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/see*, *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 enterotoxigenic character of the CNS strains, which expressed and produced the enterotoxins in vitro. The CNS strains showed multi-resistance 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-

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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; Maiques 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 (**SEI**) and showed no emetic activity in primate models or have not yet been tested (Lina et al., 2004). To date, 21 SE and SEI 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, SEI, SER, SES, and SET) and 10 new types of SEI (SEIJ, SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, SEIQ, SEIU, and SEIV) 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 (Brasil, 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 prod-

uct 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 (Cichoski 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/dfbmd/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 phylogenic 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 (A_w) 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 serially diluted from 10^{-6} to 10^{-1} and 100 μ L of each dilution was inoculated onto 20 mL of Baird-Parker agar containing egg yolk tellurite emulsion (BPA β RPF, bioMérieux, Marcy l'Etoile, France) and incubated at 37°C for 24 h. Conventional microbiology procedures were performed following instructions to the Bergey Manual of Systematic Bacteriology (Garritty 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 $5,700 \times g$ 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 μ L of primer set of forward and reverse 16S rDNA (Table 1) in a final volume of 20 μ L. 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 strain 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 Sequences, Target Genes, and Species.

Primer sets flanking the *sea*, *seb*, *sec*, *sed*, *see*, *selg*, *selh*, *seli*, *selk*, *selm*, *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 *see*, *selg*, *selh*, *seli*, *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*, *selg*, *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*,

Table 1. Primers for the V5 region of the 16S rDNA sequencing and PCR or real time reverse transcription-PCR tests targeting the classical and newly described staphylococcal enterotoxin genes

Item ¹	Sequence (5'–3')	Gene	Amplicon (bp)	Reference
SEA forward	TTGGAAACGGTTAAACGAA	<i>sea</i>	120	Johnson et al., 1991
SEA reverse	GAACCTTCCCATCAAAAACA			
SEB forward	TGCGATCAAACGTGACAAACG	<i>seb</i>	478	Johnson et al., 1991
SEB reverse	GCAGGTACTCTATAAGTGCC			
SEC forward	GACATAAAAGCTAGGAATTT	<i>sec</i>	257	Johnson et al., 1991
SEC reverse	AAATCGGATTAACATTATCC			
SED forward	CTAGTTTGGTAATATCTCCT	<i>sed</i>	317	Johnson et al., 1991
SED reverse	TAATGCTATATCTTATAGGG			
SEE forward	TAGATAAAGTTAAAACAAGC	<i>see</i>	170	Johnson et al., 1991
SEE reverse	TAACTTACCGTGGACCCCTC			
SEG forward	TGCTATCGACACACTACAACC	<i>seg</i>	704	McLauchlin et al., 2000
SEG reverse	CCAGATTCAAATGCAGAACC			
SEH forward	CGAAAGCAGAAGATTTACACG	<i>seh</i>	495	McLauchlin et al., 2000
SEH reverse	GACCTTTACTTATTTGCGTGTG			
SEI forward	GACAACAAAACGTGCGAAACTG	<i>sei</i>	630	McLauchlin et al., 2000
SEI reverse	CCATATCTTTGCGCTTACCAG			
SEJ forward	CAGCGATAGCAAAAATGAAACA	<i>selj</i>	426	Rosec and Gigaud., 2002
SEJ reverse	TCTAGCGGAACAACAGTTCTGA			
SEM forward	CCAATTGAAGACCACCAAAG	<i>selm</i>	517	Omoe et al., 2005
SEM reverse	CTTGTCCTGTTCCAGTATCA			
SEN forward	ATTGTTCTACATAGCTGCAA	<i>seln</i>	682	Omoe et al., 2005
SEN reverse	TTGAAAAAACTCTGCTCCCA			
SEO forward	AGTCAAGTGTAGACCCTATT	<i>selo</i>	534	Omoe et al., 2005
SEO reverse	TATGCTCCGAATGAGAATGA			
SEK forward	ATGAATCTTATGATTTAATTTTCAAGATCAA	<i>selk</i>	545	Sergeev et al., 2004
SEK reverse	ATTTATATCGTTTCTTTATAAGAAATATCG			
SELQ forward	GGAAAATACACTTTATATTCACAGTTTCA	<i>selq</i>	539	Sergeev et al., 2004
SELQ reverse	ATTTATTCAGTTTCTCATATGAAATCTC			
SELR forward	AATGGCTCTAAAATTGATGG	<i>selr</i>	363	Holtfreter et al., 2007
SELR reverse	TCTTGTAACCGTAACCGTTTT			
SELU forward	AATGGCTCTAAAATTGATGG	<i>selu</i>	215	Holtfreter et al., 2007
SELU reverse	ATTTGATTTCCATCATGCTC			
TSTH1 forward	ATGGCAGCATCAGCTTGATA	<i>tstH1</i>	350	Johnson et al., 1991
TSTH1 reverse	TTTCCAATAACCACCGTTT			
16S rDNA forward	ATA AGA CTG GGA TAA CTT CGG G	<i>16SrDNA</i>	500	Mason et al., 2001
16S rDNA reverse	CTT TGA GTT TCA ACC TTG CGG TCG			

