

*Research article*

## OCCURRENCE AND CHARACTERIZATION OF ENTEROTOXIGENIC STAPHYLOCOCCI ISOLATED FROM SOFT CHEESES IN SERBIA

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A total of 415 cheese samples produced with raw or cooked milk collected from local markets were analysed for the presence of coagulase-positive staphylococci. In 85 (20.48%) samples the presence of coagulase positive staphylococci was detected. The ELFA technique VIDAS SET2 (BioMerieux, France) was used for testing coagulase-positive staphylococci strains to produce classical enterotoxins (SEA, SEB, SEC, SED, SEE), and to determine the enterotoxins in cheese samples. The number of coagulase-positive staphylococci in cheese samples ranged from 1-5.79 log CFU g<sup>-1</sup>. Out of 85 coagulase-positive strains 26 (30.59%) produced enterotoxins. The presence of genes for the synthesis of staphylococcal enterotoxins (SE) in the obtained extracts of DNA from 26 enterotoxigenic strains was detected by conventional multiplex PCR technique (for genes *sea* and *seb*) i.e. the Real-Time PCR technique for genes *sec*, *sed* and *see*. In all 26 strains of coagulase-positive staphylococci (originating from cheeses produced from raw or cooked milk, which were enterotoxin producers) *sea* was present, and in 24 strains in addition to *sea* gene, *seb* was detected. None of the isolates possessed genes for the synthesis of enterotoxin C (SEC), D (SED) and E (SEE). Out of 26 tested cheese samples positive for enterotoxigenic coagulase-positive staphylococci, enterotoxin was detected in 2 (7.69%) samples of sweet-coagulating cheese, in which the number of enterotoxigenic coagulase-positive staphylococci exceeded 5 log CFU g<sup>-1</sup>. In sweet-coagulating cheeses in which the number of coagulase-positive staphylococci exceeds 5 log CFU g<sup>-1</sup> and the pH value was higher than 5.0, enterotoxins may be present in amounts sufficient to cause intoxication.

**Key words:** coagulase-positive staphylococci, enterotoxins, raw milk, cheeses

### INTRODUCTION

Cheese as food takes a very important part in the human diet due to its high nutritional value. Cheeses have been traditionally produced in Serbia for centuries, representing

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a cultural heritage and accumulated experimental knowledge, passed from generation to generation. Monasteries and feudal states were the main places where cheese production took place in the Middle Ages. The development of industrial production of cheese began in the early twentieth century, but still a significant portion of cheese, which can be found nowadays at greenmarkets in the Republic of Serbia, is produced in a traditional manner in small households. The individual producers use raw milk, cooked, or their mixture from the evening and/or morning milking. The coagulation process is carried out by addition of rennet, and no commercial starter cultures are used in the cheese production process. The native lactic acid bacteria are the main microbiota in the fermentation process and the ripening period usually lasts 7 to 10 days. Since a number of cheeses are produced from raw milk as part of the tradition, there is a possibility that pathogenic microorganisms, such as coagulase-positive staphylococci, pass from the milk to the cheese. Approximately 10% of cheese in Europe is made from raw milk [1] presenting a considerable potential risk to public health. Most cheeses at local markets in Serbia belong to the group of soft cheeses, which constitute, according to the Serbian legislation, the diversified cheese category which includes cheeses with >67% water content in total solid matter without fat). The cheeses are divided in two groups: soft cheeses without ripening (aged to 7 days) and soft cheeses with ripening (longer than 7 days). Since the raw milk is used for production of soft cheeses present at the markets in Serbia, the data from the literature show that coagulase-positive staphylococci may be present in raw milk and there are no epidemiological reports that soft cheeses are reported as a cause of intoxication we undertook to examine the occurrence of enterotoxigenic coagulase-positive staphylococci in soft cheeses produced in small households in the Republic of Serbia.

Staphylococci are aerobic, facultative anaerobic bacteria, taxonomically belonging to the family *Staphylococcaceae*, genus *Staphylococcus* [2]. To date, more than 50 species and subspecies of staphylococci have been described. According to their potential to produce the enzyme coagulase they are divided into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). The main representative of the coagulase-positive staphylococci is *Staphylococcus aureus* subsp. *aureus*. As a ubiquitous microorganism *Staphylococcus aureus* (*S. aureus*) lives on the skin of humans and animals, and often colonizes the ductus papillaris of the mammary glands and may cause subclinical mastitis. In properly drawn milk, the typical counts of *S. aureus* are 100-200 CFU/ml. In the case of subclinical mastitis, the counts may increase up to 10<sup>4</sup> CFU ml<sup>-1</sup>. The natural ecological niches of *S. aureus* are the nasal cavity and skin of warm-blooded animals. The skin, mucosa membranes, teats and udders of dairy animals are the most important reservoir of this contaminant. It is responsible for approximately 30-40% of all mastitis cases in the world an important characteristic of the microorganism ability to produce extracellular enzymes and toxins, many of which are pathogenic for humans and animals. From the view point of milk hygiene, the ability of *S. aureus* to synthesize thermostable enterotoxins, that can cause food-borne poisoning of people, is important. Staphylococcal food poisoning (SFP) is one of the most common food-

borne intoxication diseases, caused by enterotoxins produced mainly by *Staphylococcus aureus* (*S. aureus*) strains. SFP is an intoxication resulting from the consumption of food containing sufficient amount ( $1\mu\text{g kg}^{-1}$  bodyweight consumer) of one or more enterotoxins.

