Identification and molecular phylogeny of coagulase-negative staphylococci isolates from Minas Frescal cheese in southeastern Brazil: Superantigenic toxin production and antibiotic resistance

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ABSTRACT

Minas Frescal is a typical Brazilian fresh cheese and one of the most popular dairy products in the country. This white soft, semiskimmed, nonripened cheese with high moisture content is obtained by enzymatic coagulation of cow milk using calf rennet or coagulants, usually in industrial dairy plants, but is also manufactured in small farms. Contamination of Minas Frescal by several staphylococci has been frequently reported. Coagulase-negative staphylococci (CNS) strains are maybe the most harmful, as they are able to produce heat-stable enterotoxins with super antigenic activities in food matrices, especially in dairy products such as soft cheeses. The aim of the present study was to investigate the presence of CNS strains in Minas Frescal marketed in southeastern Brazil concerning the risk of staphylococci food poisoning by the consumption of improperly manufactured cheese and the possibility of these food matrices being a reservoir of staphylococcal resistance to antimicrobials. Ten distinct CNS strains were found in 6 cheeses from distinct brands. The most frequent species were Staphylococcus saprophyticus (40%), Staphylococcus xylosus (30%), Staphylococcus sciuri (20%), and Staphylococcus piscifermentans (10%). Three strains were identified to the Staphylococcus genera. Three major species groups composed of 3 refined clusters were grouped by phylogenetic analyses with similarities over to 90%. All CNS strains carried multiple enterotoxin genes, with high incidence of sea and seb (90 and 70%, respectively), followed by sec/sev, seh/sei, and sed with intermediate incidence (60, 50, and 40%, respectively), and, finally, seg/selk/selq/selr and selu with the lowest incidence (20 and 10%, respectively). Real-time reverse transcription PCR and ELISA assays confirmed the enteroxigenic character of the CNS strains, which expressed and produced the enterotoxins in vitro. The CNS strains showed multiresistance to antimicrobial agents such as β-lactams, vancomycin, and linezolid, which have therapeutic importance in both human and veterinarian medicines. The risk of staphylococci food poisoning by the consumption of improperly manufactured Minas Frescal was emphasized, in addition to the possibility of these food matrices being a reservoir for antibiotic resistance. More effective control measures concerning the presence and typing of staphylococci in raw milk and dairy derivatives should be included to prevent the spread of pathogenic strains.

Key words: classical and newly described enterotoxins, enterotoxin-encoding gene transcription and expression, multiresistance to antimicrobials, real-time reverse transcription PCR

INTRODUCTION

Bacteria belonging to the Staphylococcus genus can cause a multitude of diseases in humans. As essentially opportunistic microorganisms, these pathogens can cause severe infections, especially among immunocompromised people, and are often difficult to treat because of the relatively high prevalence of multiresistant strains (Zell, et al., 2008). Healthy people can be affected by staphylococci if they are transmitted by food products, as these microorganisms have the ability to secrete several types of toxins, many of which are responsible for food poisoning.

According to the Brazilian Ministry of Health (Ministério da Saúde), staphylococcal poisoning is the second most common food-borne disease in Brazil, only behind outbreaks involving Salmonella spp. (ICMSF, 2006). Staphylococcal food poisoning is a gastrointestinal ill-
ness caused by the ingestion of contaminated food containing enterotoxins produced by bacteria belonging to this genus. These enterotoxins exhibit super antigenic activities and are heat stable proteins that may not be destroyed under inadequate cooking.

Among the pathogens belonging to *Staphylococcus* genus, those organisms classified as coagulase positive, such as *Staphylococcus aureus*, are considered potential food enterotoxin-producing species (ICMSF, 1983). However, the possibility of CNS being involved in food poisoning has been also raised (Veras et al., 2008) after it was shown that these microorganisms can also carry enterotoxin-encoding genes (*se* genes) in their genomes (Rall et al., 2010); these have been found in processed meat (Pereira et al., 2009) and in dairy products, especially cheeses (Senger and Bizani, 2011).

The enterotoxin (*SE*) genes are encoded in mobile genetic elements, such as plasmids, prophages, and *Staphylococcus* pathogenic islands. Those mobile genetic elements are responsible for the horizontal transfer of virulence or antibiotic resistance genes between *S. aureus* strains (Ubeda et al., 2005; Maïques et al., 2006), and it has also been suggested that the exchange of mobile DNA elements between microorganisms might occur during food processing (Virdis et al., 2010).

Staphylococcal toxins are designated as *SE* with demonstrated emetic activity, whereas staphylococcal-like toxins are named *SE*-like (*SEL*) and showed no emetic activity in primate models or have not yet been tested (Lina et al., 2004). To date, 21 *SE* and *SEL* toxins have been reported. In addition to the five well-characterized, classical staphylococcal enterotoxins—SEA, SEB, SEC,SED, and SEE—6 new types of *SE* (SEG, SEH, see, SEJ, SEK, SEL, SEM, SEI, SEO, SEP, SEQ, SEU, and SEV) have been introduced (Argudin et al., 2010).

The *Minas Frescal* (fresh) is a typical Brazilian cheese produced since the 18th century (Campos, 2001); even now it is one of the most popular cheeses consumed in southeastern Brazil. It is a white soft cheese, produced from cow milk, and its major characteristic is a pleasant, slightly acid taste and rich flavor (Kamiyama and Otenio, 2013). A method to avoid *Minas Frescal* adulteration via replacement of milk by whey has been developed (Magenis et al., 2014).

According to the current Brazilian legislation (Brazil, 1997), the *Minas Frescal* cheese is a fresh cheese obtained by enzymatic coagulation of cow milk using calf rennet or coagulants. Specific lactic acid bacteria can complement the enzymatic milk coagulation. *Minas Frescal* cheese is a semiskimmed product with low saturated fat content. It is considered a lean protein source with high digestibility, and it is the dairy-derived product most often consumed in energy-restricted diets for weight loss (Moraes et al., 2009). In addition, *Minas Frescal* can be considered a functional food when lactic acid bacteria are used in manufacturing because of its health benefits, including reducing the risk of human diseases (Stringheta et al., 2007; Almeida et al., 2008).

