese manufacturing phase	Nr. of staphylococci*
Milk	3.17 (1.44)
Gel of early coagulation	3.73 (1.43)
Cheese curd	4.53 (0.80)
Cheese aged (after) 3 days of maturation	4.23 (0.93)
Cheese aged (after) 7 days of maturation	3.21 (0.69)

Milk coagulation and curd formation conditions were favorable to CoPS growth but as the cheese matrix became more restrictive due to establishment of LAB in high numbers and during subsequent competition and lactic acid development we noticed a decrease in CoPS population.

As coagulase positive staphylococci, 26 out of 36 isolates suspected to belong to the CoPS group by observation on Baird Parker plates, were determined. Enterotoxigenic potential was detected in 12 (46,15%) isolates.

By applying the API identification system, enterotoxigenic isolates of staphylococci were identified as *Staphylococcus intermedius* (11) and *Staphylococcus aureus* (1).

Sjenica cheese is made from raw milk, therefore, the main biochemical activities responsible for conversion of milk to the end product with desired and unique sensory properties are governed by autochthonous lactic acid bacteria. Taking into account this fact, the biodiversity of autochthonous LAB can be considered as the crucial factor for the maintenance of typical features of artisanal cheeses. The mean count (expressed as log cfu/g) of LAB in Sjenica cheese throughout the 90 days of ripening is presented in Table 4.

Table 4. Mean counts of presumptive lactococci, lactobacilli and enterococci in Sjenica cheese *log cfu/g mean values (standard deviation)

Cheese aged	Lactic acid bacteria *		
	<i>Lactobacillus</i> spp.	<i>Lactococcus</i> spp.	<i>Enterococcus</i> spp.
30 days	7.53(0,28)	7.47 (0,80)	4.64 (0,36)
60 days	6.02 (0,57)	7.96 (0,33)	4.55 (0,54)
90 days	6.30 (0,30)	7.21 (0,09)	4.93 (0,48)

Results of the LAB study showed that *Lactococcus* spp. and *Lactobacillus* spp. were the dominant populations, followed by *Enterococcus* spp. The number of *Lactococcus* spp. reached level of 10^7 cfu/g and *Lactobacillus* spp. and *Enterococcus* spp. attained 10^6 and 10^4 cfu/g, respectively. Enterococci have been reported to be one of the most resistant microbial groups to restrictive conditions in the cheese matrix such as salt and acidity which may be the explanation for the slight increase in the enterococcal number.

CoPS isolates were screened for antimicrobial susceptibility/resistance profile by applying the agar disc diffusion method (Figure 1.)

In our study, we noticed that all tested staphylococci showed phenotypic resistance to at least one antibiotic. Multiresistance profile which was defined as resistance to 2 or more of antibiotics tested was described in 33.33 % isolates of coagulase positive staphylococci. None of the CoPS isolates showed resistance to oxacillin. It is worth mentioning that 83.33% CoPS isolates were characterized by vancomycin resistance.

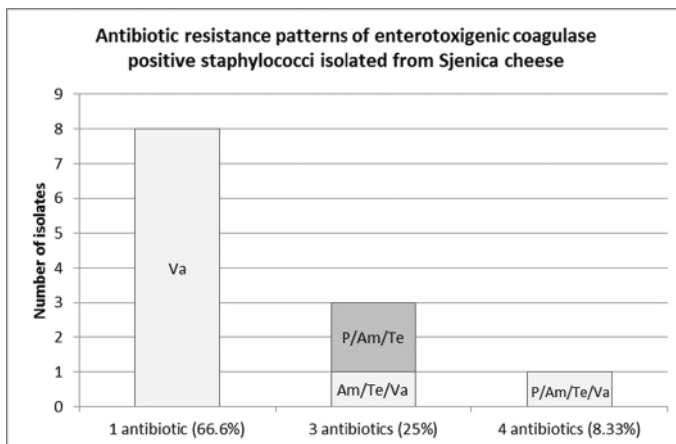


Figure 1. Antimicrobial resistance patterns of enterotoxigenic coagulase positive staphylococci isolated from Sjenica cheese (n=12) (Va – vancomycin, P – penicillin, Am – ampicillin, Te – tetracycline)

