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Detection and enterotoxin production of *Staphylococcus aureus* isolates in artisanal cheese made from raw milk

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ABSTRACT

The aim of the study was to determine whether the physicochemical factors of the matrix and the traditional acid-set cheese-making conditions allow the growth of coagulase-positive staphylococci (CoPS) and the synthesis of enterotoxins, which should contribute to an objective risk assessment in cheese production related to CoPS. CoPS were isolated from 72% of acid-set cheeses ranging from 1.70 to 5.15 $\log_{10}$ CFU g^{-1}. CoPS in a number ≥ 4 $\log_{10}$ CFU g^{-1} were determined in 5.56% of the acid-set cheese samples. Out of the total number of CoPS isolated from cheese, 37.62% of the isolates have been shown to produce enterotoxins. All isolated strains that produced enterotoxins were identified as *Staphylococcus aureus* based on the detection of *spa* gene by PCR. For cheese-derived isolates with CoPS number ≥ 4 $\log_{10}$ CFU g^{-1}, it has been proven that they possess *sec* gene encoding staphylococcal enterotoxin C. According to our results, during the proper fermentation process of artisanal acid-set cheese, the conditions do not support the growth of a critical level of staphylococci or the production of enterotoxins.

KEYWORDS

acid-set cheese, safety assessment, coagulase-positive staphylococci, enterotoxins

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1. INTRODUCTION

Traditional cheeses produced from raw milk, due to production procedures, are predisposed to the risk of the presence of enterotoxigenic coagulase-positive staphylococci (CoPS). In the case of fresh cheese, belonging to the group of acid-set cheeses, coagulation is caused by the activity of lactic acid bacteria (LAB) found in raw milk and in the environment (non-starter LAB) (European Commission, 2003). The produced lactic acid lowers the pH of milk to the isoelectric point where casein becomes unstable and coagulates. After manufacturing the cheese at a temperature of 22 ± 1 °C, it is sent to the market as a fresh cheese, without ripening. The human factor and conditions during the manufacturing process play a significant role in enabling staphylococcal growth, as well as possible production of staphylococcal enterotoxins (SE) (Medved'ová et al., 2017). Numerous human manipulations during production and transport, the temperature during cheese manufacturing and storage, duration of the storage period, and the properties of the cheese (concentration of NaCl, pH, and a_w) are factors that affect the growth of staphylococci and the production of enterotoxins (Schelin et al., 2011).

Acid-set cheeses represent an unfavourable environment for the growth of *Staphylococcus aureus* and enterotoxins production. *S. aureus* does not grow well in the presence of competing flora, and the staphylococcal growth inhibition factors are the number of competing bacterial species in relation to the number of *S. aureus* as well as to the incubation temperature (Zeaki et al., 2019). In fresh cheese, when the number of natural microflora is low, enterotoxins can be produced. In artisanal cheese with a high LAB number, the number of present staphylococci drops suddenly, and the production of enterotoxin is prevented even if a high concentration of staphylococci (10^3–10^5 CFU mL^{-1}) were present at the beginning of the production process (Medved'ová et al., 2020). Enterotoxins are produced only when fermentation is disturbed due to the inactivity of the starter culture and if a high inoculum of *S. aureus* is present (>10^4 CFU mL^{-1} of milk) (Choi et al., 2016).

The European Commission legislation recommends enumeration of CoPS and the detection of staphylococcal enterotoxins in order to define process hygiene and food safety criteria. For raw milk cheese, the highest allowed limits (M) for CoPS range from 10^2 to 10^4 CFU g^{-1}. The presence of SE must be analysed only in soft cheese, raw milk cheese, and cheese produced from thermally processed milk, when the number of CoPS exceeds 10^5 CFU g^{-1} (European Commission, 2005). Microbiological criteria regarding *S. aureus* should prevent the occurrence of SE during cheese production, i.e. its presence in the final product.

The aim of the study was to determine whether the physical and chemical factors of the cheese matrix or the traditional acid-set cheese production conditions enable the growth and reproduction of CoPS and the synthesis of enterotoxins. These findings should contribute to an objective cheese production risk assessment in relation to the presence of enterotoxigenic staphylococci. For the realisation of the set test objectives, two tasks were established: isolation and identification of CoPS from the acid-set cheese and determination of the enterotoxigenic potential of the CoPS strains. This study is presenting the first result on the subject in the Republic of Serbia, contributing to prevention and public health monitoring.

