
CHARACTERIZATION OF *STAPHYLOCOCCUS* SPECIES ISOLATED IN PRODUCTION STAGES OF BRAZILIAN COLONIAL CHEESE

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ABSTRACT

Colonial cheese is a culturally and economically important product from the south of Brazil. As most of its production is artisanal, the technology employed is mostly knowledge passed down from one generation to the next according to family tradition and may be produced with raw or pasteurized milk. It is noted for its spicy flavour and variable composition and is often classified as a medium to high-moisture cheese. This intrinsic feature increases the risk of microbial spoilage and food poisoning. One of the main bio-indicators of contamination in colonial cheese is coagulase positive *Staphylococcus*. The purpose of this study was the phenotypic identification of *Staphylococcus* species isolated from the products and surfaces in the main production stages of colonial cheese. *Staphylococcus* sp. isolates from the food and the production environment were obtained from two colonial cheese-production agro-industries in Rio Grande do Sul. Samples of fresh milk, curd, ripening and final colonial cheese were collected. In addition, surface sampling was performed on the coagulation tanks, production tables, molds, cheese ripening shelves and on the hands of the handlers. *Staphylococcus* sp. isolates in the cheese and the production environments tested in this study were identified by phenotypic techniques through biochemical and MALDI-TOF MS analyses. These isolates were subjected to gene expression analysis for enterotoxins A, B, C, D, and E. All isolates (72) were identified as *Staphylococcus* sp., and 43% of the total isolates tested were coagulase positive. *Staphylococcus aureus* was the predominant species in the raw milk and production tanks. Regarding coagulase negative staphylococci isolates, *S. warneri* and *S. sciuri* were most abundant. The *sea* and *seb* genes were detected in 4% of the *Staphylococcus* isolates. The results indicate eleven different species of *Staphylococcus* present in the colonial cheese production environments studied. The predominant presence of *S. aureus* in the different samples of milk, curd, ripened cheese, ready-to-eat cheese and hands of the handlers indicates that there are issues with the selection of milk-producing animals, pasteurization process and/or hygiene control of handlers. The *sea* and *seb* genes were detected in samples of raw milk and colonial cheese. No enterotoxin genes were detected in coagulase negative staphylococci.

KEY WORDS: Enterotoxins; polymerase chain reaction; time-offlight mass spectrometry; coagulase negative staphylococci; phenotypic identification; genotypic analysis; colonial cheese.

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Received for publication: 10/9/2019. Reviewed: 13/1/2020. Accepted: 1/4/2020.

INTRODUCTION

Colonial cheese is a culturally and economically important product from the south of Brazil. As most of its production is artisanal, the technology utilized is mostly knowledge acquired through regional family tradition and it can be produced with raw or pasteurized milk. This cheese has a spicy flavour and variable composition, often classified as medium or high moisture and known for its soft curd. This intrinsic feature increases the risk of microbial spoilage and food poisoning. One of the main contamination bio-indicators investigated in colonial cheese is coagulase positive staphylococci (CoPS). The presence of these microorganisms in food may be related to the poor quality of the raw material and inadequate hygiene practices during production, reducing the quality of the final product (Ries et al., 2012; Dantas et al., 2013; Melo et al., 2013; Coelho et al., 2014; Amorim et al., 2014; Tesser, 2014).

The contamination of cheese by enterotoxigenic CoPS and coagulase negative staphylococci (CoNS) is a public health issue due to the risk of food poisoning. The pathogenic potential of staphylococci is attributed to a combination of properties that contribute to bacterial colonization and permanence in the host and environment. These properties include the ability to produce hemolysins, nucleases, proteases, exoproteins, and antimicrobial resistance proteins, as well as their ability to form biofilms, contributing to their permanence on abiotic surfaces (Andrade et al., 2011; Galinari et al., 2014; Friedriczewki et al., 2018).

Staphylococcal food poisoning, caused by ingestion of enterotoxins, mainly produced by *S. aureus*, is one of the most common foodborne diseases. Regarding the production of enterotoxins, studies show that enterotoxin A (SEA) is most often an enterotoxin related to intoxication outbreaks followed by enterotoxin D (SED), enterotoxin C (SEC) and enterotoxin B (SEB) and rarely enterotoxin E (SEE) (Brazil, 2016; Rola et al., 2016; Wu et al., 2016). Although the main pathogen, *S. aureus*, is coagulase positive, the pathogenic potential of staphylococci is not unique to this group. Other species, lacking the ability to synthesize the coagulase enzyme, have long been of minor medical relevance. The phenotypic distinction of coagulase negative and positive was considered sufficient for the determination of the clinical course of action, since *Staphylococcus* sp. pathogenicity appeared to be linked to coagulase production. However, with the increasing significance of CoNS and the number of new species described, this differentiation has become increasingly questionable, requiring an investigation regarding the toxigenic capacity of all *Staphylococcus* species (Piessens et al., 2011; Costa et al., 2012; Moura et al., 2012).

