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Enterotoxigenic potential of *Staphylococcus* spp. isolates recovered from raw milk and artisanal cheese

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Abstract: In this work, we investigated the phenotypic profile of *Staphylococcus* spp. isolates recovered from raw milk and artisanal cheese, and their enterotoxigenic potential through the detection of classical enterotoxin genes (*sea, seb, sec, sed* and *see*). A total of 104 isolates (58 coagulase-positive *Staphylococcus* – CoPS; and 46 coagulase-negative *Staphylococcus*- CoNS) were used, of which 33 were retrieved from raw milk and 71 from artisanal cheese produced in the Serrana region of Santa Catarina. Identification of CoPS was conducted via biochemical tests. Detection of the genes *sea, seb, sec, sed,* and *see* was carried out by multiplex PCR technique. Among the 58 CoPS analyzed, 64% were identified as *S. aureus*, 22% as *S. scheiferi coagulans*, 12% as *S. hyicus* and as 2% *S. intermedius*. In the present study was noted that 40% of CoPS isolates retrieved from milk harbored *seb* gene, while only one from artisanal cheese was positive for gene *sea*. In this study all CoNS samples investigated were negative for enterotoxins genes. The enterotoxigenic potential of CoPS, is an issue of great importance for public health. For that reason, it is necessary that cheese factories strictly follow the safety processes involved in manufacturing.

Key words: *Staphylococcus* spp., staphylococcal enterotoxins, foods, PCR.

INTRODUCTION

S. aureus is considered the most important species among *Staphylococcus* spp. due to its versatile feature to produce several endotoxins, especially the staphylococcal enterotoxins (SE). However, Valle et al. (1990) described that other coagulase-producing staphylococci exhibited the same characteristic. Later, Pereira et al. (2001) demonstrated that some coagulase-negative staphylococci (CoNS) were also able to produce toxins under laboratory conditions.

Enterotoxins are proteins of low molecular weight, which are resistant to inactivation by stomach proteases and withstand temperatures up to 126.7°C/6.2 min. For that reason, in some cases they are able to survive in food even after thermal treatment (thermoresistant toxins) (Santana et al. 2010).

The genes encoding staphylococcal enterotoxins can be present in bacteriophages (*sea* and *sec*), plasmids (*seb, sed* and *selj*), staphylococcal chromosome (*seb, sec, seh, sei, selk, sell, selm, seln, selo, selp* and *selq*) and pathogenicity islands (*sec, seb, selk* and *selq*) (Jarraud et al. 2001, Zhang et al. 1998).

According to Sanitary Surveillance Agency (Serviço de Vigilância Sanitária) from Ministry of Health, *S. aureus* was the third most frequent pathogen associated to foodborne disease

outbreaks of known causative agent between the years of 2007 and 2017 in Brazil (Brasil 2017). This is an information that raises concern, as it represents a potential risk for public health, especially due to the production of enterotoxins, which are capable of inducing intoxication within few hours after their ingestion.

Raw milk and dairy products are among the most commonly contaminated food by *Staphylococcus* spp. (Santana et al. 2010), especially those that are handcrafted such as artisanal cheese (Argudin et al. 2010, Loir et al. 2003).

In this context, the objective of this work was to characterize phenotypically the isolates of CoPS and to verify the enterotoxigenic potential of *Staphylococcus* spp. isolates recovered from milk and serrano artisanal cheese samples, produced in the southern region of Brazil, through the detection of staphylococcal enterotoxin genes (*sea, seb, sec, sed* and *see*).

MATERIALS AND METHODS

Bacterial sample collection

For this study we tested 58 coagulase-positive (CoPS) and 46 coagulase-negative (CoNS) staphylococci bacterial samples recovered from raw milk (n=33) and serrano cheese (n=71), which is produced in the Serrana region of Santa Catarina region.

These isolates were previously collected during the study conducted by (Pontarolo et al. 2017) at Centro de Diagnóstico Microbiológico Animal (CEDIMA) at Universidade do Estado de Santa Catarina (UDESC) in cooperation with Empresa de Pesquisa Agropecuária e Extensão Rural of Santa Catarina (EPAGRI).

Samples were stored at -20°C in brain heart infusion broth (BHI) with 20% glycerol. Samples were recovered and purified in BHI agar, and consequently, incubated in a bacteriological incubator for 24h at 37°C.

Phenotypic characterization

For phenotypic characterization of CoPS coagulase isolates, a series of tests, such as coagulase, Vogues-Proskauer, urease, resistance to polymixin B, fermentation (mannitol, trehalose and sucrose) were performed (Bannoehr & Guardabassi 2012, Markey et al. 2013).

DNA extraction and detection of enterotoxin genes

Genomic DNA extraction of the samples followed the protocol described by (Doyle & Doyle 1987) with some modifications. A volume of 200 µL of each bacterial inoculum (colonies were previously incubated in 200 µL of BHI broth for 24h at 37°C) was mixed with 500µL of chloroform: isoamyl alcohol (24:1) and pre-heated in water-bath for 30 min at 56°C. After that, samples were centrifuged for 10 min at 12,000 rpm. We transferred supernatants to new sterile microtubes and supplemented them with 600 µL of cold 70% alcohol. Then, the microtubes, containing the mixture, were further centrifuged for 20 min at 13,500 rpm. Subsequently, supernatant of each sample was discarded, and microtubes were let to dry completely at 56°C. DNA samples were resuspended in 200 µL of ultra-pure water and stored at -20°C until use.

