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Virulence characterization of *Staphylococcus* spp. isolated from mozzarella cheeses and cheese-slicers

Caracterização da virulência de *Staphylococcus* spp. isolados de queijo mussarela e fatiadores de queijos

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Different species of the *Staphylococcus* genus can be found as contaminants in foods and may carry virulence factors that could potentially make them The enterotoxigenic potential ofpathogenic or transfer genes to other bacteria. Therefore, the aim of the present study was to isolate and identify species of *Staphylococcus* genus isolated from sliced mozzarella cheeses and cold-cut slicers and characterize biofilm formation in vitro, presence of enterotoxins and antibiotic resistance genes, and β-lactamase production. Sampling was carried out in 44 retail markets and isolates identified on species level. Resistance genes *blaZ* and *mecA*, as well as enterotoxin genes *sea*, *seb*, *sec*, and *sed*, were identified using PCR. Phenotypic evaluation of biofilm formation and detection of β-lactamase enzyme were also analyzed. Nine species of coagulase-negative *Staphylococcus* (CNS) were identified from 85 bacterial isolates. Of this total, 67.0% were positive for the presence of one or both antimicrobial resistance genes *blaZ* and *mecA*, while the β-lactamase enzyme was detected in 52.9% isolates. Regarding biofilm production, 48.2% of isolates were positive and 51.8% were negative. However, none of the isolates were identified with the enterotoxin genes. The results demonstrate the potential risk of cross-contamination, as biofilm-producing CNS could persist in the cheese retail environment, highlighting the danger of resistance genes transferring to other commensal or pathogenic bacteria.
Palavras-chave: contamination, enterotoxin gene, methicillin-resistant.

Diferentes espécies do gênero *Staphylococcus* podem ser encontradas como contaminantes em alimentos e podem carregar fatores de virulência, o que pode torná-los potencialmente patogênicos ou transferir genes para outras bactérias. Desta forma, o objetivo do presente estudo foi identificar e caracterizar bactérias do gênero *Staphylococcus* isoladas de queijo mussarela fatiado e fatiadores de frios, bem como produção de β-lactamase, formação de biofilme *in vitro* e presença genes de enterotoxinas e de resistência a antibióticos. A amostragem foi realizada em 44 mercados varejistas e os isolados identificados em nível de espécie. A identificação dos genes de resistência *blaZ* e *mecA*, e dos genes de enterotoxina *sea*, *seb*, *sec* e *sed* foi realizada por PCR. A avaliação fenotípica da formação do biofilme e a detecção da enzima β-lactamase também foram analisadas. Nove espécies de *Staphylococcus* coagulase negativa (SCN) foram identificadas a partir de 85 isolados bacterianos. Desse total, 67,0% dos *Staphylococcus* spp. foram positivos para a presença de um ou ambos os genes de resistência antimicrobiana *blaZ* e *mecA*, enquanto a enzima β-lactamase foi detectada em 52,9% dos isolados. Em relação à produção de biofilme, 48,2% dos isolados foram positivos e 51,8% negativos. No entanto, nenhum dos isolados foi identificado com os genes das enterotoxinas. Os resultados demonstram o risco potencial de contaminação cruzada, já que SCN produtores de biofilme podem ser persistentes no ambiente de varejo de queijo, apontando o perigo de passagem de genes de resistência para outras bactérias comensais ou patogênicas.
Palavras-chave: contaminação, gene da enterotoxina, resistente à meticilina.

1. INTRODUCTION

Species of staphylococci can be found in foods and on food processing surfaces, where they may form biofilms [1]. In this situation, the bacterial population is surrounded by a matrix of

exopolysaccharides, which give attached bacteria resistance to sanitizing and antimicrobial substances [2]. Additionally, microbial biofilm communities allow microorganisms to persist in the environment and contaminate other foods, endangering consumer health [3, 4].