The purpose of this study was to conduct the phenotypic identification of *Staphylococcus* species isolated in the main production stages as well as on surfaces used in the production of colonial cheese. *Staphylococcus* sp. isolates were genetically evaluated for the presence of genes involved in the production of the main enterotoxins associated with foodborne outbreaks.

MATERIAL AND METHODS

Staphylococcus sp. isolates from the cheese and the production environments were obtained from two colonial cheese production agro-industries in Rio Grande do Sul, named here agro-industries A and B. Fresh milk, curd, 7-day ripened cheese and colonial cheese were sampled between August 2014 and November 2015.

Sampling of the production surfaces and hands of the handlers was performed during the production process with the aid of swabbing moistened with 0.1% peptone water (AP-Himedia®- India) for subsequent collection and storage in 10 mL of this solution. The following production surfaces were sampled: coagulation tanks, production tables, molds and cheese ripening surfaces. The coagulation tanks and production tables were made of stainless steel, molds were made of polyethylene, and the surfaces of the cheese ripening shelves were made of wood in both agro-industries. The sampled areas were the equivalent of 50 cm². The palms and fingers of the handlers were also analysed. This study was evaluated and approved by the UFRGS Research Ethics Committee, under No. 32369514.2.0000.5347. Surface sampling was performed by swabbing the equipment and hands of the handlers, following the procedure described by Silva et al. (2010).

Coagulase positive staphylococci and CoNS from the present study were quantified according to the procedure described in Brazil (2003). Three typical and atypical colonies were isolated on blood agar (agar base from Oxoid®- England with desfibrinated sheep blood) for purity analysis and verification of hemolysis production. The colonies were cultured on brain heart infusion agar (BHI- Oxoid®- England) for the following tests: Gram staining, catalase, slide coagulase, tube coagulase and carbohydrate metabolism (mannitol, trehalose, xylose, maltose, lactose, sucrose and mannose) (Brazil, 2003; MacFaddin, 2000). After phenotypic confirmation, pure CoPS and CoNS cultures were stored in BHI Broth (Oxoid®- England) supplemented with 30% glycerol at -18°C for subsequent identification by the MALDI-TOF MS method (Microflex LT instrument, Bruker Daltonics, Bremen, Germany) and genotypic analyses for enterotoxin gene detection.

MALDI-TOF MS for these isolates was performed in duplicate by the extraction technique according to the manufacturer's instructions (Bruker, 2015). Scores were interpreted as described by Wieser et al. (2012).

DNA was extracted by the boiling method according to Hassanzadeh et al. (2016) for detection of enterotoxin genes in *Staphylococcus* sp. Gene detection for *Staphylococcus* enterotoxins was performed by polymerase chain reaction (PCR) using primers for the *sea*, *seb*, *sec*, *sed* and *see* genes as described by Moura et al. (2012), Table 1.

Table 1. Nucleotide sequences and annealing temperatures of the primers used for the amplification of staphylococci genes.

Gene Enterotoxins	Nucleotide (5'-3')	Annealing temperature (°C)	Amplicon (bp)
<i>Sea</i> ¹	5'-CCT TTG GAA ACG GTT AAA ACG- 3'	54	126
	5'-CTG AAC CTT CCC ATC AAA AAC- 3'		
<i>Seb</i> ²	5'-GGT ACT CTA TAA GTG CCT GC- 3'	55	475
	5'-TTC GCA TCA AAC TGA CAA ACG- 3'		
<i>Sec</i> ³	5'-AGA ACT AGA CAT AAA AGC TAG G- 3'	55	267
	5'-TCA AAA TCG GAT TAA CAT TAT CC- 3'		
<i>Sed</i> ⁴	5'-TTT GGT AAT ATC TCC TTT AAA CG- 3'	55	309
	5'-CTA TAT CTT ATA GGG TAA ACA TC- 3'		
<i>See</i> ⁵	5'-CCT ATA GAT AAA GTT AAA ACA AGC- 3'	55	173
	5'-TAA CTT ACC GTG GAC CCT TC- 3'		

1. staphylococcal enterotoxin A; 2. staphylococcal enterotoxin B; 3. staphylococcal enterotoxin C; 4. staphylococcal enterotoxin D; 5. staphylococcal enterotoxin E.