To date, 23 staphylococcal enterotoxins (staphylococcal enterotoxins-SEs) and enterotoxin-like toxins (staphylococcal enterotoxin-like toxins-SEL) have been described [3-5]; enterotoxin A (SEA), B (SEB), C1 (SEC1), C2 (SEC2), C3 (SEC3), D (SED), E (SEE), G (SEG), H (SEH), I (SEI), J (SEJ) [6], K (SEIK) [7], L (SEIL), M (SEIM), N (SEIN), O (SEIO) [8], P (SEIP) [9], Q (SEIQ) [7], R (SEIR) [10], S (SEIS), T (SEIT) [5], U (SEU) [11] and U2 and V, which are located on the cluster *egc* which encodes enterotoxin-like toxins synthesis [12]. Among the 23 SEs reported in literature only five SEA, SEB, SEC, SED, and SEE, different in antigenic reaction recognized as a classic enterotoxins can be identified with commercially available immunoassay kits. Staphylococcal enterotoxins have been the cause of 6.4% of food-borne outbreaks in the European Union (EU) in 2012, placing bacterial toxins as the third most common outbreak causative agent in the EU [1]. In the USA, *S. aureus* was ranked as one of the five most frequent causes of food-borne outbreaks with more than 240,000 illnesses annually. According to the EFSA record from 2014 year 777 outbreaks in 2012 were caused by the toxins of *Bacillus* spp., *Clostridium* spp. and coagulase-positive staphylococci. SFP takes the second place among food borne diseases. Out of the total number of recorded disorders 346 were caused by staphylococcal enterotoxin and cheese caused 20% of alimentary intoxications. The food, associated with staphylococcal enterotoxin poisoning is the one rich in proteins, which is produced in artisanal conditions and when the production process is followed by manual manipulation, often in combination with inadequate thermal processing and storage. Often many of these cases of poisoning remained undetected because the incubation period was short, small outbreaks are not reported, due to mistakes in the diagnosis, irregularities during sampling, i.e. errors in laboratory diagnostics. For the formation of a sufficient amount of enterotoxins able to cause intoxication must be more than  $10^5$  CFU *S. aureus*  $\text{g}^{-1}$  of cheese [13,14].

According to the official record of the Health Statistical Yearbook [15] during 2013, in Serbia, 749 cases of bacterial food intoxication were reported, the ethological agents not being specified. Since the data are insufficient in number of SFP and cheeses as food rarely reported as the cause of food poisoning even they are often purchased at markets and consumed fresh in accordance to consumers' habits, the aim of this study was to examine the occurrence of enterotoxigenic coagulase-positive staphylococci in soft cheeses.

The aim included: (1) to determine the number of coagulase-positive staphylococci in cheese samples traditionally produced in small households originating from different geographical localities in the Republic of Serbia collected at green markets in Belgrade, (2) to identify isolated strains of coagulase positive staphylococci and characterize them on the basis of their ability to produce staphylococcal enterotoxins (ses), (3)

to determine the presence of genes for enterotoxin synthesis (4) to determine the presence of enterotoxins in the cheese matrix.

## MATERIAL AND METHODS

### Sampling

The total of 415 cheese samples produced with raw or cooked cows' milk was collected at 17 green markets in the city of Belgrade. The cheeses of different age, were traditionally produced in individual households without the addition of commercial starter cultures originating from different geographical localities (Figure 1). The samples were aseptically collected in sterile plastic bags according to ISO 707:2008 (IDF 50:2008) standard and transferred under refrigeration (at about 4°C) to the laboratories where they were immediately analysed.