The production of *Minas Frescal* cheese has increased from 42,700 tons in 2000 to 63,555 t in 2011, placing it third among cheeses produced in the country, behind only Mozzarella and Prato cheese, a Brazilian semihard cow variety of cheese (Cichoscki et al., 2002). The manufacturing of *Minas Frescal* cheese may occur in dairy industrial plants, but it is also commonly manufactured by small producers with inadequate manufacturing practices and can undergo excessive manipulation (Bulhões and Rossi Junior, 2002).

Soft cheeses do not undergo any treatment while ripening to ensure their safety before consumption. *Staphylococci* can contaminate soft cheeses by improper handling during processing, inadequate storage conditions, use of contaminated milk, or even due to the physico-chemical features of the cheese matrix, such as high pH, high water activity, and low salt concentrations, which are favorable to the growth of these microorganisms (Viana et al., 2009). Outbreaks caused by enterotoxigenic *Staphylococcus* strains would hardly be associated with the contamination of *Minas Frescal* soft cheese, as it is considered a healthy and harmless food for the Brazilian population (Silva and Souza, 2006).

The main species of CNS prevailing in dairy products are *Staphylococcus sciuri*, *Staphylococcus xylosus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus lentus* (Soares et al., 2011), as well as several strains isolated from different sources shown to be resistant to antibiotics of therapeutic relevance (Kästner et al., 2006; Martin et al., 2003), mainly penicillin and synthetic β-lactams represented by oxacillin, vancomycin, and linezolid (Virdis et al., 2010). Many of these oxacillin-resistant microorganisms also present resistance to other antimicrobials, thus limiting the use of glycopeptides such as vancomycin and linezolid (Resch et al., 2008).

For a long time, *S. aureus*, a coagulase-positive staphylococci, has always been thought to be the only pathogenic species among *Staphylococcus* spp., whereas CNS are, even today, classified as contaminant agents. In Brazil, current legislation (Brasil, 2001) establishes $10^3$ cfu/g as the upper tolerance limit count for coagulase-positive staphylococci in soft cheese matrices, whereas CNS are neglected as contaminant microorganisms in food.

*Staphylococcal* enterotoxin producers are a public health problem, as food processing does not inactivate...
these toxins. According to the Centers for Disease Control and Prevention (http://www.cdc.gov/nczved/divisions/dfbmdl/diseases/staphylococcal/), staphylococcal food poisoning can cause a brief illness of 1 to 3 d, but highly susceptible patients, such as the young and the elderly, are more likely to have severe illness requiring intravenous therapy and hospital care.

The aim of the present study was to evaluate the CNS contamination of Minas Frescal cheeses marketed in Rio de Janeiro, Brazil. The identification of the main CNS strains was performed by DNA sequencing and their phylogenetic relationships were established. The presence of multiple genes encoding the classical and newly described enterotoxins in CNS genomes was investigated. The risk of food poisoning was assessed by evaluating the ability of the CNS strains in transcribing and expressing the classical and newly described enterotoxins in vitro by using real-time reverse transcription PCR and ELISA assays. The resistance of the isolated strains to antimicrobial agents of therapeutic importance in staphylococcal infections was also evaluated.

**MATERIALS AND METHODS**

**Isolation of Bacterial Strains and Growth Conditions**

A bulk sample consisting of 6 Minas Frescal cheeses from distinct brands inspected by municipal, state, or federal food inspection services and collected in the municipality of Rio de Janeiro were stored in low-density polyethylene bags. Samples showed acidic pH of 5.6 to 5.8, 3.0 to 4.0 mg of sodium, and water activity (Aw) of 0.93 to 0.96. Twenty-five grams of cheese was added to 225 mL of 0.1% peptone water. The suspensions were transferred to homogenizer bags (Interscience, Saint Nom, France) and coupled to a Stomacher 400 circulator (Seward, Worthing West Sussex, UK) at 260 rpm for 1 min. The suspensions were serial diluted from 10−6 to 10−1 and 100 μL of each dilution was inoculated onto 20 mL of Baird-Parker agar containing egg yolk tellurite emulsion (BPA+ RPF, bioMerieux, Marcy l’Etoile, France) and incubated at 37°C for 24 h. Conventional microbiology procedure were performed following instructions to the Bergey Manual of Systematic Bacteriology (Garrity et al., 2005), where 80 colonies presumptive of CNS (3–10 colonies of each sample) were tested by Gram staining and catalase, coagulase, and thermostable DNAase activities. The 60 presumptive CNS strains were stored at −80°C in tryptone soy agar (BD BBL, Franklin Lakes, NJ) plus 45% vol/vol glycerol. Cultures were grown in brain heart infusion (BD BBL) medium at 37°C for 24 h.

**DNA Preparation**

The isolated strains were cultured overnight aerobically in 10 mL of brain heart infusion broth (BD BBL) at 37°C for 24 h. The suggestive CNS colonies were grown up to optical density at 600 nm (OD_{600} = 0.5), which corresponds approximately to a concentration of 10^6 cfu/mL, were harvested by centrifugation at 5,700 × g for 1 min at room temperature. The cell pellet was used for DNA extraction using the DNeasy blood and tissue kit (Qiagen, Dusseldorf, Germany), following the manufacturer’s instructions. Genomic DNA was quantified using the Qubit fluorimeter and Qubit assay kits (Invitrogen, Grand Island, NY).

**16S rDNA Sequencing**

Amplification of the V5 region of 16S rDNA was performed using 50 ng of DNA templates from the 60 strains isolated from Minas Frescal cheeses. A PCR was performed under the following conditions: 95°C for 10 min, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were purified using the PCR DNA Purification kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) and sequenced using 20 ng of purified DNA and 13 μL of primer set of forward and reverse 16S rDNA (Table 1) in a final volume of 20 μL. After the amplification, products were purified according to the protocol of the BigDye Terminator Purification X kit (Applied Biosystems) and sequenced in a 3130 sequencer Genetic Analyzer (Applied Biosystems). *Staphylococcus* species identification was performed by comparing the partial sequences of 16S rDNA to the sequences available at GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). The nucleotide sequences were then submitted to GenBank, which provided new accession number to each isolated strains of *Staphylococcus* species available in the GenBank database. Multiple sequence alignments were performed using ClustalW (Kyoto University, Bioinformatics Center; http://www.genome.jp/tools/clustalw/).