Some species of *Staphylococcus*, classified as coagulase-negative *Staphylococcus* (CNS), may have the ability to form biofilms, facilitating their adhesion and colonization on food handling surfaces [5-7]. Moreover, bacterial biofilms have been found in different types of foodstuffs, including fermented foods like cheeses. Therefore, this highlights the importance of scientific research related to the presence of this pathogen in this type of food [7-9].

The enterotoxigenic potential of CNS in food outbreaks has not yet been fully established, but studies have point out this possibility since enterotoxin genes have been detected in CNS species [8, 10, 11]. Consequently, enterotoxigenic CNS strains may contribute to food poisoning [11]. Additionally, some CNS can carry antimicrobial resistance genes, passing these genes to other non-resistant bacteria. The resistance genes *mecA* and *blaZ*, present in some *Staphylococci* strains, confer mechanisms of resistance to β-lactams drugs. The β-lactamases encoded by the *blaZ* gene, represent one of the main resistance mechanisms of the *Staphylococcus* genus and act by hydrolyzing the β-lactam ring of the drugs [12, 13]. The *mecA* gene encodes a modified penicillin-binding protein 2a (PBP-2a), which has a low affinity for β-lactams. Also, *mecA* has been used as a genetic marker for Methicillin-resistant *S. aureus* (MRSA) that includes the β-lactam antibiotic class, such as penicillin, methicillin, and cefazolin [14]. Thus, the aim of the present study was to investigate the prevalence of coagulase-negative staphylococci isolated from sliced Mozzarella cheeses and cheese slicers, and to characterize their capacity of biofilm formation in vitro and the genotypic virulence factors of the isolates.

2. MATERIALS AND METHODS

2.1 Food samples

Samples were obtained from 44 retail markets (15 bakeries, 24 grocery store, and 5 supermarkets), all located in the city of Garanhuns, Pernambuco state - Brazil. A total of 88 samples were obtained, being 44 samples from sliced mozzarella cheese and 44 samples of swabs from the surfaces of cheese-slicers. All visits to retail establishments to acquire samples were performed with the support of the local Sanitary Surveillance of Garanhuns. After collection, the samples were immediately transported in isothermal boxes containing recyclable ice, to the Microbiology Laboratory of the Federal University of the Agreste of Pernambuco (UFAPE).

2.2 Isolation and identification of coagulase-negative staphylococci (CNS)

Cheese samples were processed using 25g of each sample of Mozzarella cheese were homogenized in stomacher with 225 mL of 0.1% peptone water. Then, other two serial dilutions (10^{-2} and 10^{-3}) were performed using 9 mL of 0.1% peptone water for both cheese samples and surface swabs of cheese-slicers (initially diluted in 10 mL of peptone water 0.1%). After this, 100 μL of each dilution was spread, in duplicate, on the surface of Baird-Parker agar (BP; Himedia, India) and incubated at 37°C for 48h. For confirmation of the genus *Staphylococcus*, three typical colonies (bright black with opaque ring, surrounded by a clear, transparent halo and highlighted on the opacity of the medium) and three atypical colonies (gray or shiny black, halo or with only one of the halos) were collected from each BP plate and cultured in 3 mL of BHI broth (Brain Heart Infusion Broth, Himedia, India) at 37ºC/24h to perform tests of Gram, catalase and coagulase. Isolates with characteristics of *Staphylococcus* spp. were frozen at -20°C in BHI containing glycerol 20% for further phenotypic and genotypic characterization.

2.3 Characterization of species of *Staphylococcus*

The genus *Staphylococcus* was differentiated from *Micrococcus* spp. by testing the resistance to antibiotic bacitracin (0.04 U.I.). The species-level identification of CNS was performed through biochemical tests. Initially, the isolates were tested for resistance or sensitivity to the antibiotic

novobiocin (5 mcg), as recommended by the Clinical and Laboratory Standards Institute (2008) [15]. The isolates resistant to novobiocin were submitted to fermentation test of the following sugars, D-xylose, D-mannose, L-arabinose, D-cellobiose, raffinose and sucrose, in addition to nitrate reduction test. The isolates of novobiocin-sensitive were tested for fermentation of α-lactose, D-mannitol, D-trehalose, maltose, sucrose and D-mannose, as well as the use of arginine. All isolates were tested for the presence of urease [16, 17].