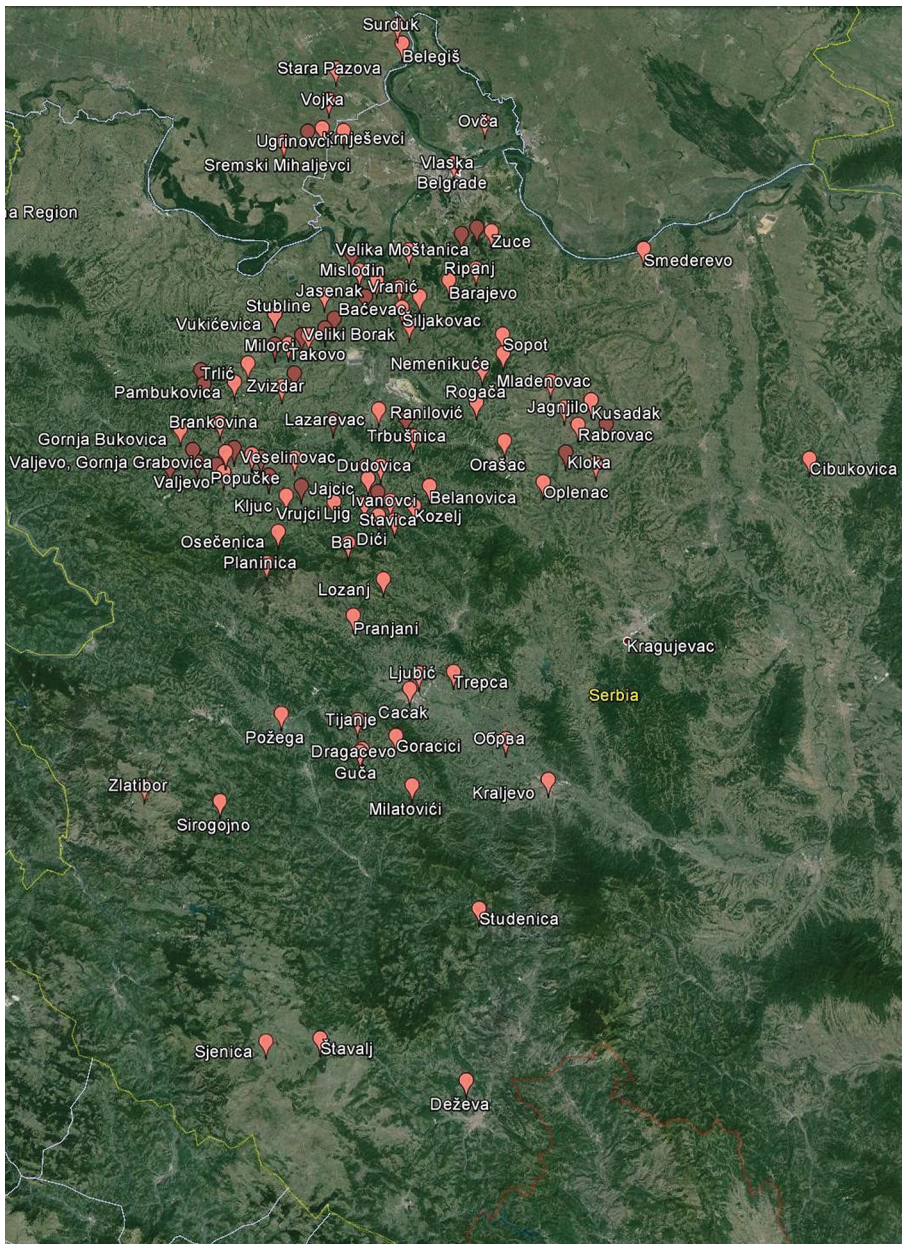
Coagulation in all cheese samples was due to the addition of rennet. The pH value of cheeses was the criteria for dividing cheese samples into 2 groups: acid-coagulating and sweet-coagulating cheeses. All cheeses in which the pH value measured was more than 4.6 were classified into the group of sweet-coagulating and pH less than 4.6 into the group of acid-coagulating cheeses [16]. Out of 415 cheese samples 126 were acid-coagulating and 289 sweet-coagulating cheeses. The age of cheese samples was determined on the basis of the results obtained from the given questionnaire to individual producers. Cheese samples aged under 7 days were classified into the group of cheeses without ripening and cheese samples aged longer than 7 days into group of cheeses with the ripening phase according to legislation [17].

### Isolation and enumeration of coagulase-positive staphylococci

Cheese samples (20 g) were diluted in 180 mL of buffered peptone water (LAB, Bury, Lancashire, U.K.), homogenized in a BagMixer (Interscience). Sample homogenates were tenfold diluted in buffered peptone water and the dilutions then plated in duplicate on selective culture media. In order to isolate and enumerate coagulase-positive staphylococci, the standard ISO 6888 method with Baird Parker medium was used [18]. The limit of detection was 100 CFU g<sup>-1</sup>. The typical five colonies (circular, black, smooth, convex, with perfect edges, surrounded by an opaque zone and/or transparent halo) per sample were subjected to Gram staining, catalase test and coagulase test. Gram, catalase and coagulase positive strains were biochemically identified, using the ID 32 Staph-Biomerieux (BioMérieux, France) to the species level on the basis of their biochemical characteristics.

### Strains

One strain of coagulase-positive staphylococci per positive cheese sample was examined to detect the ability to synthesize SEs, described as follows: 85 strains were tested for the staphylococcal enterotoxins and 26 strains enterotoxin producers were



**Figure 1.** Geographical origin of cheese samples collected at Belgrade markets

tested for presence of genes for enterotoxin synthesis. Each strain was stored in nutrient agar slant prior to the analysis. Type strain *Staphylococcus aureus* ATCC 25923, as well as strains *Staphylococcus aureus aureus* CIP 67.8, enterotoxin A i enterotoxin B producers were kindly provided by The Institute of Meat Hygiene and Technology, Belgrade and used as control.