PCR reactions were optimised in 25µL reactions using 1mM MgCl₂ (Invitrogen®-USA), 10 pmol of each primer, 1U TaqDna polymerase (Invitrogen®-USA), 1x Buffer (Invitrogen®-USA), 200µM deoxynucleotides (ABgene®), and ddH₂O (Milli Q). Amplification cycles were performed on Veriti 96 Thermal Cycler (Applied Biosystems Inc., Norwalk, CT, USA), using the following parameters for the detection of the *sea* gene: Initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, 54°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 5 min.

For the detection of *seb*, *sec*, *sed*, and *see* genes, the following program was used: 94°C for 5 min, 30 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 5 min. Strains of *Staphylococcus aureus* ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), ATCC 23235 (*sed*) and ATCC 27664 (*see*) were used as positive controls. PCR products were electrophoresed in 10% polyacrylamide gel and stained with silver nitrate.

RESULTS AND DISCUSSION

All isolates (72) were identified as *Staphylococcus* sp. (Table 2). 43% (31/72) of the total isolates tested, were coagulase positive. Among these CoPS, *S. aureus* was predominant (93%) in the raw milk and production tank in agro-industry B. The presence of CoPS in colonial cheese is a constant concern. Some authors observed that 25% of colonial cheese samples presented CoPS, therefore not in concordance with the legislation (Lucas et al., 2012; Pinto et al., 2011). Casaril et al. (2017) and Komatsu et al. (2010) noted non-concordance with the legislation in 50% and 80% of the samples analysed, respectively. A different result was obtained in the present study. Despite the identification of CoPS in the production stages, in all the colonial cheese analysed in the agro-industries the quantifications of CoPS were within current legislative standards (up to $3\log_{10}$ CFU g⁻¹) (Brazil, 2001).

The presence of *S. aureus* in raw milk samples may be indicative of mastitis in the herd, since the prevalence of *S. aureus* is high in this form of disease (Hogeveen et al., 2011). In the agro-industries analysed, milking was performed mechanically and the equipment is another possible source of contamination (Silva et al., 2018). Moreover, the presence of *S. aureus* on the surface of the production tank suggests that the heating may not have been efficient, there may have been contamination from the handlers, and/or there may be a biofilm in this structure, a characteristic evidenced by Galinari et al. (2014).

In the agro-industries analysed, the milk thermal processing was not frequently controlled. In the study by Andrade et al. (2019), the authors also suggest that the high incidence of *S. aureus* in samples of handmade cheese may be associated with the contamination of raw milk, re-contamination after pasteurization, inadequate storage conditions and also by handlers, favouring the dissemination of this bacterium in food. It is noteworthy that *S. aureus* is the main species causing staphylococcal food poisoning outbreaks due to the production of staphylococcal enterotoxin (Kadariya et al., 2014.).

Table 2. *Staphylococcus* species isolated in several stages and surfaces during colonial cheese manufacturing process at two agro-industries in Rio Grande do Sul, Brazil.

	<i>S. aureus</i>	<i>S. capuae</i>	<i>S. epidermidis</i>	<i>S. equorum</i>	<i>S. haemolyticus</i>	<i>S. hominis</i>	<i>S. intermedius</i>	<i>S. saprophyticus</i>	<i>S. scituri</i>	<i>S. warneri</i>	<i>S. xyloso</i>
Raw milk	A ¹ (3) B(12)	**	-	-	A(1)	-	A(1)	-	B(2)	-	-
Production tank	B(6)	-	-	-	-	-	-	-	-	-	-
Production table	-	-	-	B(1)	B(1)	-	-	A(1)	A(1)	A(1)	-
Curd	B(1)	A(1)	-	B(1)	-	-	-	-	B(1)	A(1)	-
Forms	-	-	-	-	-	-	-	-	-	A(2)	-
Ripening cheese	A(1) B(1)	-	-	-	-	-	B(1)	B(1)	B(3)	A(1)	B(1)
Ripening shelves	-	-	-	A(2) B(1)	-	-	-	B(1)	A(1) B(2)	B(2)	-
Colonial cheese	A(2)	-	A(1)	B(2)	B(1)	-	-	-	B(4)	-	-
Hands of Handlers	A(2) B(1)	-	-	A(1)	-	B(1)	-	-	-	A(2)	-
Total of isolates	29	1	1	8	3	1	2	3	14	9	1

1. A: Agro-industry A; B: Agro-industry B; * The number of isolates of the indicated species are mentioned in parentheses; ** Species that were not isolated from colonial cheese or surface.