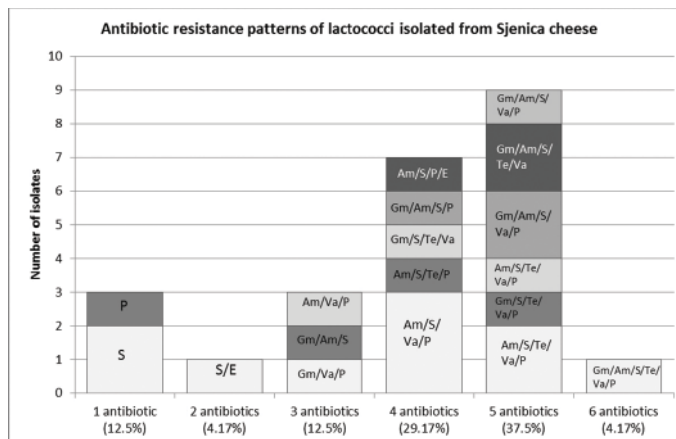


Figure 2. Antimicrobial resistance patterns of lactococci isolated from Sjenica cheese (n=24) (Am – ampicillin, E – erythromycin, S – streptomycin, Gm – gentamicin, Va – vancomycin, Te – tetracycline, P – penicillin)

LAB microbiota of Sjenica cheese were screened for antimicrobial resistance phenotypes by disc diffusion test (Figure 2, 3, 4). The screened LAB population demonstrated different profiles of phenotypic antibiotic resistance and multiple resistance to the tested antibiotics.

The most prevalent resistance phenotype among lactococci was resistance to streptomycin (83.33%), ampicillin and penicillin (70.83%). Resistance to vancomycin was detected in 16 (66.66%) isolates of tested lactococci.

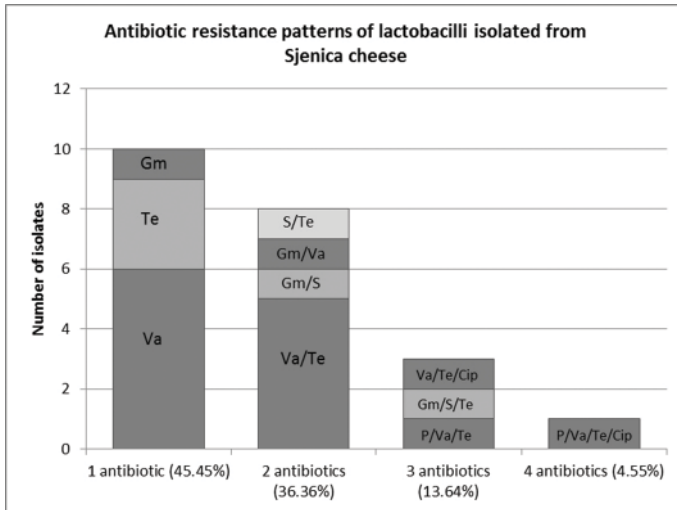


Figure 3. Antimicrobial resistance patterns of lactobacilli isolated from Sjenica cheese (n=24) (Gm – gentamicin, Va – vancomycin, Te – tetracycline, P – penicillin, S – streptomycin, Cip – ciprofloxacin)