## Enumeration of lactic acid bacteria

Determination the number of *Lactococcus* spp. and *Lactobacillus* spp. was performed by the standard ISO27205 (IDF 149:2010) method [19].

## Chemical analysis

The pH value was measured with a pH-meter (pH-Vision 246071 Extech instruments), the water activity by  $a_w$ -meter (GBX Scientific Instruments, FA-st/1 tastatura: Model MX 3700/ML 4700). Sodium chloride (NaCl) content in cheese samples was determined by the titrimetric method [20].

## Determination of staphylococcal enterotoxins

The ELFA technique VIDAS® Staph enterotoxin II (SET2; bioMérieux, REF 30 705, 2004) was used for the detection of classical enterotoxins SEA, SEB, SEC, SED and SEE, using the equipment Mini VIDAS (bioMérieux, France), with the detection limits of 0.5 ng ml<sup>-1</sup> for SEA and SEB, and of 1.0 ng ml<sup>-1</sup> for SEC–SEE. The isolates were cultured for 24 h aerobically in 10 mL BHI broth (Merck, Germany) at 37°C. Bacterial culture supernatants were collected by centrifugation at 7000 g for 10 min. and the pH value was adjusted to 7.5–8. The aliquot (500µL) was transferred directly into the well of the VIDAS SET2 strip. As the ELFA technique does not allow the quantitative detection of SEs, the results are expressed as either positive or negative.

## PCR amplification

The presence of *se* genes encoding the synthesis of staphylococcal enterotoxins (SE) in the obtained extracts of DNA from 26 enterotoxigenic coagulase-positive isolates was detected by the conventional multiplex PCR technique (for genes *sea* and *seb*) i.e. the Real-Time PCR technique (for genes *sec*, *sed* and *see*). The primers used in this work, their sequences, and size of fragments are summarised in Table 1.

**Table 1.** List of primers used for determination of genes for staphylococcal enterotoxins

Primer	Target gen	Length of amplified sequence (bp)	Sequence
sea-f sea-r	<i>sea</i>	93	5'-TCAATTTATGGCTAGACGGTAAACAA-3' 5'- GAAGATCCAACCTCCTGAACAGTTACA -3'
seb-f seb-r	<i>seb</i>	85	5'- AACAACTCGCCTTATGAAACGGGAT -3' 5'- CTCCTGGTGCAGGCATCATGTCA -3'
sec-f sec-r	<i>sec</i>	284	5'- CGTATTAGCAGAGAGCCAACCA -3' 5'- GTGAATTTACTCGCTTTGTGCAA -3'
sed-f sed-r	<i>sed</i>	150	5'- AAACGTTAAAGCCAATGAAAACA -3' 5'- TGATCTCCTGTACTTTTATTTCTCCTA -3'
see-f see-r	<i>see</i>	171	5'- TACCAATTAACCTTGTGGATAGAC -3' 5'- CTCTTTGCACCTTACCGC -3'

## RESULTS

This study included cheese samples of different ages, produced in small households from different geographical localities in Serbia (Figure 1). Bacteriological findings of cheese samples with coagulase-positive staphylococci are presented in Table 2. Out of 415 cheese samples in 85 (20.48%) cheese samples were detected coagulase-positive staphylococci. The number of coagulase-positive staphylococci in cheese samples ranged from 1-5.79 log CFU g<sup>-1</sup> (Table 3).

**Table 2.** Occurrence of coagulase-positive staphylococci in cheese samples

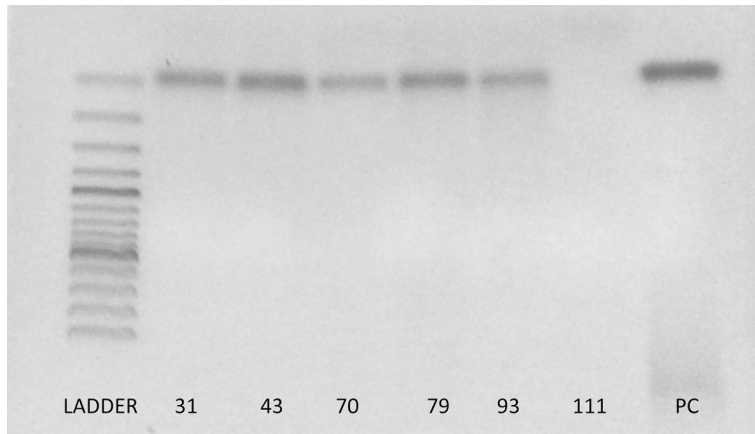
Type of cheese	Number of samples (n)	Positive for coagulase-positive staphylococci					
		Cheeses produced with cooked milk		Cheeses produced with raw milk		Total	
		Number	%	Number	%	Number	%
Acid-coagulating	126	1	0.79	18	14.29	19	15.08
Sweet-coagulating	289	23	7.96	43	14.89	66	22.84
Total	415	24	5.78	61	14.70	85	20.48