2.4 Biofilm production assays

Evaluation of *in vitro* biofilm production was performed following recommendations of Stepanovic et al. (2003) [3] and Darwish and Asfour (2013) [5]. Frozen CNS isolates were reactivated and transferred to Tryptone Soy Broth (TSB) supplemented with 0.25% glucose (TSB-Glucose 0.25%) [18] and incubated for 24 hours at 37°C. The inoculum was diluted in TSB-Glucose 0.25% (1:40) and 200 µL of each isolate was added in triplicate into 96-well flat-bottomed polystyrene microtiter plates and incubated at 37°C for 24 hours. Sterile TSB-Glucose 0.25% was used as a negative control. After incubation, the volume of all wells was removed by aspiration and the wells were washed three times with 250 µL of sterile saline. Subsequently, each well was filled with 250 µL of methanol and the plate maintained at room temperature for 15 minutes. Afterwards, the methanol was discarded, 250 µL of aqueous crystal violet 0.5% solution was added and the plate incubated for 5 minutes at room temperature. Finally, the crystal violet was discarded and 250 µL of ethanol was added to each well to remove the unbound dye and then immediately discarded, keeping the plate at room temperature until the wells were completely dry. The reading was performed on a microplate reader using OD 620 nm.

The interpretation of the results was performed according to the arithmetic mean of the optical densities (ODs), test samples (ODt) and negative control (ODc), where they were used to classify each sample according to the following categories: No biofilm producer = ODt≤ODc; weak biofilm producer = ODc<ODt≤ (2xODc); moderate biofilm producer = (2xODc) <ODt≤ (4xODc); strong biofilm producer = (4xODc) <ODt.

2.5 PCR for detection of staphylococcal enterotoxins

Genomic DNA from CNS isolates was extracted and purified using the heating method described by Hassanzadeh et al. (2016) [19] and frozen at -20°C for further use. The primers used for PCR are described in Table 1.

Table 1. Primers sequences used for detection of the classical staphylococcal enterotoxin, resistance genes and specific staphylococcal genus 16S rRNA.

Primers	Sequence 5' - 3'	Genes	Amplicon (pb)	Reference
***sea* 1**	TTG GAA ACG GTT AAA ACG AA	*sea*	120	Cunha et al., 2006
***sea* 2**	GAA CCT TCC CAT CAA AAA CA			
***seb* 1**	TCG CAT CAA ACT GAC AAA CG	*seb*	478	Cunha et al., 2006
***seb* 2**	GCA GGT ACT CTA TAA GTG CC			
***sec* 1**	GAC ATA AAA GCT AGG AAT TT	*sec*	257	Cunha et al., 2006
***sec* 2**	AAA TCG GAT TAA CAT TAT CC			
***sed* 1**	CTA GTT TGG TAA TAT CTC CT	*sed*	317	Cunha et al., 2006
***sed* 2**	TAA TGC TAT ATC TTA TAG GG			
***mec*A-F**	GTA GAA ATG ACT GAA CGT CCG ATA A	*mec*A	310	Fontes et al., (2013)
***mec*A-R**	CCA ATT CCA CAT TGT TTC GGT CTA A			
***bla*Z-F**	AAG AGA TTT GCC TAT GCT TC	*bla*Z	517	Sawant et al., (2009)
***bla*Z-R**	GCT TGA CCA CTT TTA TCA GC			
16S - 1	GTA GGT GGC AAG CGT TAT CC	16S rRNA	228	Monday and Bohach, 1999
16S -2	CGC ACA TCA GCG TCA G			