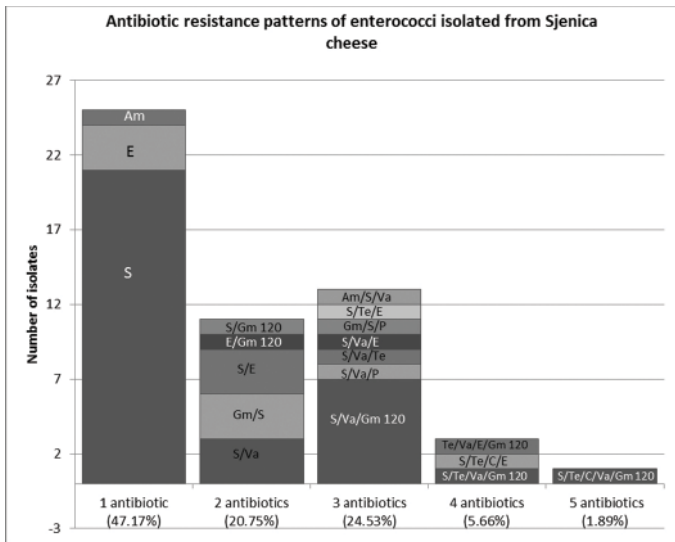


Figure 4. Antimicrobial resistance patterns of enterococci isolated from Sjenica cheese (n=57) (Am – ampicillin, E – erythromycin, S – streptomycin, Gm – gentamicin, Gm 120 – gentamicin 120 µg, Va – vancomycin, Te – tetracycline, P – penicillin, C – chloramphenicol)

The tested lactobacilli isolates were susceptible to erythromycin, clindamycin, chloramphenicol and ampicillin. The most prevalent resistance phenotype was resistance to vancomycin (62.5%), followed by resistance to tetracycline (54.17%).

Out of 57 tested isolates of enterococci, 47 (82.46%) isolates exhibited resistance to streptomycin. Vancomycin resistance was detected in 17 (29.82%) enterococcal isolates. None of the tested isolates of enterococci showed resistance to clindamycin and chloramphenicol.

As phenotypic screening of CoPS and LAB by agar disc-diffusion method showed the tetracycline resistant subpopulation, our next step was to perform the MIC evaluation (Table 5). Currently, there is no officially recognized cut-off value for LAB, so as a reference, we used the breakpoints established by the FEEDAP Panel of the European Food Safety Authority [20].

Table 5. Resistant population of staphylococci and LAB according to results of MIC evaluation for tetracycline (*according to manufacturer's recommendations; **FEEDAP [17])

Species	Disc-diffusion test		E-test	
	No. of resistant isolates	Cut-off values	No. of resistant isolates	
<i>Staphylococcus</i> spp.	4	1*	4	
<i>Lactococcus</i> spp.	9	4**	5	
<i>Lactobacillus</i> spp.	12	4/8/32**	12	
<i>Enterococcus</i> spp.	5	2**	2	

Four *Staphylococcus* spp. isolates, 5 *Lactococcus* spp. isolates, 12 isolates of lactobacilli and 2 isolates of enterococci including all tetracycline resistant strains, were tested for the presence of RPP, *tet(M)* and *tet(K)* antibiotic resistance determinants (Table 6, Figure 5).

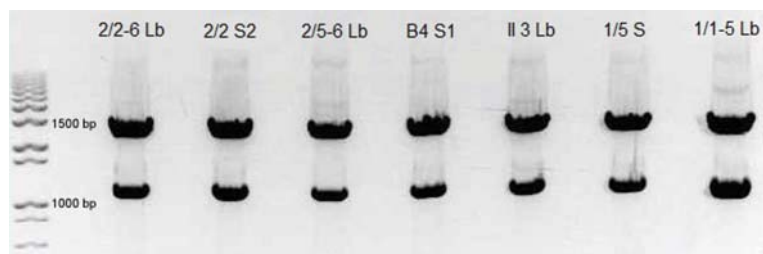


Figure 5. Gel electrophoresis analysis of *tet(M)* amplicons from tested strains of lactobacilli and CoPS (Lb- lactobacilli, S- coagulase positive staphylococci)

Table 6. Tetracycline resistance genes detected by conventional PCR in CoPS and LAB isolated from Sjenica cheese