**Phylogenetic Analyses**

Phylogenetic relationships among the CNS strains were performed by the alignment of sequences using the Clustal X 2.0 software (Larkin et al., 2007). The phylogenetic trees were constructed using the software Mega 6.0 and UPGMA methods (Sneath and Sokal, 1973).
**PCR Tests**

**Primer Sets, Sequence, Genes, and Species.**
Primer sets flanking the sea, seb, sec, selg, selh, selk, seln, selo, selq, selr, and selu sequences are listed in Table 1.

**Uniplex-Duplex and Multiplex-PCR Tests.**
Uniplex-PCR tests were performed for amplifying the test sequence and duplex-PCR was used with the sea, seb primers and sec, sed primers. The PCR mixtures contained 25 μL of 20 mM MgCl₂, 10× PCR buffer (Invitrogen), 100 mM dNTP mix (Fermentas Thermo Scientific, Vilnius, Lithuania), 0.2 mM of each primer (Table 1), 0.5 U of Taq DNA polymerase (Invitrogen), and 100 ng of DNA templates. The DNA amplification by uniplex- and duplex-PCR assays were performed under the following conditions: 94°C for 5 min followed by 35 cycles of 94°C for 2 min, 53°C for 2 min, and 72°C for 1 min for extension, ending with a final extension at 72°C for 7 min, according to the conditions described by Mehrotra et al. (2000) with modifications in the annealing temperature using a thermal cycler (MyCycler, Bio Rad, Hercules, CA). The amplified fragments were visualized on 1.0% agarose gels (Sigma-Aldrich, St. Louis, MO) stained with GelRed (dilution 1:1000; BioAmerica, Tel Aviv, Israel) and documented on a transilluminator (MiniLumi Imaging Bio-Systems, BioAmerica).

**Multiplex-PCR Tests.**
Multiplex-PCR assays were performed by simultaneous amplification of the sec, selg, selh, selj, selm, seln, selo, selk, selq, selr, and selu sequences using the primer sets listed in Table 1. Each reaction contained 50 μL of a mix containing 0.5 U/μL of Taq DNA polymerase, 10× PCR buffer, 100 mM dNTP, 0.2 mM of each primer, and 100 ng of DNA template. The DNA amplification of see, selq, selh, and seli was carried out as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 53°C for 90 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. The DNA amplification of the selj, selm, seln, and selo group and the selk, selj, selm, seln, and selo groups were documented on a transilluminator (MiniLumi Imaging Bio-Systems, BioAmerica).

<table>
<thead>
<tr>
<th>Item</th>
<th>Sequence (5'→3')</th>
<th>Gene</th>
<th>Amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA forward</td>
<td>TTTGAAACGCGTATCCCGTAACC</td>
<td>see</td>
<td>120</td>
<td>Johnson et al., 1991</td>
</tr>
<tr>
<td>SEA reverse</td>
<td>GAACCTTCCCATCAAAACCA</td>
<td>seb</td>
<td>478</td>
<td>Johnson et al., 1991</td>
</tr>
<tr>
<td>SEB forward</td>
<td>TGCGATCAAACTGACAACC</td>
<td>sec</td>
<td>257</td>
<td>Johnson et al., 1991</td>
</tr>
<tr>
<td>SEB reverse</td>
<td>CCAGTACTCTTATAGTCGCC</td>
<td>see</td>
<td>317</td>
<td>Johnson et al., 1991</td>
</tr>
<tr>
<td>SEC forward</td>
<td>GATCTAAACGCTAGGAATT</td>
<td>see</td>
<td>170</td>
<td>Johnson et al., 1991</td>
</tr>
<tr>
<td>SEC reverse</td>
<td>AAAATCGATACATCATATCC</td>
<td>sed</td>
<td>704</td>
<td>McLauchlin et al., 2000</td>
</tr>
<tr>
<td>SED forward</td>
<td>CTAGTTTGTTAATAATCATCCT</td>
<td>sed</td>
<td>495</td>
<td>McLauchlin et al., 2000</td>
</tr>
<tr>
<td>SED reverse</td>
<td>TAATGCTATATCTTATAGGG</td>
<td>see</td>
<td>630</td>
<td>McLauchlin et al., 2000</td>
</tr>
<tr>
<td>SEE forward</td>
<td>TAGATAAGTATCAAAAACG</td>
<td>see</td>
<td>426</td>
<td>Rosse and Gigaud, 2002</td>
</tr>
<tr>
<td>SEE reverse</td>
<td>TCTAGCGGAACCTGACCC</td>
<td>see</td>
<td>517</td>
<td>Omoe et al., 2005</td>
</tr>
<tr>
<td>SEL forward</td>
<td>CGAATATCGACCACAATCCAC</td>
<td>selb</td>
<td>682</td>
<td>Omoe et al., 2005</td>
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<tr>
<td>SEL reverse</td>
<td>TTTGAAAATCTCTGTCTCCA</td>
<td>selm</td>
<td>534</td>
<td>Omoe et al., 2005</td>
</tr>
<tr>
<td>SED forward</td>
<td>AGCTAAAGTATGAGCCTATT</td>
<td>selo</td>
<td>545</td>
<td>Sergeev et al., 2004</td>
</tr>
<tr>
<td>SED reverse</td>
<td>TATGTTTGCCTGAATGCAGT</td>
<td>selk</td>
<td>539</td>
<td>Sergeev et al., 2004</td>
</tr>
<tr>
<td>SELL forward</td>
<td>ATTTTTATGCTTCCTCTTATAAATATCC</td>
<td>selq</td>
<td>363</td>
<td>Holtfreter et al., 2007</td>
</tr>
<tr>
<td>SELL reverse</td>
<td>ATTTATCTGCTTCTCATAAGATATGC</td>
<td>selr</td>
<td>215</td>
<td>Holtfreter et al., 2007</td>
</tr>
<tr>
<td>TSTH1 forward</td>
<td>ATGGCATCCATGCTGATA</td>
<td>tstH1</td>
<td>350</td>
<td>Johnson et al., 1991</td>
</tr>
<tr>
<td>TSTH1 reverse</td>
<td>TTTCCCAATACCAAGCCTGTT</td>
<td>16SrDNA</td>
<td>500</td>
<td>Mason et al., 2001</td>
</tr>
</tbody>
</table>