The detection of enterotoxin genes was performed using SuperMix PCR kit (Invitrogen) following manufacturing protocol and the amplification performed in the Mastercycler® X50 Thermocycler (Eppendorf™), with the PCR program steps: initial denaturation at 94ºC for 2 min, followed by 40 cycles with denaturation at 94ºC for 30 seconds, annealing at 55ºC (*sea, seb, sec*) or 43.9°C (*sed*) for 30 seconds and extension at 72°C for 1 min, with a final extension of 10 minutes at 72°C. Positive controls used for each enterotoxin were *Staphylococcus aureus* 6+ (*sea* and *seb*, origin Fiocruz-Brazil), *S. aureus* FRI 361 (*sec*) and *S. aureus* FRI 1151 (*sed*). All isolates were tested for 16S rRNA to confirm the genus *Staphylococcus*.

2.6 Multiplex PCR for detection mecA and blaZ genes

Multiplex PCR was prepared with two pairs of oligonucleotide primers to detect *mecA* and *blaZ* genes (Table 1). The reaction was prepared using 2.5 μL (25 pmol) of *mecA* primers and 4.0 μL (40 pmol) of *blaZ* primers; 2 μL of Multiplex PCR mix (Solis Biodyne, Estonian) and 0.5 μL (~100 ng) of template DNA. The total volume was adjusted to 10 μL with sterile ultrapure water. Amplification was performed in a Mastercycler® Pro thermocycler (Eppendorf), with the following cycles: initial activation at 95°C for 12 minutes; initial denaturation at 94°C for 5 minutes, followed by 35 cycles (denaturation 94°C - 45 seconds; annealing 50.2°C - 30 seconds; extension 72°C - 30 seconds) and final extension at 72°C - 10 minutes. All reactions included positive control (DNA from *Staphylococcus capitis* subsp. *ureolyticus*, *blaZ* and *mecA* genes positive) and negative control (reaction without DNA).

The amplicons from conventional and multiplex PCR were visualized on a 2% agarose gel, stained with SYBR®Safe 10.000X Gel Stain (Invitrogen, USA), under UV transilluminator (DyNA Light UV transilluminator, LAbnet).

2.7 Confirmation of production of β-lactamase enzyme

In order to verify the production of β-lactamase enzyme, the isolates were screened with paper discs of nitrocefin-chromogenic cephalosporin (Cefinase Discs®; BD Company; USA), following the manufacturer's recommendations. *Staphylococcus aureus* ATCC 29213 and ATCC 25923 was used as a positive and negative controls, respectively [20].

3. RESULTS

A total of 88 samples were analyzed in our study and contamination with *Staphylococcus* spp. in the 44 slicer surface swab samples ranged from 1.0×10^1 to 9.4×10^4 CFU/mL, whereas in the samples of Mozzarella cheese the *Staphylococcus* spp. contamination ranged from 1.0×10^1 to 6.5×10^4 CFU/g. All staphylococci isolates from the Mozzarella cheese and swabs from cheese slicers samples were identified and classified in the present study as Coagulase Negative *Staphylococcus* (CNS).

After the biochemical tests, 85 isolates of CNS were identified on the surface of cheese slicers and sliced Mozzarella cheese samples: *Staphylococcus saprophyticus, S. xylosus, S. cohnii* subsp. *urealyticum, S. epidermidis, S. warneri, S. captis* subsp. *ureolyticus, S. chromogenes, S. caprae* and *S. simulans*. Thirty-one isolates were not identified by the conventional biochemical tests and were grouped as *Staphylococcus* spp. (Table 2).

The total of samples analyzed showed that 67.0% (57/85) were positive for *mecA* or *blaZ* genes. Among these, *mecA* gene was verified in 29.4% (25/85) and *blaZ* gene in 63.5% (54/85) of the CNS isolates. For the positive isolates, 38.6% (22/57) of the isolates were confirmed carrying both *blaZ* and *mecA* genes. When the isolates were tested for production of β-lactamase enzyme, 52.9% (45/85) were positive. Among the isolates producing β-lactamase, 35% (16/45) were positive for *blaZ*, and only one isolate (1/45; 2.2%) from the 25 isolates carrying *mec*A gene was also positive for β-lactamase. However, 16 (35%) isolates were identified as not carrying neither *blaZ* nor *mecA* genes. A total of 26% (12/45) of the isolates that were positive for both *blaZ* and *mecA* were positive as well for β-lactamase in the phenotypic test. The presence of *sea*, *seb*, *sec* or *sed* enterotoxin genes was not detected in the CNS isolates from Mozzarella cheese and cheese-slicers.