Strain designation	Tetracycline resistance genes		
	RPP	tet (M)	tet (K)
<i>Lactobacillus</i> spp.			
2/2-6 Lb	+	+	-
1/2-8 Lb	+	+	-
1-1-7 Lb	+	+	-
2/4-1 Lb	+	-	+
2/5-6 Lb	+	+	-
1/1-5 Lb	+	+	-
II 3 Lb	+	+	-
II 2 Lb	+	-	-
2/5-5 Lb	+	+	-
II 3 Lb 30C	+	-	+
2/5-5 Lb*	+	+	-
1/5-5 Lb	+	-	-
<i>Lactococcus</i> spp.			
II 3 Lc 1	+	+	-
II 3 Lc 2	+	+	-
II 3 Lc 2a	+	-	-
II 2 Lc 1	+	-	-
II 2 Lc 1a	+	-	-
<i>Staphylococcus</i> spp.			
Λ2S1+	+	-	-
B4S1	+	+	-
2/2 S2	+	+	-
1/5 S	+	+	-
<i>Enterococcus</i> spp.			
1/1 E5	+	+	-
II E2	+	-	-

All isolates were positive for RPP genes; out of 23 tested isolates, 14 (60.87%) were characterized by *tet*(M) gene, while 2 lactobacilli isolates revealed the presence of *tet*(K) resistance determinant.

DISCUSSION

Raw milk cheeses are typical dairy products associated with foodborne intoxication caused by staphylococcal enterotoxins (SE). It is generally considered that enterotoxinogenic staphylococci must reach levels of at least 10^5 - 10^6 cfu/g to produce

detectable amounts of SE which significantly correlate with a toxic dose of less than 1 μ g required for clinical manifestation of intoxication [27]. This critical level of staphylococci may be reached in the early phase of cheese manufacturing (from 5 to 48 hours) when the cheese matrix is not acidified to a growth-restricting pH value and the competing LAB population has not reached a high number. Therefore, we followed the staphylococcal population (coagulase positive staphylococci-CoPS) throughout the hazardous period from the initial phase of Sjenica cheese manufacturing from raw milk to cheese aged for 7 days.

The slight increase in number of CoPS from milk to gel is partly due the physical phenomenon of bacterial entrapment by curd particles during milk coagulation [21,22]. During the initial phase of cheese manufacturing (coagulation, curd formation and handling) we noticed an increase in staphylococcal number as acidification rate governed by LAB fermentation activity was slow. The pH value of curd amounts 5.77 ± 0.15 (results not shown). Cogan et al. [23] showed that the majority of LAB isolates originated from 25 European artisanal cheeses were not good acid producers and reduced the pH of milk to 5.3 in 6h at 30°C. This moderate acidification is not sufficient to inhibit the staphylococcal growth. It was found that the number of CoPS decreased from day 3 to day 7 and was only present in low numbers (10²cfu/g) by day 90 (results not shown). The similar dynamic of CoPS population in raw milk cheese matrix was reported by other authors [24-26] who pointed out that inhibitory effects of natural LAB microbiota, reduced water activity (a_w) resulting from whey drainage, increased salt concentration during brining and temperature profile of the ripening process generate a hostile environment suboptimal for staphylococcal growth [27].

Out of 26 CoPS isolates, 12 (46.16%) were determined as enterotoxigenic. Similar prevalence of enterotoxigenic CoPS isolates was detected by a number of authors [28-30]. We have to bear in mind the scientifically well argued fact that even the coagulase positive staphylococci characterized as SE producers, will not produce toxins in every cheese matrix. It is attributed to the fact that SE synthesis is influenced by a number of intrinsic cheese parameters such as pH, water activity, redox potential, and to a great extent by bacterial antagonism as staphylococci are quite sensitive to microbial competition. Furthermore, the technological parameters such as acidification kinetics, intensity of curd operation, temperature profile of curd handling and ripening process, as an interplay, may generate a restrictive microenvironment which does not support enterotoxins synthesis [27].

The enterotoxigenic isolates of staphylococci were identified as *Staphylococcus intermedius* (11) and *Staphylococcus aureus* (1). Lamprell et al. [31] pointed out that all CoPS counted on Baird Parker agar do not necessarily belong to the *S. aureus* species, as some colonies may be identified as *S. intermedius*. *Staphylococcus intermedius* is the predominant non-*S. aureus* species isolated from food; some strains were characterized by enterotoxigenic potential [32] and shown to be clearly involved in staphylococcal food poisoning outbreaks [33]. Capurro et al. [34] described the presence of *S. intermedius* in bovine milk in a range of 0,2 to 2%. *Staphylococcus aureus* and *Staphylococcus intermedius* were

identified as dominant species among enterotoxigenic CoPS isolates from Turkish artisanal white cheeses [35].