2. MATERIALS AND METHODS

2.1. Manufacturing process and sampling

A total of 50 samples of artisanal acid-set fresh cheese (traditionally designed as "Švapski" cheese) produced from raw cow milk, as a traditional product in our region, were tested. "Švapski" cheese is traditionally handmade on the farm or in households. Acid-set cheese belongs to fresh cheeses without ripening, and the manufacturing process lasts from 24 to 48 h. Its shelf life is 10 days at temperatures up to 8 °C. At the beginning of the cheese manufacturing, it is necessary to cool the milk to a temperature of 27 °C. The production technique consists of milk fermentation at ambient temperature (22 ± 1 °C) for 24–48 h, after which the curd is processed by heating at 35 °C for 30 min, in order to increase the separation of whey. Acid-set cheeses are aged 2–6 days.

Samples were collected aseptically in sterile plastic bags from artisanal cheese-producing country households and from the markets of the municipalities of Banja Luka and Gradiška. The samples were packed in sterile plastic bags and transferred under refrigeration (4 °C) to the laboratory, where they were immediately analysed.

2.2. Determination of pH

A potentiometric method was used to determine the pH of the samples. Briefly, 10 g of cheese sample was dispersed in 10 mL of deionised water, and the measurement was performed using a pH meter (Mettler Toledo, Greifensee, Swiss) according to the manufacturer's instructions.

2.3. Determination of a_w values

Determination of a_w values was done using the LabMaster-a_w apparatus (Novasina, Lachen, Swiss), which is based on the measurement of electrolytic resistance according to ISO 1878:2017 standard (2017). Finely chopped cheese samples were transferred into a plastic cup, which were then placed in the chamber for measurement with the selected temperature. All measurements were done in triplicate, and a_w values of the samples were read on the device's LCD.

2.4. Determination of the number of CoPS

The determination of the CoPS number was done using ISO 6888-1:1999/Amd 1:2003 standard (2003). Briefly, 10 g of cheese sample was diluted in 90 mL of buffered peptone water (Condalab, Madrid, Spain) and homogenised in a stomacher for 5 min. Decimal serial dilutions were prepared and inoculated in Baird Parker agar (Condalab, Madrid, Spain) supplemented with Tellurite Egg Yolk Emulsion (Condalab, Madrid, Spain). Plates were incubated for 48 h at 37 °C. Typical colonies were counted and further investigated according to ISO 6888-1:1999/Amd 1:2003 standard (2003).

2.5. Detection of staphylococcal enterotoxins

A Transia Plate Staphylococcal Enterotoxin enzyme immunoassay kit (BioControl, Bellevue, Washington, USA), which detects A, B, C1, C2, C3, D, and E enterotoxins, was used to detect the presence of SE. Briefly, supernatant of *S. aureus* culture grown overnight in Brain Heart Infusion broth (Condalab, Madrid, Spain) at 37 °C was used as sample in this sandwich type ELISA method according to the European Union Reference Laboratory for Coagulase-positive Staphylococci protocol (Ostyn et al., 2010).

2.6. Identification of CoPS species

Identification of CoPS species based on the presence of the *spa* gene was done by PCR using the appropriate primer and cycling condition mentioned in Table 1 (Harmsen et al., 2003). The bacterial DNA was extracted from all primo-isolates using a MasterPure Complete DNA and RNA Purification kit (Epicentre Illumina, San Diego, California, USA) according to the manufacturer's instructions. The resulting nucleic acid precipitate was dissolved in 35 µL of buffer for storage of DNA and placed in a freezer at –30 °C. A PCR assay was performed using the Amplitaq Gold PCR Master Mix (2×) (Invitrogen, Waltham, Massachusetts, USA) according to the manufacturer's instructions. Briefly, each PCR mixture had a final volume of 25 µL containing 12.5 µL of the PCR master mix, 1 pmol of each primer, and 5 µL of isolated DNA. Amplification was carried out in thermocycler Master Cycler Gradient (Eppendorf, Hamburg, Germany). Visualisation of PCR amplicons of the expected size (180–600 bp) was performed by electrophoresis in 2% agarose gel stained with Midori Green (NIPPON Genetics Europe, Düren, Germany). 100bp Ladder (Invitrogen, Waltham, Massachusetts, USA) was used to determine the size of the amplified DNA fragments.