Table 2. Absolute and relative frequency of coagulase-negative staphylococci species isolated from samples of Mozzarella cheese and the surface of slicers.

CNS species	Surface of Slicers	Mozzarela Cheese	Absolute frequency (%)
S. saprophyticus	10	12	22 (25.9)
S. xylosus	8	6	14 (16.5)
S. cohnii subsp. *urealyticum*	3	4	7 (8.2)
S. epidermidis	1	3	4 (4.7)
S. warneri	2	1	3 (3.5)
*S. captis*subsp. *ureolyticus*	1	0	1 (1.2)
S. chromogenes	0	1	1 (1.2)
S. caprae	0	1	1 (1.2)
S. simulans	0	1	1(1.2)
Staphylococcus spp.[a]	17	14	31 (36.5)
Total	42	43	85 (100)

[a] isolates not identified at the species level by the biochemical tests used.

Furthermore, in the Figure 1 and 2 is shown the biofilm production *in vitro* from the CNS isolates which were classified as weak, moderate and strong biofilm producers.

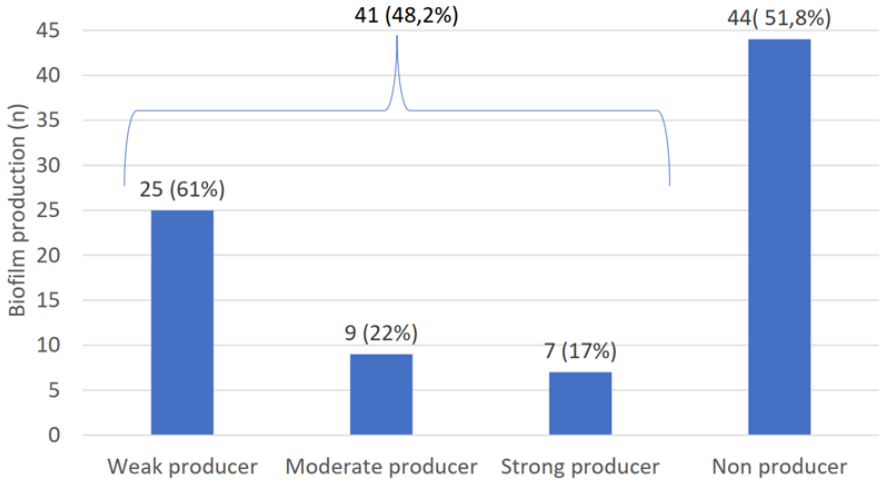

Figure 1. Samples of biofilm-producing and non-producing coagulase-negative staphylococci isolates from mozzarella cheese and slicer surfaces and their classification in level of biofilm production.