In agro-industry A, *S. aureus* was also identified in the ripened cheese and colonial cheese samples, as well as in one of the samples obtained from the handlers, a possible source of contamination in the cheese production process. Similar results were observed in agro-industry B where, although *S. aureus* strains were not isolated from colonial cheese, it was detected in the curd, ripened cheese and handler samples. During the ripening process, the cheeses were manipulated daily to invert their position on the ripening surface. Owing to this excessive handling throughout the production process, colonial cheese is likely to be contaminated. Other authors have shown that food handlers are the most frequent sources of food contamination in ready-to-eat food (Moura et al., 2012; Coelho et al., 2014). Approximately 40% of healthy people present *Staphylococcus* sp. on the nasopharyngeal mucosa and infected wounds. Moreover, the use of uniforms, hat, mask and gloves is not a

common practice in most agro-industries, including agro-industry B, a subject of this study. This fact reflects the importance of training programs and proves that hygiene practices are the most relevant factors contributing to the quality control and safety of colonial cheese (Sales et al., 2015; Roncatti, 2016).

Staphylococcus intermedius was another CoPS species identified in the raw milk and cheese ripening stage in the agro-industries studied. This species may be classified as an opportunistic pathogen in farm animals. In the present study, the occurrence of *S. intermedius* may also be related to mastitis or cross-contamination associated with the presence of domestic animals in the cheese production facilities since dogs, cats, horses and pigs were observed (Hogeveen et al., 2011, Piessens et al., 2011).

Regarding the CoNS isolates, *S. warneri* stands out in agro-industry A (7 isolates) and *S. sciuri* in agro-industry B (12 isolates). Motta et al. (2014) also noted that *S. sciuri* was the most frequently isolated CoNS in milk samples used in cheese production. CoNS can also lead to persistent infections with an increased somatic cell count and decreased milk production (Gillespie et al., 2009). Some species of CoNS identified in this study, such as *S. xylosus* and *S. equorum*, are particularly important in food production, as they cause flavour and colour stabilisation in sausages, salami and cheese. These species can be safely used as starter cultures in fermentation processes (Janssens et al., 2013).

The *sea* and *seb* were detected in 4% (3/72) of the *Staphylococcus* sp. isolates tested. No genes were detected for the other enterotoxins investigated (*sec*, *sed*, and *see*). One *S. aureus* isolate obtained from raw milk from agro-industry B showed the concomitant presence of two genes: *sea* + *seb*. In both of the analysed industries, pasteurized milk was used for the production of the colonial cheese. This procedure ensures the destruction of most vegetative cells, but is not sufficient to inactivate staphylococcal enterotoxin (ICMSF, 2015). The presence of the *sea* was confirmed in the other two *S. aureus* isolates obtained from the colonial cheese produced in agro-industry A. These cheeses had CoPS counts of 2 log₁₀ CFU·g⁻¹ and 2.3 log₁₀ CFU·g⁻¹, respectively. This seems to be insufficient to begin the production of enterotoxins considering a minimum population of 4log₁₀ CFU·g⁻¹ has been defined as the required amount for staphylococci to be able to produce staphylococcal enterotoxins (SEs) (Argudin et al., 2010; Pauli et al., 2012). However, while the count is below the minimum level for enterotoxin production at the time of our analyses (ready-to-eat cheese on the tenth day of ripening), a population growth is possible throughout the shelf life of the cheese if storage conditions are not adequate. This would still create a risk of enterotoxins being present in the product.

No enterotoxin genes were detected in the CoNS. Using the same primers as in this study, Moura et al. (2012) identified the genes for SE production in 40% of CoPS and CoNS isolates, with the *sea* and *seb* genes most frequently recorded. On the other hand, Borelli et al. (2011) did not detect genes for the same enterotoxins in Minas cheese isolates.

Our results indicate that eleven different *Staphylococcus* species were present in the colonial cheese-production environment and in the product itself. The predominant presence of *S. aureus* in the different samples of milk, curd, ripened cheese, ready-to-eat cheese, and hands of the handlers indicates that there probably are problems to be solved in the selection of milk-producing animals, pasteurization process, and hygiene control of handlers. An overall low frequency of isolates with enterotoxin genes was recorded, and no enterotoxin genes were detected in the CoNS.

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