2.7. Detection of SEs genes carriage of CoPS

All *S. aureus* strains were screened for the presence of genes (*sea, seb, sec, sed,* and *see*) encoding classical staphylococcal enterotoxins A-E. The presence of the gene for synthesis of SE in the obtained DNA extracts was examined by a conventional multiplex PCR technique (for *sea*, *seb*, and *sed* genes), or Real Time PCR technique (for *sec* and *see* genes). Primers, references, and cycling conditions are summarised in Table 2 (Monday and Bohach, 1999; Shannon et al., 2007; Duquenne et al., 2010; Pajić et al., 2016).

2.8. Statistical analysis

Descriptive statistical parameters were used as basic statistical methods. The results of the number of isolated CoPS are shown as $\log_{10}$ CFU g^{-1}.

3. RESULTS AND DISCUSSION

3.1. Determination of pH and a_w values of the cheese

Although many staphylococcal strains possess genes encoding one or more enterotoxins, it does not imply that enterotoxins production is going to happen, even if appropriate conditions for the expression of genes occur during manufacturing or storage. Physicochemical characteristics of the cheese matrix (pH, a_w value, NaCl concentration, competitive role of LAB), as well as the

Table 1. List of primers and cycling condition used in this study in order to identify the gene *spa*

Primer	Nucleotide sequence	Length (bp)	Cycling condition
spa-f	TAAAGACGATCCTTCGGTGAGC	180-600	95 °C for 5 min; 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min for 30 cycles and at 72 °C for 7 min
spa-r	CAGCAGTAGTGCCGTTTGCTT		

Table 2. Primers used in this study to identify genes responsible for the synthesis of staphylococcal enterotoxins in coagulase-positive staphylococci

Primer	Length (bp)	Sequence	Reference	Cycling conditions
sea-f	93	TCAATTTATGGCTAGACGGTAAACAA	Duquenne et al. (2010)	95 °C for 5 min; 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min for 30 cycles and 72 °C for 7 min.
sea-r		GAAGATCCAACTCCTGAACAGTTACA		
seb-f	85	AACAACTCGCCTTATGAAACGGGAT	Pajic et al. (2016)	
seb-r		CTCCTGGTGCAGGCATCATGTCA		
sec-f	284	CGTATTAGCAGAGAGCCAACCA	Shannon et al. (2007)	
sec-r		GTGAATTTACTCGCTTTGTGCAA		
sed-f	150	AAACGTTAAAGCCAATGAAAACA	Duquenne et al. (2010)	
sed-r		TGATCTCCTGTACTTTTATTTTCTCCTA		
see-f	171	TACCAATTAACTTGTGGATAGAC	Monday and Bohach (1999)	
see-r		CTCTTTGCACCTTACCGC		

environmental conditions such as temperature, have huge impact on the production of enterotoxin. In a total of 36 samples with detected CoPS, pH values varied from 4.00 to 5.59, with a medium value of 4.46 ± 0.32. Also, a_w values ranged from 0.973 to 0.988, with a medium value of 0.979 ± 0.004. A pH value higher than the isoelectric point of casein (pH > 4.60) was determined in 5 samples (16.67%) of the acid-set cheeses, out of which 2 (5.56%) samples measured pH > 5.00. According to other studies, pH appropriate for the enterotoxins production varies between 5 and 9.6 (Hennekinne, 2018), which is in compliance with our findings. Another significant parameter for CoPS growth is water activity, with a_w value adequate for enterotoxins production between 0.973 and 0.988 (Hennekinne, 2018). Our average a_w value of 0.979 fits into the specified a_w range.

3.2. Determination of CoPS quantity in acid-set cheese samples

Staphylococcal food poisoning is one of the most common food-borne diseases worldwide, and unpasteurised milk and cheese are typical milk products associated with these epidemic illnesses. Staphylococcal contamination of these products may be due to direct bacterial excretion from an udder with subclinical mastitis or may result from environmental contamination during raw milk handling and processing (Costanzo et al., 2020). This work represents the first study of risk assessment of acid-set cheese related to CoPS in our region. Out of a total 50 samples, CoPS were isolated from 36 samples of cheese, or 72% of all samples, which is in compliance with the findings of other authors, who tested other sorts of traditional cheeses made from raw milk in our region (Bulajić et al., 2015; Golić et al., 2015). The European Commission (2005) suggested that raw milk products should not contain a high bacterial concentration (10^5–10^6 CFU g^{-1} or mL^{-1}), in order to prevent production of staphylococcal enterotoxins during the manufacturing process. The average number of CoPS in our samples was 2.91 ± 0.67 $\log_{10}$ CFU g^{-1}. High bacterial presence in our study (in numbers higher than 4 $\log_{10}$ CFU g^{-1}) was detected in 5.56% of samples (Table 3). A similar result was recorded in the study of Kousta et al. (2010), who detected the presence of *S. aureus* in a number higher than 4 $\log_{10}$ CFU mL^{-1} in 4% of cheese from unpasteurised milk.