To detect enterotoxin (*sea, seb, sec, see* and *sed*) and *fem*A genes, we used multiplex PCR technique based on the standardized methodology proposed by (Freitas 2005) with modifications. PCR mixture of each sample consisted of 2 µL of genomic DNA, 0.4 pmol of each primer (Table I), 200mM of each dNTP, 1x PCR buffer, 2 mM of $MgCl_2$, 1.25 U of Platinum Taq DNA polymerase, resulting in a final volume of 25 µL. Amplification reaction was carried out as follows: initial denaturation step at 94°C for 5

min, 35 cycles of denaturation at 94°C for 2 min, annealing at 52°C for 2 min, extension at 72°C for 3 min, and final extension at 72°C for 7 min. To avoid production of unspecific amplicons, we established a set of two Multiplex-PCR assays with different primers. One of them contained the primers *sea, sec* and *see*, while the other had the primers *seb, sed* and *femA. S. aureus* ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), ATCC 23235 (*sed*) and ATCC 27664 (*see*) were used as positive quality control. Gene 16S and ultrapure water were used, respectively, as internal and negative quality controls for the amplification reactions.

RESULTS

A total of 104 *Staphylococcus* spp. (58 CoPS and 46 CoNS) isolates retrieved from milk and serrano artisanal cheese samples were used in this study. Through phenotypic analyses of CoPS, we observed a predominance of *S. aureus* (64%) in relation to the other species (Table II). In addition, all samples identified as *S. aureus* revealed the presence of *femA* gene.

Out of the 58 CoPS isolates, nine of them (15.51%) carried at least one of the enterotoxin genes investigated. Of the 20 isolates from raw milk, 8 of them (40%) harbored *seb* gene, and were characterized as: *S. aureus* (n=5), *Staphylococcus scheiferi coagulans* (n=2) and *Staphylococcus hyicus* (n=1). In relation to the 38 CoPS isolates obtained from raw milk, only one (*S. aureus*) harbored gene *sea*. It is important to mention that there was no relation between isolate origin (raw milk and cheese) and presence of enterotoxin genes. All CoNS samples investigated in this study were negative for enterotoxins genes.

Table I. Oligonucleotides used to detect enterotoxin-producing genes and *fem*A gene in *Staphylococcus* spp.

Primer	Sequence 5' - 3'	Size of amplicon	Reference
sea	F – GGT TAT CAA TGT GCG GGT GG	102pb	(Freitas 2005)
	R – CGG CAC TTT TTT CTC TTC GG		
seb	F – GTA TGG TGG TGT AAC TGA GC	164pb	
	R – CCA AAT AGT GAC GAG TTA GG		
sec	F - AGA TGA AGT AGT TGA TGT GTA TGG	451pb	
	R – CAC ACT TTT AGA ATC AAC CG		
sed	F – CCA ATA ATA GGA GAA AAT AAA AG	278pb	
	R – ATT GGT ATT TTT TTT CGT TCT		
see	F – AGG TTT TTT CAC AGG TCA TCC	209pb	
	R – CTT TTT TTT CTT CGG TCA ATC		
femA	F – AAA AAA GCA CAT AAC AAG CG	132pb	
	R – GAT AAA GAA GAA ACC AGC AG		
16S	F - AGG TGG CAA GCG TTA TCC	228pb	(Asfour & Darwish 2011) modified
	R - CGC ACA TCA GCG TCA G		

Table II. Phenotypic characterization of CoPS isolates obtained from milk and artisanal cheese produced in the *Serrana* region of Santa Catarina.

	S. aureus	***S. scheiferi coagulans***	***S. hyicus***	***S. intermedius***	**TOTAL**
Raw milk	10 (50%)	4 (20%)	6 (30%)	0	20 (34%)
Cheese	27 (71%)	9 (24%)	1 (3%)	1 (3%)	38 (65%)
Total	**37/58 (64%)**	**13/58 (22%)**	**7/58(12%)**	**1/58 (2%)**	**58**

DISCUSSION

The genus of *Staphylococcus* is subdivided into 40 species based on their ability to synthesize (or not) coagulase, an extracellular enzyme. The majority of the species is classified as coagulase-negative, except for *Staphylococcus aureus* (*S. aureus*), *Staphylococcus schleiferi subsp. coagulans*, *Staphylococcus intermedius*, *Staphylococcus hyicus*, and *Staphylococcus delphini* (Markey et al. 2013).

One of the main concerns of food microbiology is staphylococcal food poisoning, a common widespread foodborne disease caused by the ingestion of staphylococcal enterotoxins, which are usually produced by CoPS enterotoxigenic strains, mainly *S. aureus* (Borges et al. 2008), but also from other CoNS species (Pereira et al. 2001, Valle et al. 1990).