*Se = enterotoxins; SE = SE-like toxins.*

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μL of the cDNA ultrapure water were added to each
selq, selr, and selu group were carried out in the same
conditions: 95°C for 5 min, followed by 35 cycles at
95°C for 30 s, 55°C for 90 s, and 72°C for 90 s, with a
final extension at 72°C for 10 min (Omoe et al., 2005).
The PCR products were visualized by electrophoresis
on 1.2% agarose gels (Uniscience do Brasil, São Paulo,
Brazil) in 1× TAE (Tris-acetate-EDTA) buffer stained
by 0.5 μg/mL of GelRed (BioAmerica) and documented
on a transilluminator (MiniLumi Imaging Bio-Systems,
BioAmerica).

Enterotoxin Expression Assays

The isolated strains were cultivated overnight aerobi-
cally in 10 mL of brain heart infusion broth (BD BBL)
at 37°C for 72 h. Bacteria supernatants were collected
by centrifugation at 4,000 × g for 10 min at room
temperature and used for the detection of SEA, SEB,
SEC, SED, and SEE in an ELISA using a commercial
detection kit (Ridascreen Set A, B, C, D, E Art. No:
R4101, R-Biopharm AG, Darmstadt, Germany). The
assay was performed according to the manufacturer’s
recommendation and as described elsewhere (Rahimi
and Ghasemian Safai, 2010). The mean lower limit of
detection of the assay was 0.25 ng/mL. The threshold
was defined as the average optical density of 2 negative
controls plus 0.15, a constant established by the kit.
Samples containing SE showed absorbance values equal
or greater than the threshold value. All experiments
were performed in duplicate.

Real Time Reverse Transcription-PCR Assays

Total RNA was extracted by using the Qiagen Ri-
bopure TM Bacteria kit (Life Technologies, Carlsbad,
CA) following the manufacturer’s instructions and
quantified using the Qubit fluorimeter and Qubit assay
kits (Invitrogen).
The cDNA synthesis was performed by using the High Capacity cDNA Reverse Transcription Kit (Ap-
plied Biosystems) as follows: 4.0 μg of RNA, 2.0 μL of
10× reverse transcription (RT) buffer, 0.8 μL of dNTP
mix (100 μM), 2.0 μL of 10× RT random primers, 1.0
μL of MultiScribe (Applied Biosystems, Foster City,
CA) reverse transcriptase, and 4.2 μL of nuclease-free
H2O. The cDNA synthesis was performed under the
following conditions: step 1 at 25°C for 10 min, 37°C
for 120 min, and 85°C for 5 min in an ABI Prism 7500
Fast RT-PCR system (Applied Biosystems). Samples
were plated in triplicate in 96-well plates where 12 μl
of the SYBR Green PCR Master Mix, 1 μL of primer
mix (sea, seb, sec, sed, see, seg, seh, sei, sej, seh,
seh, selo, sek, selr, selu, or tstH1; Table 1), and 4.5
μL of the cDNA ultrapure water were added to each
well. Amplification was performed under the following
conditions: 95°C for 15 min, 40 cycles at 95°C for 15 s,
54°C for 30 s, and 72°C for 30 s. The dissociation
curve was performed at 95°C for 15 s, 54°C for 30 s, and 95°C
for 15 s. The DNA templates from reference strains S.
aureus ATCC 29231 (sea), S. aureus ATCC 14458 (seb,
tsst, selk, selq, selr, and selu), S. aureus ATCC 19095
(sec, seg, seh, and sei), S. aureus ATCC 13563 (sed), S.
aureus ATCC 27664 (see), and S. aureus ATCC 27154
(selj, selm, seln, and selo) were used as controls. Gene
expression was estimated by the semi quantification of
mRNA (arbitrary units) in comparison with the mRNA
content from reference strains. Messenger 16S rDNA
was used as an internal control to normalize enterotox-
in expression levels. Cycle threshold value estimations,
the standard deviations, and the cDNA quantification
were calculated using the GraphPad Prism 5 software
package (GraphPad Software Inc., La Jolla, CA).
Calibration curves based on 5 points were constructed
in triplicate corresponding to serial dilutions (1, 1:10,
1:100, 1:1,000 and 1:10,000) from 100 ng of a DNA
template stock solution.

Antibiotic Susceptibility Tests

An inoculum of each isolated strain equivalent to 0.5
McFarland scale was swabbed onto the Muller Hinton
agar plate (BD BBL) and the antibiotic disc was then
placed on the plate followed by overnight incubation
at 37°C. The inhibition zone was interpreted according
to the Clinical Laboratory Standards Institute (CLSI,
2014) guidelines (formerly known as the National Com-
mittee for Clinical Laboratory Standards). The tested
antibiotics were penicillin G (10 μg), oxacillin (1 μg),
neomycin (30 μg), trimethoprim (5 μg), clindamycin (2
μg), gentamicin (10 μg), cefotaxin (30 μg), rifampicin
(5 μg), erythromycin (15 μg), tetracycline (30 μg), van-
comycin (30 μg), ciprofloxacin (5 μg), sulfazathrim (23
μg), cefepime (30 μg), and linezolid (30 μg).

MIC Determinations

Minimum inhibitory concentrations for vancomycin,
linezolid, methicillin, and ampicillin were determined
by the macrodilution broth method based on CLSI
recommendations, using in-house-prepared panels (NC-
CLS, 2003). Antibiotic concentrations of 0.03, 0.06,
0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL were tested. One
milliliter of broth was transferred to the tubes and 100
μL of bacteria suspension was adjusted to 106 cfu/mL
in saline solution 0.85% according to 0.5 McFarland
scale and transferred to tubes containing 1 mL of each
diluted antimicrobial. Isolated strains were grown in
Mueller-Hinton broth (BD BBL) and the MIC was

estimated as the lowest antibiotic concentration that inhibits visible growth after 24 h (NCCLS, 2003).