Table 3. Distribution of CoPS counts in positive samples of acid-set cheeses

Number of CoPS	Number of samples	Percentage
≥5 $\log_{10}$ CFU g^{-1}	1	2.78%
4-5 $\log_{10}$ CFU g^{-1}	1	2.78%
3-4 $\log_{10}$ CFU g^{-1}	14	38.88%
2-3 $\log_{10}$ CFU g^{-1}	19	52.78%
<2 $\log_{10}$ CFU g^{-1}	1	2.78%
Total	36	100%

3.3. Identification of CoPS isolates and detection of enterotoxin-producing strains

Among 101 CoPS strains isolated from cheese samples, 38 (37.62%) enterotoxins-producing (SEA - SEE) isolates were confirmed. All 38 isolates were identified as *S. aureus*, based on the presence of the *spa* gene. Our analysis showed that 2 out of 38 *S. aureus* enterotoxins-producing isolates reached a number ≥ 4 $\log_{10}$ CFU g^{-1}. Both of them carry *sec* gene, only.

SE primarily are produced by CoPS, most often *S. aureus* subsp. *aureus*, a common human and animal pathogen. *S. aureus* strains, isolated from humans, typically produce SEA, while cow strains produce SEC or SED, and sheep strains SEC (EC Opinion, 2003; Rola et al., 2016). According to the literature, production of SEC is most often confirmed in *S. aureus* strains isolated from cows and sheep with mastitis (Schelin et al., 2017). Data showed that the prevalence of enterotoxigenic strains in raw milk samples ranged from 25.5% to over 72%, and SEA and SEC were determined as dominant enterotoxins (Carfora et al., 2015; Riva et al., 2015). European Union Milk Hygiene Directive 92/46 established microbial standards for CoPS in raw milk, where limits for CoPS ranged from 5×10^2 CFU mL^{-1} to 2×10^3 CFU mL^{-1} (Council of the European Communities, 1992). In our state there are no such standards for CoPS in raw milk, and Regulation (EC) No 853/2004 (European Commission, 2004) defines the highest limit for total bacterial concentration in raw milk as 10^5 CFU mL^{-1}. Also, the European Commission (2005) defines only a criterion for food safety as the absence of staphylococcal enterotoxin in 25 g for cheese, milk powder, and whey powder placed on the market during the shelf life, if CoPS numbers ≥ 10^5 CFU g^{-1} are found in these foods. According to these regulations, only 1 sample with CoPS number ≥ 10^5 CFU g^{-1} was detected (Table 3). The prevalence of enterotoxigenic strains among CoPS isolates from cheeses, whey, butter, and some other meat and egg products was higher than 30%, and the most commonly found genes were *sea* and *sec* (Basanisi et al., 2016; Mehli et al., 2017). Our results are in agreement with these studies.

The artisanal acid-set cheese analysed in our study was produced from raw milk at 22 ± 1 °C with LABs originating from the environment in which the process occurred. Although the extrinsic and intrinsic factors in our research did not represent favourable conditions for the multiplication of staphylococci, nevertheless, they allow for bacterial growth and enterotoxin production. Knowing this, as well as the fact that they are made from raw milk, acid-set cheeses generally can be a threat regarding CoPS growth. Traditional cheese production happens at temperatures that favour the growth of mesophilic LABs. The role of LABs in staphylococcal growth during raw milk cheese production is very important, since metabolic activity of LABs leads to the pH reduction of cheese matrix, decreasing of staphylococcal quantity and disabling enterotoxin production. In fresh cheese, enterotoxins can be produced when the number of

natural microflora is low. In fresh cheeses, with a numerous LABs, the number of staphylococci is decreasing rapidly, and the production of enterotoxins is prevented, even if a high amount of staphylococci (10^3–10^5 CFU mL^{-1}) is present at the beginning of the manufacturing process (Ołdak et al., 2020). Our study showed that basic characteristics of raw milk cheese, its production technology, and storage conditions (shelf life of 10 days at a temperature below 8 °C) contribute to the reduction of staphylococcal growth.

4. CONCLUSION

We can conclude that, during the proper fermentation process of artisanal acid-set cheese, the conditions do not support the growth of a critical level of staphylococci or the production of enterotoxins.

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