In this study, a total of 104 *Staphylococcus* spp. (58 CoPS and 46 CoNS) recovered from raw milk and artisanal cheese were investigated. In relation to CoPS identification, we observed that the majority of the samples were identified as *S. aureus* (63.79%). In addition, all these *S. aureus* isolates exhibited the presence of gene *femA*. As described above and in other studies (Senger & Bizani 2011) also found similar results to ours. The authors investigated a total of 60 samples of Minas Frescal cheese (half of them handcrafted and the other half industrially manufactured) commercialized in the city of Canoas, Rio Grande do Sul, Brazil. They also observed that 40% of bacterial isolates retrieved from artisanal cheese, and 23.3% from industrial cheese, were contaminated with *S. aureus*. Interestingly, we also identified the species *S. scheiferi coagulans* (22%), *S. hyicus* (12%) and *S. intermedius* (2%) among the isolates. Interestingly, a study conducted in Sweden revealed that 69% of milk samples and 6% of dairy products were contaminated by CoPS (Rosengren et al. 2010).

Ingestion of food containing preformed staphylococcal enterotoxins causes sudden and rapid onset illness with symptoms like nausea, vomiting, abdominal cramps, and diarrhea. For that reason, the presence of enterotoxic staphylococci in foods is an important health problem, especially for consumers of raw dairy products (Santana et al. 2010), In this study, nine (15%) of the 58 CoPS isolates were positive for at least one of the five enterotoxin genes investigated.

In relation to the origin of the samples, 40% of them recovered from milk showed the presence of gene *seb*, while 3% of them retrieved from artisanal cheese harbored *sea* gene. The *sea* gene is carried by a prophage and can be easily disseminated among *Staphylococcus* spp. strains. Its product, enterotoxin A, is frequently associated with food poisoning since it is toxic at low concentrations. However, the *seb* gene can be carried by a plasmid, chromosome and islands of chromosomal pathogenicity (Jarraud et al. 2001). In their study (Ercoli et al. 2017), on the Investigation of a Staphylococcal Food Poisoning Outbreak from a Chantilly Cream

Dessert, in Umbria (Italy), harbored *sea, seg, sei* and *seh* genes in *Staphylococcus aureus* strains isolated from food, environment and human samples.

Like this study (Gucukoglu et al. 2012) demonstrated that 13.7% of *S. aureus* isolates recovered from raw milk were positive for *sea* and *seb* genes. A study conducted in East Slovakia reported a high rate (47.4%) of enterotoxigenic *Staphylococcus* spp. in sheep cheese isolates, especially by the presence of the genes *seb* (36.8%), *sea* (5.3%), and *sea + seb* (5.3%) (Holecková et al. 2002). In their study (Mello et al. 2016) performed in different Brazilian States, including the South, Southeast and Northeast, observed the prevalence of the *sea* gene in 18.2% followed by the *seb* gene in 7.7% of cow's milk isolates with subclinical mastitis.

On the other hand, none of the five enterotoxin genes investigated were found in the CoNS isolates from this work. As reported to (Rosec & Gigaud 2002) reported in their study a low incidence of classic enterotoxin genes in 332 *Staphylococcus* spp. isolates recovered from different food samples in France. Similar to our results (Borelli et al. 2011) also did not detect the presence of the genes *sea, seb, sec* and, *sed* in staphylococcal isolates recovered from Minas cheese produced with raw milk. In addition (Hunt et al. 2012) demonstrated that 83.2% of isolates recovered from raw milk and cheese did not harbor the enterotoxin-coding genes studied. It is important to note that growth and enterotoxin production by enterotoxic strains of *Staphylococcus* sp. in foods are influenced by varied factors, such as temperature, pH, water activity (aw), inoculum size, atmospheric composition, carbon and nitrogen sources, salt levels, and competing microflora. In addition, lactic acid bacteria, present in the natural microflora of raw milk, are able to influence not only the synthesis of staphylococcal enterotoxins (they can reduce pH of milk, produce bacteriocins and hydrogen peroxide), but they are also considered nutritional competitors (Novick 2003). This could explain the low incidence of staphylococci carrying enterotoxin genes, especially in cheese, since the acidification of the substrate as consequence of lactic bacteria proliferation.

Although the presence of genes encoding enterotoxins does not necessarily means that they will be produced, it does emphasize the potential of the bacteria to trigger enterotoxin production under optimal conditions in food (Santana et al. 2010).

The presence of enterotoxin genes, even at low quantity, emphasizes the enterotoxigenic potential of CoPS, which is an important public health issue. Therefore, it is necessary that cheese and dairy factories strictly follow all processes and flows influencing food safety, in order to assure a bacteriological safe and stable product. Although the enterotoxin genes were not detected in the CoNS isolates, we expect that these data will assist the current legislation to establish a minimum detection limit of CoNS in foods.

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F.D.M. conceived the study, designed the experiments, coordinated the investigation, analyzed the data and wrote the paper. R.A.P. S., K.A.D., P.W and L.P. performed laboratory analysis and drafted the experimental section. S.R.W., U.M.C. and S.M.F. critically reviewed the manuscript. All the authors have read and approved the final manuscript.

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