RESULTS AND DISCUSSION

Isolation and Identification of CNS from Minas Frescal

Sixty suggestive CNS microorganisms were isolated from Minas Frescal cheese by colony morphology, the coagulase slide test, subsequent tube test, and biochemical tests. After these tests, 45 strains were submitted to sequencing of the V5 region of the 16S rDNA, and the alignment of sequences to those available in GenBank was discriminative enough to differentiate 42 Staphylococcus strains isolated from Minas Frescal at the species level and 1 to the subspecies level with similarities >98%. Three strains were confirmed as belonging to the Staphylococcus genera (similarity = 95%). Some of the 42 staphylococci were identical to each other and 10 distinct strains were considered. The 10 distinct strains were grouped into 4 Staphylococcus species, where the predominant species was S. saprophyticus with 4 strains (40%), followed by 3 of S. xylosus strains, (30%), 2 of S. sciuri strains (20%), and 1 of S. piscifermentans strain (10%). A single isolated strain, Staphylococcus saprophyticus bovis KT955013, was identified to the subspecies level (98% similarity; Table 2).

According to previous reports, the predominant species found in Minas Frescal cheese are Staphylococcus equorum, S. lentus, Staphylococcus simulans, S. sciuri, and S. xylosus (Ruaro et al., 2013), but S. saprophyticus, S. epidermidis, in addition to S. equorum, were also found in the dairy products of a small Portuguese ewes (Soares et al., 2011). However, S. equorum, S. xylosus, S. saprophyticus, and S. succinus were the most prevalent species in French cheeses (Coton et al., 2010). In the present study, Staphylococcus equorum, S. lentus, S. simulans, S. epidermidis, and S. succinus were not found in Minas Frescal cheese, but as 3 staphylococci strains were not identified up to the species level, perhaps some of these could be among the species isolated from Minas Frescal cheese. The stability of the biodiversity of the consortia found in Minas Frescal cheese is in accordance with the one already described for red-smeared cheese (Rea et al., 2007), but great diversity among CNS can be found if the microbiota of distinct cheese matrices are compared (Dojranic et al., 2013).

Phylogenetic Relationships of CNS Isolated from Minas Frescal Cheese

The 10 distinct CNS strains isolated from Minas Frescal cheese showed little diversity when compared with staphylococci microbiota from different food matrices (Figure 1), perhaps due to the unique features of the cheese studied. The 10 distinct CNS strains can be grouped in 3 species sets: saprophyticus, sciuri, and simulans. Frequently, the saprophyticus species group includes S. xylosus and S. saprophyticus; the simulans species group comprises S. carnosus and S. piscifermentans; and the sciuri species group contains S. sciuri, S. lentus, and S. pasteuri (Lamers et al., 2012). In the present study, the main cluster comprised S. saprophyticus KT955013, KT955010, KT955007, and S. xylosus KT955011, both grouped with S. piscifermentans KT955006, a species that is phylogenetically related to the simulans species group, as already established. Staphylococcus sciuri KT955004, a species belonging to the sciuri species group, was clustered with S. xylosus KT955012. The third cluster includes S. sciuri JX966436.1, S. xylosus KT955008, and S. saprophyticus KT955005 (Figure 1).

The close similarities between S. sciuri KT955004 and S. xylosus KT955012 as well as S. saprophyticus KT955005 and S. xylosus KT955008 are supported by a bootstrap value of 100%. The interspecies similarities were superior to 90%, which demonstrates the close phylogenies among the CNS strains isolated from Minas Frescal cheese.

Genotypic and Phenotypic Characterization of CNS Strains from Minas Frescal

In the present study, 9 distinct combinations of staphylococcal enterotoxin genes were found in the 10 CNS strains, comprising the SE A to E, G to J, as well as the SEI K to R and U (Table 2). The sea and seb genes showed high prevalence, in which the sea gene was the most predominant, carried by 90% of strains, followed by seb, carried by 70% of the genomes. Genes sec, sed, see, she, and sei showed intermediary prevalence, where see and sec were carried by 60% of the isolated strains, seh and sei were found in 50% of the strains, and sed in 40% of the strains. Finally, seg, selk, selq, sebr, and selu showed low prevalence, being carried by 20% of the CNS strains (Table 2); no tstH1 gene was found.

Although studies on the emerging enterotoxins in Brazilian milk or dairy-derived products have not yet been reported, the frequency of the enterotoxin genes encoding the classical enterotoxins set SEA to SED in Minas Frescal cheeses can be compared with those described for the CNS microbiota of bulk bovine milk from a recent study (de Freitas Guimarães et al., 2013), which found that the sea gene is carried by 35.1% of the strains, seb by 7.1%, and sec by 6.5%. Even considering only the classical enterotoxin genes, Minas Frescal cheese seems to comprise a CNS microbiota with higher
Table 2. Genotypic and phenotypic characterization of CNS strains from Minas Frescal cheese