Antibiotic resistance is a major public health concern as it is an ecological phenomenon with resistant bacteria persisting and circulating in the environment with possible transmission to humans via contaminated food. In our study, 12 cheese-associated CoPS isolates were investigated for their resistance to antibiotics (Figure 1.) Multiresistance is a common characteristic of 33.33 % CoPS isolates. None of the tested CoPS isolates were characterized by oxacillin resistance. Nevertheless, the scientific community agrees that accurate detection of oxacillin/methicillin resistance can be difficult due to the presence of two subpopulations – one susceptible and the other resistant- coexisting within a culture of staphylococci. This phenomenon is called heteroresistance and frequently occurs in oxacillin-resistant populations of staphylococci [36]. Vancomycin resistance is coded by genes carried on genetic mobile elements such as plasmids and transposons. The transmissible nature of vancomycin resistance contributes to the dissemination of this resistance profile among staphylococcal populations. The incidence of vancomycin resistant *Staphylococcus aureus* in raw milk, cheeses and biofilms has been reported worldwide [37-39]. Abulreesh and Organji [40] noticed remarkable resistance to β -lactams and vancomycin among *Staphylococcus aureus* and coagulase negative staphylococci recovered from raw milk, cheese samples, potable water and biofilms. Our results indicated vancomycin resistance in 83.33% CoPS isolates originated from autochthonous Sjenica cheese. On the contrary, Spanu [41] pointed out that all tested staphylococci isolated from raw sheep's milk cheese were susceptible to vancomycin.

There is a growing scientific evidence that commensal bacteria, especially lactic acid bacteria established at high numbers in fermented dairy ecosystems, may serve as reservoirs of antibiotic resistance genes potentially transferable to human and animal pathogens [42,43]. Hence, the investigation of LAB antibiotic resistance patterns may represent an efficient tool in predicting the antibiotic resistance among clinical pathogens. Extensive literature data pointed out that genes conferring resistance to several antimicrobials (i.e., chloramphenicol, erythromycin, streptomycin, tetracycline, and vancomycin), hosted on mobile genetic elements, have been characterized in lactococci [44], lactobacilli [45,46] and enterococci [47,48] from food.

Pioneer antibiotic susceptibility studies have showed *Lactococcus* strains to be susceptible to most antimicrobial agents [49-51]. Conversely, most recent studies have confirmed isolates of *Lactococcus lactis* resistant to chloramphenicol, erythromycin, streptomycin and tetracycline [10,52]. In our study, the majority of lactococci isolates were resistant to streptomycin (83.33%), ampicillin and penicillin (70.83%). Noteworthy, vancomycin resistance has been shown by 66.66% of tested lactococci. Contrary to our results, no *Lactococcus* isolates originated from traditionally fermented Indian food showed phenotypic resistance to tested antibiotics including the most representative ones among aminoglycosides, beta-lactams, cephalosporins, chloramphenicol, glycopeptides, lincosamides, macrolides and tetracyclines [53]. Toomey et al. [54]

reported on phenotypic resistance screen of *Lactococcus lactis* isolates to 6 common antibiotics (ampicillin, chloramphenicol, erythromycin, streptomycin, tetracycline, and vancomycin), although molecular characterization of streptomycin resistance failed to give amplicons corresponding to any of the known streptomycin resistance genes.

Our results showed a high level (62.5%) of phenotypic resistance to vancomycin among lactobacilli isolated from Sjenica cheese. This type of resistance associated to many *Lactobacillus* spp. has been often described as intrinsic [13]. However, in spite of the propulsion of vancomycin resistance among lactobacilli, it has also been suggested that it cannot be an intrinsic feature of the species due to the variability occurring among *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. johnsonii*, and *L. crispatus* strains [55,56]. All tested lactobacilli showed susceptibility to ampicillin and also high susceptibility toward penicillins which coincides with the general observation that lactobacilli are sensitive to the inhibitors of cell wall synthesis [13].