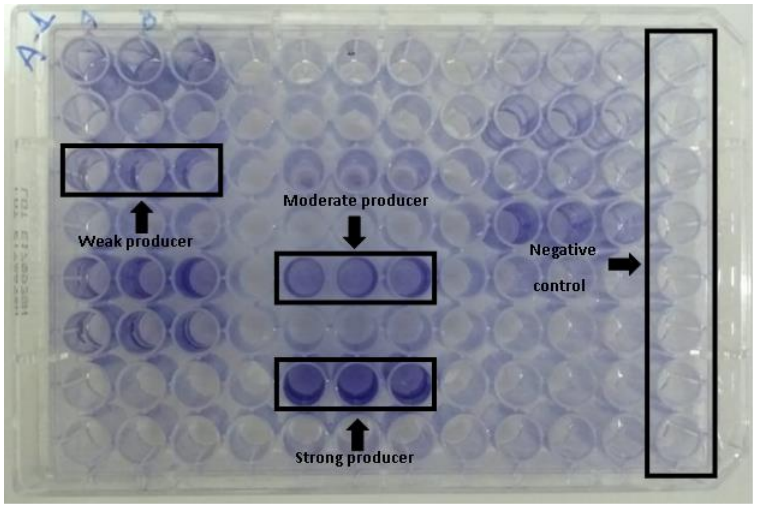

Figure 2. Microtiter method in polystyrene plates showing coagulase-negative staphylococci differentiating in non-producing, weak, moderate and strong biofilm producers by staining with crystal violet 0.5%.

Table 3 shows the relationship between the species of SNC identified and the levels of biofilm producer and non-biofilm producer, demonstrating that all identified species were able to produce biofilm. The isolates strongly producing biofilms belonged to the species *S. saprophyticus* (3), *S. cohnii* subsp. *urealyticum* (1) and *Staphylococcus* (3).

Table 3. Biofilm formation according to species of coagulase-negative staphylococci.

CNS species	Total	Biofilm production			
		Strong [n° (%)]	Moderate [n° (%)]	Weak [n° (%)]	Non[b] [n° (%)]
S. saprophyticus	22	3	5	6	8
S. xylosus	14	0	0	4	10
S. cohnii*subsp. *urealyticum	7	1	1	2	3
S. epidermidis	4	0	0	0	4
S. warneri	3	0	0	0	3
S. captis*subsp. *ureolyticus	1	0	0	1	0
S. chromogenes	1	0	0	1	0
S. caprae	1	0	0	0	1
S. simulans	1	0	0	0	1
***Staphylococcus* spp.[a]**	31	3	3	11	14
Total	85	7 (17)	9 (22)	25 (61)	44

[a]isolates not identified at the species level by the biochemical tests used. [b]Non-producer of biofilm.

In our study, the mecA and blaZ genes were detected in the isolates as shown in Table 4.

Table 4. Absolute and relative frequency of presence of blaZ and mecA genes and phenotypic expression of β-lactamase.

Gene	Absolute Frequency	Relative Frequency	β-lactamase positive (n= 45)	
			Absolute Frequency	Relative Frequency
***bla*Z+**	32	37.6%	16	18,8%
***mec*A+**	3	3.5%	1	2,2%
***bla*Z+*mec*A+**	22	25.9%	12	14,1%
***bla*Z$^-$/*mec*A$^-$**	-	-	16	18,8%
Total	57	67.6%	45	52,9%

4. DISCUSSION

The result found in the present study is highly relevant due to the important epidemiological significance of the presence of these *Staphylococcus* in foods, since different researchers have observed characteristics of virulence in the CNS group, such as biofilm-producing isolates, antibiotic resistance genes and presence of enterotoxin encoding genes [8, 10, 21].

Taking this into consideration, it is also worth noting that CNS can be an important source of dissemination of virulence genes such as biofilm, antimicrobial and enterotoxin genes [21]. Therefore, it would be interesting to consider a reassessment of the microbiological standards established by the Brazilian legislation for enterotoxigenic *Staphylococcus* in foods, which only requires analysis in food for coagulase positive staphylococci.

In our study, the presence of genes encoding the enterotoxins SEA, SEB, SEC and SED was investigated in all 85 CNS and the presence of *sea*, *seb*, *sec* or *sed* genes was not detected in any of the isolates. A similar result was obtained by Wang et al. (2018) [21], who investigated among other genes, *sea*, *seb*, *sec* and *sed* in CNS isolated from samples of raw chicken meat products, indicating the negative potential for food poisoning by CNS in the samples of their work. Even though there were negative results for the classical enterotoxins search in our study, the occurrence of CNS with enterotoxigenic potential in food has been reported. Among the CNS species isolated from food and capable of producing enterotoxins are *S. epidermidis*, *S. xylosus*, *S. warneri*, *S. saccharolyticus*, *S. hominis*, *S. saprophyticus*, *S. sciuri*, *S. carnosus*, *S. succinus*,

S. lentus, *S. simulans*. In these species, the main enterotoxin genes found were *sea*, *seb*, *sec*, *sed*, *see*, *seh*, *sei* and *sel* [8, 10].