**Table 3.** Contamination level of cheese samples with coagulase-positive staphylococci

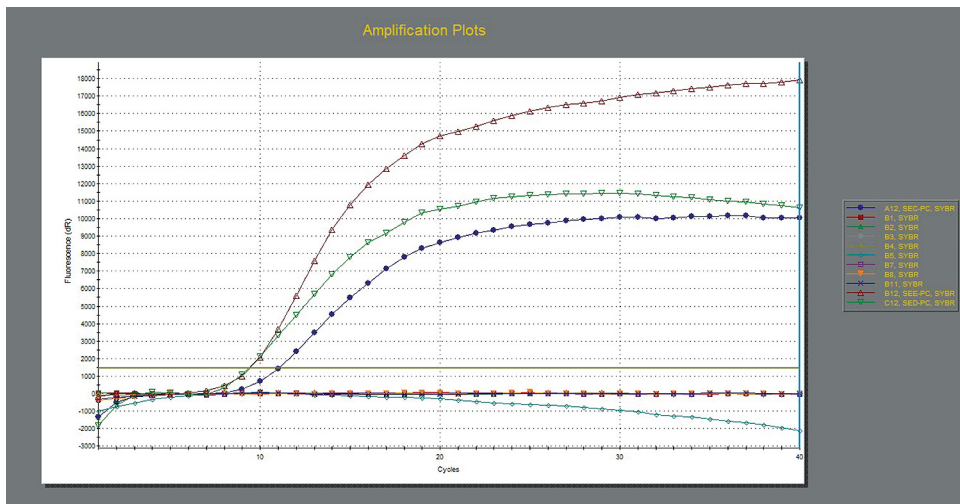
Level of contamination	Number of samples (n)	Sweet-coagulating cheeses				Acid-coagulating cheeses				
		Cooked milk		Raw milk		Number of samples (n)	Cooked milk		Raw milk	
		n	%	n	%		n	%	n	%
≤ 2 log cfu/g	6	4	66.67	2	33.33	3	1	33.33	2	66.67
2-5 log cfu/g	47	20	42.55	27	57.45	13	0	0.00	13	100
> 5 log cfu/g	13	3	23.08	10	76.92	3	0	0.00	3	100
Total	66	27	40.91	39	59.09	19	1	5.26	18	94.74

Out of 85 cheese samples positive for coagulase-positive staphylococci 26 (30.59%) were positive for enterotoxigenic staphylococci from which 20 (23.53%) were produced of raw milk and 6 (7.06%) of cooked milk (Table 4). All 26 isolates (one per positive sample) were identified by mPCR reaction as belonging to *S. aureus*. The ID 32 Staph-Biomerieux identification was in agreement with that the PCR reaction. Since the ELFA technique used in the experiment, detected the group of classical enterotoxins (SEA-SEE), it was decided to identify *se* genes encoding the synthesis of one or more toxins. All 26 isolates produced classical enterotoxins (SEA-SEB). DNA extracted from those staphylococcal isolates was used for testing for enterotoxin genes using the conventional multiplex PCR for genes *sea* and *seb* (Figure 2), or the

technique of Real-Time PCR for genes *sec*, *sed* and *see* (Figure 3). Origin of isolates positive to enterotoxin genes revealed that more isolates came from sweet coagulating cheeses, than acid and more came from raw than cooked milk (Table 4). In all 26 isolates of coagulase-positive staphylococci originating from cheeses produced from raw/cooked milk (which were enterotoxin producers) the gene for enterotoxin A (*sea*) was detected, and in 24 isolates in addition to the *sea* gene, the gene for the synthesis of enterotoxin B (*seb*) was detected. None of the isolates possessed the genes for the synthesis of enterotoxin C (*sec*), D (*sed*) and E (*see*) (Table 6). Out of 26 tested cheese samples positive for enterotoxigenic coagulase-positive staphylococci, enterotoxin was detected in 2 (7.69%) samples which were sweet-coagulating cheese, made from raw milk, and the number of coagulase-positive staphylococci exceeded 5 log CFU g<sup>-1</sup>.



**Figure 2.** Results of PCR electrophoresis for products obtained by primers for *seb* gen. M marker (GeneRuler 100 bp Plus DNA Ladder). PC-positive control



**Figure 3.** Amplification curve of genes *sec*, *sed* and *see* of samples and positive control



**Table 4.** Origin of enterotoxigenic coagulase-positive staphylococci isolates in cheese samples

Type of cheese	Finding of coagulase-positive Staphylococci	Origin of coagulase-positive staphylococci positive to enterotoxine genes (n)				
		(n)	Cooked milk		Raw milk	
			Number	%	Number	%
Acid-coagulating	19	6	1	3.85	5	19.23
Sweet-coagulating	66	20	5	19.23	15	57.69
Total	85	26	6	23.08	20	76.92