<table>
<thead>
<tr>
<th>CNS strain</th>
<th>Accession number and similarity (%)</th>
<th>Number of strains found (n = 45)</th>
<th>Presence of enterotoxin genes&lt;sup&gt;2&lt;/sup&gt;</th>
<th>mRNA detection&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Cycle threshold values</th>
<th>mRNA semiquantitative evaluation&lt;sup&gt;4&lt;/sup&gt; (arbitrary units)</th>
<th>Enterotoxin production (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staphylococcus spp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>KT955005(98) 3</td>
<td>sea, sed, seh, sli</td>
<td>sea</td>
<td>31 ± 0.4</td>
<td>1.3</td>
<td>0.55 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>KT955007(98) 5</td>
<td>sea, seb, see, seh, seh, sei</td>
<td>sea, seb, seh</td>
<td>27.0 ± 0.5, 28 ± 0.3, 31 ± 1.0</td>
<td>1.6; 0.8; 4.5</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus bovis</em></td>
<td>KT955013(98) 5</td>
<td>sea, seb, see, seh, seh, sei</td>
<td>sea, seb</td>
<td>19.7 ± 0.7; 26 ± 0.5</td>
<td>1.2; 0.5</td>
<td>0.74 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>KT955010(98) 6</td>
<td>see, seh, sei, sed, seli, sej, selj, selk, selm, seln, selo, selq, selr, selu</td>
<td>see, selr, selu</td>
<td>27.0 ± 1.0</td>
<td>4.8; 0.1; 0.8</td>
<td>0.81 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>KT955008(98) 3</td>
<td>sea, see</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.02 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>KT955011(98) 3</td>
<td>sea, seb, see, seh, seh, sei</td>
<td>sed</td>
<td>27.6 ± 1.0</td>
<td>3.7</td>
<td>1.0 ± 0.03</td>
<td></td>
</tr>
<tr>
<td><em>S. xylosus</em></td>
<td>KT955012(98) 4</td>
<td>sea, seb, see, seh, seh, sei</td>
<td>sea, seb, seh, see, sed, seli, selj, selk, selm, seln, selo, selq, selr, selu</td>
<td>33 ± 0.4; 29 ± 0.1; 33 ± 1.8; 25 ± 0.2; 29.3 ± 1.8; 35 ± 1.0; 34 ± 1.0; 29 ± 0.7</td>
<td>3.6; 2.2; 1.8; 0.7; 1.0; 0.1; 0.1; 1.0</td>
<td>0.71 ± 0.08</td>
<td></td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>KT955004(98) 4</td>
<td>sea, seb, see, seh, seh, sei, sed, seli, selj, selk, selm, seln, selo, selq, selr, selu, tstH1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.12 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>KT955009(98) 3</td>
<td>sea, seb, see, seh, seh, sei</td>
<td>seb</td>
<td>33 ± 0.8</td>
<td>3.2</td>
<td>0.26 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>S. piscifermentans</em></td>
<td>KT955006(98) 3</td>
<td>sea, seb, see, seh, seh, sei</td>
<td>sea, sed</td>
<td>33 ± 1.8; 19 ± 0.4</td>
<td>9.2</td>
<td>0.36 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are displayed as the means ± SD of assays performed in duplicate.<br><sup>2</sup>The presence of enterotoxin genes sea, seb, see, seh, sei, sed, seli, selj, selk, selm, seln, selo, selq, selr, selu, and tstH1 was tested by PCR using specific sets of primers.<br><sup>3</sup>mRNA transcripts for sea, seb, see, seh, sej, seli, selj, selk, selm, seln, selo, selq, selr, selu, tstH1 All enterotoxins genes were evaluated in triplicate by real time RT-PCR tests.<br><sup>4</sup>mRNA semi quantification was performed in arbitrary units in comparison with the mRNA level for the same gene estimated using the reference strains. Messenger rDNA was used to normalize mRNA levels. SEA-SEE enterotoxin production was evaluated by ELISA using a detection kit (RIDASCREEN SET A, B, C, D, E; R-Biopharm AG, Darmstadt, Germany).
Figure 1. Phylogenetic trees generated from the multiple alignment of 16S rDNA sequences from the 42 CNS strains found in Minas frescal cheese. The Clustal X 2.0 software (Larkin et al., 2007) using Mega 6.0 and the unweighted pair group method (UPGMA) were used. Bootstrap values ranged from 0.0 to 0.3.
biodiversity and superantigenic toxin genes and, at the same time, with high phylogenetic relationships between them. This disagreement regarding the CNS microbiota of bulk bovine milk and Minas Frescal cheese may be due to contamination with other staphylococci strains from the environment or human origin acquired during the cheese manufacturing process.

Following the identification of the staphylococcal enterotoxins SEG and SEI by Munson et al. (1998), Monday and Bohach (2001), and Jarraud et al. (2001), it has been reported that the genes encoding these enterotoxins are part of a chromosomal operon, termed the enterotoxin gene cluster (egc), comprising 5 genes designated seI, selm, sei, seln, and seg. Two CNS *S. xylosus* strains in the present study—KT955011 and KT955012—were shown to carry the seg and sei genes, and in 2 strains of *S. saprophyticus*, KT955005 and KT955010, a single sei gene sequence was detected. The presence of part of the genes and not the entire egc operon may be due to the high degree of genetic polymorphism in the chromosomal assembly (Thomas et al., 2006). Subsequently, Letertre et al. (2003) demonstrated that some egc clusters possess an additional gene designated selu. Indeed, the *S. saprophyticus* KT955010 strain carries the selu gene clustered to the sei gene. The selm, seln, and selo genes were not found in the CNS genomes of Minas Frescal cheese strains. The CNS strains clustered in groups near the bottom of the phylogenetic tree carried the classical enterotoxin genes and the species at the top of the tree showed high diversity among enterotoxin genes, combining the presence of classical and the newly described in their genomes (Figures 1 and 2 and Table 2).

To assess the risk of Minas Frescal related to staphylococcal food poisoning, the ability of those strains carrying the se and sel genes in their genome in expressing and producing enterotoxins was evaluated. The mRNA transcripts for each enterotoxin gene was evaluated by real-time reverse transcription PCR assays and the enterotoxin production was estimated by a sandwich enzyme immunoassay for the combined detection of *Staphylococcus* enterotoxins A, B, C, D and E.

Nine CNS strains from Minas Frescal cheese were able to produce the classical enterotoxins SEA, SEB, SEC, SED, and SEE during growth, in concentrations ranging from 0.12 to 1.8 ng/mL (Table 2). Although the sandwich enzyme immunoassay is considered the most sensitive method to detect SEA to SEE enterotoxins, able to detect 0.125 ng/mL, differences in the specificity and sensitivity of the assays for the detection of staphylococcal enterotoxins from foods are expected (Pereira et al., 2009). Only a single strain, *S. saprophyticus* KT955005, was unable to produce SEA to SEE enterotoxins when evaluated by this assay.