Enterococci are considered intrinsically resistant to beta-lactams [57], but our results do not support this observation since only two isolates of enterococci were characterized by penicillin and ampicillin resistance. Similar findings were recorded by other authors [58,59]. We observed a high prevalence of streptomycin resistance (82.46%). Using a 120 µg gentamicin disc, as a reliable indicator of high level gentamicin (HLG) resistance [60], we also found out that 12 (21.05%) enterococcal isolates showed HLG resistance. Similar results were obtained by other authors [61-63]. Prevalence of vancomycin resistance among enterococcal isolates was 29.82%. Bulajić & Mijačević [64] pointed out that among enterococcal strains isolated from autochthonous Sombor cheese, only one strain showed vancomycin resistance. In contrast, Citak et al. [65] have shown resistance to vancomycin among the population of enterococci isolated from Turkish white cheeses and was found in 96.8% of *E. faecalis* isolates, and 76% of *E. faecium* strains. Having in mind the fact that aminoglycosides (in combination with glycopeptides) are considered antimicrobials of choice for the treatment of enterococcal infections, the worst case scenario related to possible dissemination of this resistance through the food chain highlights the biological hazard.

Tetracycline resistance in most bacteria is governed by acquisition of new genetic material. Currently, there are more than 40 different genes coding for tetracycline resistance [66], mediated mainly by two mechanisms: protection of ribosomes and energy-dependent efflux of tetracycline. The variety of tetracycline genes and their localization on the mobilome, mainly conjugative plasmids and transposons [67,68] explains the wide distribution of TCr among foodborne LAB [42,13] and other bacterial genera.

Literature data showed the existence of intergenus and interspecies differences and also species dependency of lactobacilli resistance to various antibiotics [69]. Hence in performing MIC evaluation of lactobacilli, we applied group-specific or species specific MICs values. By applying the API identification system, the lactobacilli isolates were identified as *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum*

and *Lactobacillus brevis* (results not shown), so we used the cut-off value specific for corresponding species.

The MIC evaluation of presumptive tetracycline resistant populations of CoPS and LAB showed that majority of 30 tested isolates should be considered resistant to tetracycline (23 isolates). The discrepancy between the results obtained by disc-diffusion and E-test may be due to several factors: insufficient growth of LAB on standard test media; undesirable interaction between complex media components and tested antibiotics, but also a recognizable effect of inoculum size, temperature and time of incubation. At last but not least, no validated breakpoints for the discrimination of resistant and susceptible strains are defined, which additionally complicates the antimicrobial susceptibility assessment.

In order to clearly identify the risk of antibiotic resistance through the food chain, we need to characterize the genetic base of tetracycline resistance and therefore to complete the picture about the nature of antibiotic resistance. As the first step, we used tetracycline resistance genes encoding the ribosomal protection proteins (RPPs). As phylogenetic analysis confirmed the monophyletic origin of these determinants, it is possible to design a set of PCR primers which in positive samples give the amplicons of RPP genes in general [70]. After the isolates were subjected to PCR amplification with the universal primer set, we exposed the tested isolates to PCR screening with class-specific primers Tet M and Tet K. Tet K protein revealed the different mechanisms of tetracycline resistance- i.e. energy-dependent efflux of tetracycline.