**Table 5.** Number of coagulase-positive staphylococci, *Lactococcus* spp, *Lactobacillus* spp. and physico-chemical parameters (pH,  $a_w$  and NaCl content)

	n	$\bar{X} \pm SD$ (log cfu/g)	Xmin	Xmax	Cv(%)
Coagulase-positive staphylococci (log cfu/g)	85	3,60±1,19	1,00	5,79	33,27
<i>Lactococcus</i> spp. (log cfu/g)	85	8,33±0,55	7,02	9,80	6,58
<i>Lactobacillus</i> spp. (log cfu/g)	85	6,62±0,95	4,00	9,19	14,43
pH	85	4,98±0,50	4,30	6,25	10,16
$a_w$	85	0,95±0,02	0,82	0,977	2,42
NaCl (%)	85	1,10±0,71	<0,01	3,48	64,31

**Table 6.** The presence of genes encoding the synthesis of enterotoxins in coagulase-positive staphylococci isolates from samples of soft cheeses

Species of staphylococci	Number of isolates	<i>sea</i> gen		<i>seb</i> gen		<i>sec</i> gen		<i>sed</i> gen		<i>see</i> gen	
		Br.	%	Br.	%	Br.	%	Br.	%	Br.	%
<i>S. aureus</i>	26	26	100	24	92.31	0	0	0	0	0	0

## DISCUSSION

The bacteriological analysis of 415 soft cheese samples showed the presence of coagulase-positive staphylococci in 85 (20.48%) samples of soft cheeses (Table 2). Our results are in accordance with the results of Araújo et al. [21], who detected coagulase-positive staphylococci in 20% of soft cheese samples in Brazil. Similar results were obtained by De Luca et al. [22] while El-Sharoud and Spano [23] did not detect *S. aureus* in soft cheese samples from local markets in Egypt. On the other hand,

in Rosengren *et al.* [24] survey, coagulase-positive staphylococci were detected in 69% (38/55) of the raw milk cheese samples whereas the occurrence in cheeses made from pasteurized milk was 6% (6/96). The number of *S. aureus* ranges from 100-200 cfu/ml in the milk obtained by proper milking, while this number increases up to  $10^4$  CFU ml<sup>-1</sup> in the case of latent infection in the mammary glands [25]. The number of coagulase-positive staphylococci greater than  $10^4$  CFU g<sup>-1</sup> cheese produced from raw milk was the result of primary contamination of milk due to latent infection or subclinical mastitis. The presence of coagulase-positive staphylococci in cheese made of raw milk points to subclinical mastitis or poor hygiene during the cheese making process. The control of *S. aureus* growth during the fermentation of raw milk cheese means prevention against staphylococcal enterotoxin production. In addition to the origin of coagulase-positive staphylococci are data from previous findings and the frequent occurrence of coagulase-positive staphylococci in milk [26-32]. The occurrence of coagulase-positive staphylococci in cheeses produced from cooked milk can be explained by the poor hygiene and subsequent contamination from the environment, from dirty hands of workers and equipment, whereas a high water activity (0.94 to 0.96) and high pH value (6.0- 6.2) in the cheese enable their growth. In addition to the contamination originating from humans are data of the frequency of enterotoxigenic staphylococci in food processing workers of 17% [33]. The SEA toxin was found to be more prevalent in food processing human carriers than in food itself, but other forms of toxin are found to have opposing prevalence [34].

Cheese production process is a complex one. Behaviour of *S. aureus* in cheese depends on the production process and the capacity of the microorganism to survive stress in the cheese matrix [35]. In the Republic of Serbia soft cheeses are produced in a traditional manner in individual households from cooked or raw milk. The milk from the morning, evening, or mixture from both milkings is used. If the cheese is produced with the mixture from evening and morning milking, evening milk is kept in a cool state during the night, but small producers often keep the milk at room temperature which are conditions that are favourable for the growth of coagulase-positive staphylococci. Observing the soft cheese production process, conditions for *S. aureus* growth, especially in the early stages (pH value and temperature) are favourable. The growth of the microorganism is possible until the physico-chemical parameters (pH, water activity, NaCl content) and the presence of competitive microbiota begin to affect the growth of *S. aureus*. Environmental conditions in which cheeses are produced, especially during the warm months of the year (Spring and Summer) favour the growth of coagulase-positive staphylococci. Thus during the manufacture and storage of soft cheese coagulase-positive staphylococci can multiply and produce enterotoxins, especially in the case of cheese production without lactic acid fermentation, such as it is in sweet-coagulating cheeses. The conditions during the production of soft cheeses with fresh coagulation, when the pH of the cheese is over 4.6 favoured the growth and multiplication of coagulase-positive staphylococci.

According to the literature [36], the growth of this microorganism is in a pH range from 4 to 10, and optimally at a pH of 6-7 which is weakly acidic.