The mRNA for at least 1 enterotoxin gene was detected in 7 strains (70%) and, additionally, 5 of them (50%) were able to transcribe multiple enterotoxin genes in vitro. Strains *S. saprophyticus* KT955007, *S. bovis saprophyticus* KT955013, *S. saprophyticus* KT955010, *S. xylosus* KT955012, and *S. piscifermens tans* KT955005 expressed mRNA for multiple se genes. A differential transcription among the se genes was observed, where the most frequently transcribed among the classical were sea and selh, transcribed by 4 strains, and, among the newly described, selk, selr, selu, were transcribed by 2 strains each; no mRNA tstH1 was detected.

No mRNA transcripts were detected in the *S. saprophyticus* KT955005, *S. xylosus* KT955008, or *S. sciuri* KT955004 strains assayed, although the enterotoxins were detected by the enzyme-linked immune-sorbent tests. The genotypic and phenotypic analyses pointed to a high risk of food poisoning in consuming Minas Frescal cheese contaminated by the CNS strains identified in the present study. The food poisoning status of Minas Frescal cheese can be acquired even during cheese manufacturing, because CNS strains are able to express and produce enterotoxins at the early stage of logarithmic growth. The presence of enterotoxigenic strains in a ready-to-eat dairy product is of concern and underlines the need for standardized diagnostic methods to screen for and quantify the presence of classical and also emergent enterotoxins directly in the food matrices.

The use of real-time reverse transcription PCR assays conferred sensitivity and specificity for the determination of the enterotoxin gene transcription, besides the additional ability to evaluate the expression of all enterotoxin genes encoding both the classical and newly described super antigenic enterotoxins. The real-time reverse transcription PCR tests also avoided the false positives that occur in immunoassays by cross-reactions between the classical polyclonal antibodies and the newly described enterotoxins.

**Multiresistance of CNS Strains to Antimicrobial Agents**

The 10 distinct CNS strains (plus *Staphylococcus* spp.) from Minas Frescal cheese showed multiresistance to several antimicrobial agents. Five strains showed multiple antimicrobial resistance (MAR) indexes from 0.73 to 0.93, two strains presented MAR indexes of 0.53 and 0.40, and the remaining 3 strains showed a MAR index of 0.26 (Table 3).

The resistance of the 10 multiresistance of CNS strains (MRCNS) was distributed as follows: 6 strains (60%) were resistant to sulfamethoprim, 5 strains (50%)
to aminoglycosides (ciprofloxacin, neomycin, or gentamicin), 6 strains (60%), to chloramphenicol, 8 strains (80%) to vancomycin, 5 strains (50%) to clindamycin, 10 strains (100%) to β-lactams (oxacillin, penicillin, or cefoxitin), 6 strains (60%) to tetracyclines (tetracycline or rifampicin), 3 strains (30%) to cefepime, 5 strains to erythromycin (50%), and 3 strains (30%) to linezolid (Table 3).

The percentage of MRCNS resistant to β-lactams was superior to that found by Sampimon et al., (2011), which showed that only 14% of the 170 staphylococci from raw milk obtained from various ruminant species were resistant to penicillin. It should be considered that the contamination by resistant strains could occur later, during cheese processing, by improper handling or sanitization of the production line in industrial plants.

Figure 2. Uniplex, duplex and multiplex-PCR screening to detect enterotoxin genes in CNS strains from Minas Frescal cheese. The DNA templates obtained from 11 CNS strains isolated from Minas Frescal cheese were tested by PCR using the primer set targeting for sea, seb, sec, sed, see, seh, sei, selk, selq, selr, and selu genes. (A) Lane M 100 bp = DNA ladder plus (Fermentas, Foster City, CA); lane ATCC 29231 = Staphylococcus aureus ATCC 29231 carrying sea gene; lane ATCC19095 = S. aureus ATCC19095 carrying the sec gene; lane ATCC13563 = S. aureus ATCC 13563 carrying the sed gene; lane ATCC27664 = S. aureus ATCC27664 carrying the see gene. (B) Lane M 100 bp = DNA ladder plus; lane ATCC 19095 = S. aureus ATCC 19095 carrying seg seh sei genes. (C) Lane M 100 bp = DNA ladder plus; lane JX114791.1 = Staphylococcus spp. JX114791.1; lanes KT955005 = Staphylococcus saprophyticus KT955005; lane KT955007 = S. saprophyticus KT955007. (D) Lane M 100 bp = DNA ladder plus; lane KT955007 = S. saprophyticus KT955007; lane KT955004 = Staphylococcus sciuri KT955004; lane KT955009 = S. sciuri KT955009. (E) Lane M 100 bp = DNA ladder plus; lane KT955013 = S. saprophyticus KT955013; lane KT955012 = S. xylosus KT955012. (F) Lane ATCC 14458 = S. aureus ATCC 14458 carrying selk, selq, selr, and selu gene; lanes KT955010 = S. saprophyticus KT955010.
reinforcing the difference between the CNS microbiota from bulk milk and dairy-derived products, as discussed previously.

Surprisingly, the resistance to chloramphenicol was very similar to that estimated for MRCNS strains isolated from human clinical samples (Knauer et al., 2004), reinforcing the fact that the multi-resistance character of the food matrices strains must be taken in consideration when evaluating the safety hazards of food poisoning.

The MRCNS isolated in the present study showed superior resistance to β-lactam compared with other antimicrobials, as all isolates were resistant to at least 1 antimicrobial agent belonging to this class of compounds. Fifty to sixty percent of the strains were resistant to aminoglycosides, clindamycin, ciprofloxacin, and sulfamethoxprim.

To determine the MIC for ampicillin and methicillin, oxacillin, or cefoxitin, all susceptible CNS strains found in Minas Frescal cheese were tested using the macrodilution broth method. The resistant strains were tested against concentrations ranging from 0.03 to 2 mg/mL of each antimicrobial agent.

Concerning methicillin-resistance, *S. saprophyticus* KT955010, *P. piscifermentans* KT955006, *S. saprophyticus* bovis KT955013, and *S. xylosus* KT955012 showed MIC values of 1.0 mg/mL. *Staphylococcus xylosus* KT955012 and *P. piscifermentans* KT955006 strains presented a MIC value of 0.5 mg/mL, whereas *S. sciuri* KT955009 and *S. xylosus* KT955011 strains had a MIC value of 0.06 mg/mL. The remaining strains were susceptible to 0.03 mg/mL of methicillin, ampicillin, and vancomycin.