All isolates were positive for RPP genes; out of the 23 tested isolates, 14 (60.87%) were characterized by *tet(M)* gene presence, while 2 lactobacilli isolates revealed the presence of *tet(K)* resistance determinant. In our study, PCR amplicons of some isolates revealed the presence of RPP genes, but not amplicons for *tet(M)* suggesting that other tetracycline resistance genes are present. There is an observation that *tet(M)* gene is the most frequently found tetracycline resistance determinant in LAB [71]. The widespread distribution of *tet(M)* among members of *L. plantarum*, *L. curvatus*, *L. casei*, *L. acidophilus*, *L. gasseri* and *L. crispatus* was reported by several authors [46,71,72]. Ammor *et al.* [73] confirmed coexistence of two tetracycline resistance genes *tet(M)* and *tet(K)* in a foodborne strain of *Lactobacillus sakei*. Our results did not support this finding. Molecular characterization of tetracycline resistance genes among staphylococci and LAB population originated from Spanish and Italian retail cheeses detected *tet(S)*, *tet(W)* and *tet(M)* as the most common resistance genes (12). Florez *et al.* [12] reported the presence of the *tet(M)* gene in two *L. lactis* strain isolated from an artisanal starter-free cheese. We confirmed the presence of the *tet(M)* gene in 2 out of 5 tested lactococcal isolates. The general observation pointed out that the presence of *tet(M)* has not been as frequently associated with lactococci as to other LAB such as *E. faecalis* and *Lactobacillus* spp. [74-76]. The transmissible nature of *tet(M)* and *tet(K)* genes originated from food-borne lactobacilli were confirmed as it has been shown their association to host a Tn916 transposon [76] and a plasmid [73] respectively.

In conclusion, the presence of coagulase positive enterotoxigenic staphylococci in Sjenica cheese does not present the objective risk as compliance with the principles of Good Hygienic Practice and Good Manufacturing Practice allows reliable control of contamination level and growth of staphylococci. Although the small proportion of CoPS and LAB isolated from Sjenica cheese were characterized by the presence of *tet* genes, the well-documented transmissible nature of these resistance determinants justified the need for further investigation in order to completely characterize the molecular mechanism of possible lateral transfer and consequently resistance dissemination through the food chain.

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PROCENA BEZBEDNOSTI SJENIČKOG SIRA U ODNOSU NA KOAGULAZA POZITIVNE STAFILOKOKE I ANTIBIOTSKU REZISTENCIJU BAKTERIJA MLEČNE KISELINE I STAFILOKOKA

BULAJIĆ Snežana, MIJAČEVIĆ Zora, LEDINA Tijana, GOLIC Bojan

Sjenički sir je autohtoni sir u salamuri koji se proizvodi od sirovog mleka na Sjeničko-peštarskoj visoravni.

Cilj ovog ispitivanja je bila procena bezbednosti sjeničkog sira sa aspekta prisustva koagulaza pozitivnih stafilocoka i profila rezistencije na antibiotike kod autohtone mikroflora bakterija mlečne kiseline i stafilocoka.

Tokom praćenog perioda proizvodnje sira, broj koagulaza pozitivnih stafilocoka nije dostigao vrednost od 10^5 cfu/g. Enterotoksogenu aktivnost je pokazalo 12 (46,15%) izolata koagulaza pozitivnih stafilocoka i oni su identifikovani kao *Staphylococcus intermedius* (11 izolata) i *Staphylococcus aureus* (1 izolat). Najčešći fenotip rezistencije među ispitivanim koagulaza pozitivnim stafilocokama je rezistencija na vankomicin.

Dominantnu populaciju među bakterijama mlečne kiseline činile su laktokoke. Kod laktokoka najprisutnija je bila rezistencija na streptomycin (83.33%), ampicilin i penicilin (70.83%); kod laktobacila rezistencija na vankomicin (62.5%) i tetraciklin (54.17%), a kod enterokoka rezistencija na streptomycin (82.46%).

Izolati koagulaza pozitivnih stafilocoka i bakterija mlečne kiseline koji su na disk-difuzionom i E-testu pokazali fenotipsku rezistenciju na tetraciklin, ispitivani su na prisustvo gena ribozomalnih zaštitnih proteina, *tet(M)* i *tet(K)* gena. Kod svih izolata je utvrđeno prisustvo gena ribozomalnih zaštitnih proteina; 14 od 23 (60.87%) je pokazalo prisustvo *tet(M)* gena, a 2 prisustvo *tet(K)* gena.