Our results showed that out of 85 isolates of coagulase-positive staphylococci 26 (30.59%) isolates produced enterotoxins (Table 4). Since we detected a group of classical enterotoxins (SEA-SEE) by application of screening method VIDAS SET 2 (BioMerieux, France), we were unable to determine which of five classical enterotoxins produce isolates of coagulase-positive staphylococci, so we decided to identify genes for the synthesis of enterotoxin. Out of 85 enterotoxigenic isolates in 26 (30.59%) isolates were detected sea; while in 24 isolates, in addition to sea gene, seb was detected. In none of the isolates sec, sed and see were detected (Table 6). Our results are in accordance to the results of Medvedova et al. [37], who detected genes for the synthesis of enterotoxins (SEA-SEE) in 32% *S. aureus* isolates originating from milk and other dairy products. Among se gens, sea was the predominant, but the authors did not detect seb gen in contrast to our results. Our results agree with literature data for the predominance of enterotoxin A (SEA) recorded in different countries of the world. In the UK, during an extensive monitoring of the epidemic in the period from 1969 to 1990, 79% of the isolates of *S. aureus* were SEA producers [38]. In France the enterotoxin A (SEA) is cited as the most common cause of poisoning (69.7%) in 31 outbreaks, reported in the period 1981-2002, and caused by different food, such as milk, milk products, meat and salads. The most common in the se outbreaks were sea, then sed, seg, sei and seh, rarely seb and sec; while the see gene was not detected [39], and our results agree with this findings of sea gene, but differ in terms of finding other genes because sec and sed genes were not detected.

Examining the presence of enterotoxins in 26 cheese samples positive for eneterotoxigenic staphylococci enterotoxins were detected in two cheese samples in which the number of coagulase-positive staphylococci exceeded 5 log CFU g<sup>-1</sup>. In coagulase-positive staphylococci isolates, originating from these two cheese samples sea and seb gens were detected. The isolates were identified as *Staphylococcus aureus* based on biochemical characteristics. The pH value in cheese samples was 5.08 and 5.35, and water activity was 0.960 and 0.962, respectively, which are also growth conditions for *S. aureus*. The microorganism had enough time and favourable conditions to multiply to the levels above 5 log CFU g<sup>-1</sup> and synthesize sufficient amount of the detected enterotoxin, The NaCl content in 2 cheese samples, positive for eneterotoxins was 0.497 and 1.872%. Since *S. aureus* can grow at 20% NaCl mentioned values were not inhibitory for the growth and multiplication of the microorganism. Although the number of *Lactococcus* spp. was more than 7 log CFU g<sup>-1</sup> in cheese samples (Table 5) it had no effect in the reduction of the number of *S. aureus*. Low pH can cause prophage induction, leading to the expression of gene sea [40] which explains the frequent finding of sea and SEA toxin. Cretenet et al. [35] showed that *Lc. lactis* can positively or negatively modulate the expression of the genes in the matrix of the cheese. The expression of sea is slightly increased in the presence of *Lc. lactis*, compared to a strong suppression of sec observed in a cheese matrix in the absence of *Lc. lactis*. In

the presence of *L.lactis* agr control system is reduced with regard to the decrease in the pH value. A common finding of SEA can be explained by the influence of water activity. The values of water activity ( $a_w$ ) for *S. aureus* growth differ from the values for enterotoxin production. The minimum value of  $a_w$  for growth is 0.83 to 0.86, which is equivalent to the concentration of 20% NaCl. The optimal value for *S. aureus* growth is  $a_w > 0.99$ . However, the synthesis of SEA and SED is less susceptible and is possible at approximately the same values of water activity, enabling the growth of *S. aureus* when other conditions are optimal. In contrast, the synthesis of enterotoxin SEB is very sensitive to lower values of water activity and SEB synthesis at the value of 0.93 is difficult, despite the intensive growth of the microorganism [41], which explains our findings. Water activity influences the enterotoxin C (SEC) synthesis. In addition,  $a_w$ , low pH may cause the induction of prophage, resulting in an increase in the expression of genes of sea [40].

Contrary to our results are the results of Rola *et al.* [42], who proved the presence of *S. aureus* in 56% of cheese samples produced from raw milk. Although the number of *S. aureus* was  $\geq 10^5$  log CFU g<sup>-1</sup>, and the maximum value of  $2.6 \times 10^7$  log CFU g<sup>-1</sup> the authors did not detect the enterotoxin in cheese samples. Cremonesi *et al.* [43] showed that none out of 33 cheese samples tested by immunological assays contained SEs but in 14 of 33 samples a mixture of *se* positive (*sea*, *sec*, *sed*, *seg*, *sel*, *sej*) isolates was identified. Even, Rosengren *et al.* [24] found levels  $> 5$  log<sub>10</sub> CFU g in 16% (6/39) of raw milk cheeses, the highest level, 6.56 log<sub>10</sub> CFU g, was detected in raw milk cheese with no starter culture added, SEs (SEA-E) were not detected in any cheese sample. One or more *se* genes were detected in 50% of *S. aureus* isolates from traditionally produced raw milk cheese by RT PCR in Turkey. The most frequently SEE (75 %), thus *see* gene (61.5 %) was detected, whereas SED and *sed* gene were not detected in any isolates [44].