Concerning resistance against ampicillin, the *S. xylosus* KT955008 strain was shown to be resistant to 0.5 mg/mL, whereas *S. xylosus* KT955012, *S. saprophyticus* bovis KT955013, and *P. piscifermentans* KT955006 were resistant to 0.125 mg/mL, and *S. sciuri* KT955004, *S. xylosus* KT955011, and *S. xylosus* KT955012 were resistant to 0.06 mg/mL. The MIC estimated in our study were in accordance to a previous evaluation of the resistance of *S. aureus* strains isolated from meat, dairy products, and ready-to-eat food (Aydin et al., 2011), which showed MIC values of 0.03 mg/mL for penicillin estimated for 91 isolated strains.

The high resistance observed in the present study for cheese staphylococci can be due to the inappropriate use as growth promoters of some antimicrobial agents, such as oxacillin, vancomycin, chloramphenicol, neomycin, and erythromycin, which are commonly used in veterinary medicine to treat infections (Phillips et al., 2004). Antimicrobial resistance of staphylococci in dairy products could be the result of the incorrect use of penicillin in the treatment and prevention of mastitis in different ruminant animals (Sampimon et al., 2011).

The resistance of the isolated CNS to antibiotics of therapeutic importance has become a public health problem, as the manufacturing processes of Minas Frescal cheese cannot kill the strains or inactivate the toxins. This makes an apparently safe food the cause of serious gastroenteritis with the ability to disseminate pathogens with multiresistance to antibiotic therapy.

### Table 3. Multiple antimicrobial resistance of CNS strains found in Minas Frescal cheese

<table>
<thead>
<tr>
<th>CNS strain</th>
<th>Antimicrobial agent resistance</th>
<th>Multiple antimicrobial resistance (MAR) index</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus spp.</em></td>
<td>SXT, CIP, NEO, GEN, CLO, VAN, CLI, TET, CPM, OXA, PEN, CFO, ERI, LZD</td>
<td>0.93</td>
</tr>
<tr>
<td><em>S. saprophyticus</em> KT955005</td>
<td>SXT, CIP, NEO, GEN, CLO, VAN, CLI, TET, CPM, OXA, PEN, CFO, ERI, LZD</td>
<td>0.93</td>
</tr>
<tr>
<td><em>S. saprophyticus</em> KT955007</td>
<td>CIP, NEO, GEN, CLO, VAN, OXA, PEN, CFO, ERI, LZD</td>
<td>0.53</td>
</tr>
<tr>
<td><em>S. sciuri</em> KT955004</td>
<td>OXA, PEN, TET, ERI</td>
<td>0.26</td>
</tr>
<tr>
<td><em>S. sciuri</em> KT955009</td>
<td>SXT, VAN, OXA, PEN</td>
<td>0.26</td>
</tr>
<tr>
<td><em>S. saprophyticus</em> KT955013</td>
<td>CL0, VAN, OXA, PEN, CFO, ERI</td>
<td>0.40</td>
</tr>
<tr>
<td><em>S. piscifermentans</em> KT955006</td>
<td>SXT, CLO, CLI, TET, RIF, OXA, PEN</td>
<td>0.53</td>
</tr>
<tr>
<td><em>S. xylosus</em> KT955011</td>
<td>SXT, CIP, NEO, CLO, VAN, CLI, TET, RIF, CPM, OXA, PEN, CFO, ERI, LZD</td>
<td>0.93</td>
</tr>
<tr>
<td><em>S. xylosus</em> KT955008</td>
<td>SXT, CIP, NEO, CLO, VAN, CLI, TET, RIF, CPM, OXA, PEN, CFO, ERI, LZD</td>
<td>0.86</td>
</tr>
<tr>
<td><em>S. saprophyticus</em> KT955010</td>
<td>SXT, CIP, NEO, GEN, CLO, VAN, CLI, RIF, OXA, PEN, CFO</td>
<td>0.73</td>
</tr>
</tbody>
</table>

1SXT = sulfamethoprim; CIP = ciprofloxacin; NEO = neomycin; GEN = gentamicin; CLO = chloramphenicol; CLI = clindamycin; VAN = vancomycin; TET = tetracycline; RIF = rifampicin; CPM = cephalaxin; OXA = oxacillin; PEN = penicillin; CFO = cefoxitin; ERI = erythromycin; LZD = linezolid.

2The MAR index of an isolate is defined as a/b, where a represents the number of antimicrobials to which the isolate was resistant to and b represents the number of antimicrobials to which the isolate was subjected. *Staphylococcus aureus* strains ATCC WB81 (*sea*), ATCC 13563 (*sed*), ATCC 27664 (*see*) with MAR index = 0.5 and *S. aureus* strains ATCC14458 (*seb*), ATCCWB72 (*see*) and *S. xylosus* ATCC 29971 with MAR index = 0.3 were used as reference strains.
The safety of Minas Frescal cheese consumption could also be enhanced by the inclusion of a microbial barrier, such as the inclusion of probiotic strains producing natural antibiotics, competitive flora, or even the addition of natural bioagents against spoilage or pathogenic microorganisms. In situ bacteriocin production by lactic acid bacteria, acting as adjunct or protective culture, could be an effective strategy to avoid staphylococcus food poisoning in Minas Frescal cheese. The use of a probiotic bacteria in the cheese processing should enhance the cost of the product and at the same time add value to the cheese, as it becomes richer in organoleptic and nutritional properties but can be priced as being naturally preserved.

**CONCLUSIONS**

The staphylococci contamination of Minas Frescal cheese can occur by inadequate manufacturing practices or inadequate storage conditions, use of contaminated milk, or even due to the physico-chemical features of the cheese matrix favorable to the growth of these microorganisms. In addition, the CNS strains are also able to produce enterotoxins. The additional safety hazards associated with these strains mostly include antimicrobial resistance to the compounds commonly used to treat staphylococci infections. Antimicrobial resistance of CNS strains from food matrices can spread in populations by the consumption of an apparently safe food. The detection and quantification of enterotoxins in cheese matrices produced by those CNS strains should be evaluated.

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**REFERENCES**