In a study published by Darwish and Asfour (2013) [5], of the 68 (63%) CNS isolates from the milk of cows with subclinical mastitis, 30 (44%) were strong biofilm producers, 21 (30.9%) were moderate biofilm producers and 13 (19.2%) were weak biofilm producers, whereas only 4 (5.9%) isolates did not produce biofilm. Likewise, Friedriczewski et al. (2018) [1], found 20 isolates of *S. aureus* in samples of Mozzarella cheese produced with buffalo milk. Of these isolates, 2 (10%) were classified as strong biofilm producers, 7 (35%) as moderate biofilm producers, 10 (50%) as weak biofilm producers and one (1) did not produce biofilm. According to the data of our study, a considerable number of samples with the capacity to produce biofilm were observed (Table 3). Thus, it is possible to conclude that this characteristic causes concern, since this is a persistent pathogen in the environment and can contaminate cheeses and other food products.

Hence, regardless of the samples collected and the sampling locations (cheese or surface swab), it was observed that the contamination with microorganisms of *Staphylococcus* genus is present in the area covered by this study, indicating that all places subjected to analysis showed bad hygienic conditions in the processing and marketing of Mozzarella cheese. Therefore, this data will be able to support the decisions of the supervisory agency, and assist in carrying out training actions, to improve the quality of this product that is highly consumed by the population.

The resistance to antimicrobial agents is a huge problem faced today all over the world. Issues related to the antimicrobial resistance of coagulase negative staphylococci has been related by different researchers [21, 22]. It is important to emphasize that both are important virulence genes for microorganisms, which act on the β-lactam ring of penicillin compounds, leading to the failure of these antimicrobial agents over the bacteria [20]. In the study published by Pizauro et al. (2019) [22], among the 84 CNS isolated from buffalo milk and the processing environment of this milk, only one isolate was found carrying the *mecA* gene. This information reinforces the importance of our findings, since the number of their isolates is similar to that analyzed in the present study.

Robles et al. (2014) [23], performed a study to detect β-lactamase in *S. aureus* and CNS isolated from bovine mastitis. These researchers observed that several of their isolates did not have the *blaZ* gene, although phenotypic tests showed enzyme production activity. This is due to the fact that the β-lactamase phenotype may result from the expression of more than one gene, or even because there is more than one mechanism that confers resistance to this group of microorganisms, in addition to the expression of the *blaZ* gene [24].

On the other hand, 18,8% (16/85) of the positive samples for the *blaZ* gene confirmed by PCR, were also positive for the enzyme production by the phenotypic confirmation tests. In addition, 26,7% (12/85) of the positive samples for both *mecA* and *blaZ* genes by the genotypic method, were also positive for β-lactamase production. Regarding the *mecA* gene, only 1,2% (1/85) of the samples that amplified the gene were positive to produce the enzyme in vitro. Thus, due to the confirmation of biofilm formation and the presence of resistance genes by these isolates, it is likely that this microorganism may be persistent in the environment as well as transfer genes to other non-staphylococci bacteria.

5. CONCLUSION

The results found indicate a public health concern, since coagulase negative staphylococci isolates also have the ability to form biofilms on food surfaces, and thus can become a source of contamination for people. Although in this study CNS isolates were not identified as carriers of the *sea*, *seb*, *sec* and *sed* genes, different reports indicate that CNS isolates can carry these enterotoxins genes, consisting of an important subject for further research. For these reasons, it is necessary to emphasize the importance of good handling and hygiene practices for equipment and utensils used with Mozzarella cheese, in order to minimize the risk of food poisoning caused by *Staphylococcus* genus.

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