The studies conducted in Norway indicated that 14.7% of CPS isolated at different stages of cheese production were *se* gene positive (*seg* and *sej*) [29].

Although the number of coagulase-positive staphylococci in soft cheese is high it does not mean that expression of genes for enterotoxin synthesis will be possible since cheese is a complex matrix. The different storage conditions after cheese purchasing, as well as temperatures in the refrigerators and short time after the cheeses are consumed do not allow enterotoxigenic staphylococci to produce enough amount of enterotoxins, since out of 26 cheese samples positive for enterotoxigenic staphylococci only in two cheese samples (7.69%) enterotoxins were detected. Two cheese samples in which enterotoxin presence was detected were aged 3-4 days and 7 days, the number of coagulase-positive staphylococci exceeded 5 log CFU g<sup>-1</sup>.

## CONCLUSION

Out of 415 cheese overall samples from traditional production, coagulase-positive staphylococci were detected in 85 samples (20.48%). The potential for synthesis of classical enterotoxins (SEA-SEE) was detected in 26 (30.58%) coagulase-positive staphylococci isolates, isolated mainly from raw milk cheese samples. Gen *sea* were present in all, while *seb* in all but 2 isolates, while *sec*, *sed*, *see* genes were not detected. Enterotoxin was detected in 2 (7.69%) sweet-coagulating raw milk cheese samples in which number of staphylococci exceeded 5 log CFU g<sup>-1</sup>.

Strains of *S. aureus* capable of enterotoxin production are highly present. In sweet-coagulating cheeses, made from raw milk in which the number of coagulase-positive staphylococci exceeds 5 log CFU g<sup>-1</sup> and the pH value is higher than 5.0, enterotoxins may be present in the amounts sufficient to cause intoxication, thus they may represent a risk for human health. The control of *S. aureus* growth during the fermentation of raw milk cheese means prevention of staphylococcal enterotoxin production.

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## Authors' contributions

RSR have made substantial contributions in design of the study; collected samples and did isolation and enumeration of coagulase-positive staphylococci and lactic acid bacteria from cheeses; Identification examining the phenotypic characteristics using ID 32 Staph–Bioemerieux and chemical analysis as well; Writting the manuscript, interprating and representing the obtained results in the tables and figures; She has given final approval of the version after corrections to be published. BV carried out molecular techniques such as conventional multiplex PCR technique and Rel-Time PCR. Determination of staphylococcal toxins using ELFA technique VIDAS Staph enterotoxin SET 2 carried, as well; He has given final approval of the version to be published. NZ participated in the design of the study, performed the statistical analysis and written the part of manuscript.

## Declaration of conflicting interests

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

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## **NALAZ I KARAKTERIZACIJA ENTEROTOKSOGENH STAFILOKOKA IZOLOVANIH IZ MEKIH SIREVA U SRBIJI**

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Ukupno 415 uzoraka sireva proizvedenih od sirovog ili kuvanog mleka, prikupljenih sa pijaca ispitano je na prisustvo koagulaza pozitivnih stafilocoka. U 80 (20,48%) uzoraka je dokazano prisustvo koagulaza pozitivnih stafilocoka. Za ispitivanje sposobnosti koagulaza pozitivnih stafilocoka da stvaraju enterotoksine (SEA, SEB, SEC, SED, SEE) i dokazivanje enterotoksina u uzorcima sireva korišćena je ELFA tehnika VIDAS SET 2 (*BioMérieux*, Francuska). Broj koagulaza pozitivnih stafilocoka u zorcima sireva se kretao od 1-5,79 log CFU g<sup>-1</sup>. Od 85 izolata koagulaza pozitivnih stafilocoka



26 (30,59) je stvaralo enterotoksine. Prisustvo gena za sintezu enterotoksina (SE) u dobijenim ekstraktima DNK iz 26 enterotosogenih izolata je dokazano konvencionalnom multipleks PCR tehnikom (za gene *sea* i *seb*), dok je za gene *sec*, *sed* i *see* korišćena Real-Time PCR tehnika. Kod svih 26 izolata koagulaza pozitivnih stafilokoka (poreklom iz sireva proizvedenih od sirovog ili kuvanog mleka, koji su stvarali enterotoksine) dokazano je prisustvo *sea*, i kod 24 izolata pored *sea* gena dokazan je i *seb*. Nijedan izolat nije imao gene za sintezu C (SEC), D (SED) i E (SEE). Od 26 uzoraka sireva pozitivnih na prisustvo enterotoksogenih koagulaza pozitivnih stafilokoka, enterotoksin je dokazan u 2 (7,69%) uzorka kiselo-koagulišućeg sira u kojima je broj enterotoksogenih koagulaza pozitivnih stafilokoka bio više od 5 log CFU g<sup>-1</sup>. U kiselo-koagulišućim sirevima u kojima je broj koagulaza pozitivnih stafilokoka veći od 5 log CFU g<sup>-1</sup> i pH viši od 5, enterotoksin može biti prisutan u količini dovoljnoj da izazove intoksikaciju.