¹Se = enterotoxins; SEI = SE-like toxins.

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 90s, 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 cultured overnight aerobically in 10 mL of brain heart infusion broth (BD BBL) at 37°C for 72 h. Bacteria supernatants were collected by centrifugation at $4,000 \times g$ 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 RiboPure 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 (Applied Biosystems) as follows: 4.0 µg of RNA, 2.0 µL of 10× reverse transcription (RT) buffer, 0.8 µL of dNTP mix (100 mM), 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*, *selj*, *selm*, *seln*, *selo*, *selq*, *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 enterotoxin 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 Committee 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), cefetoxin (30 µg), rifampicin (5 µg), erythromycin (15 µg), tetracycline (30 µg), vancomycin (30 µg), ciprofloxacin (5 µg), sulfazothrim (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 (NCCLS, 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-smear cheese (Rea et al., 2007), but great diversity among CNS can be found if the microbiota of distinct cheese matrices are compared (Dobranic 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. pasteurii* (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*, *selr*, 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 cheese¹

CNS strain			Characterization				Phenotypic
Accession number and similarity (%)	Number of strains found (n = 45)	Presence of enterotoxin genes ²	mRNA detection ³	Cycle threshold values	mRNA semiquantitative evaluation ⁴ (arbitrary units)		
<i>Staphylococcus</i> spp.	(95)	3	<i>sea, sed, seh, sli</i>	<i>sea</i>	31 ± 0.4	1.3	0.55 ± 0.01
	KT955005(98)	5	<i>sea, sec, sed, see, seh, sei</i>	—	—	—	1.8 ± 0.04
<i>S. saprophyticus</i>	KT955007(98)	6	<i>sea, seb, see, sch</i>	<i>sea, seb, seh</i>	27.0 ± 0.5; 28 ± 0.3; 31 ± 1.0	1.6; 0.8; 4.5	—
	KT955013(98)	5	<i>sea, seb, sec</i>	<i>sea, seb</i>	19.7 ± 0.7; 26 ± 0.5	1.2; 0.5	0.74 ± 0.01
	KT955010(98)	6	<i>see, seh, sei, selk, selq, selr, selu</i>	<i>see, selr, selu</i>	—	4.8; 0.1; 0.8	0.81 ± 0.07
<i>S. xyloso</i>	KT955008(98)	3	<i>sea, see</i>	—	—	—	1.02 ± 0.05
	KT955011(98)	3	<i>sea, seb, sec, sed, see, seg, seh, sei</i>	<i>sed</i>	27.6 ± 1.0	3.7	1.0 ± 0.03
	KT955012(98)	4	<i>sea, seb, sec, sed, see, seg, sei, selk, selq, selr, selu</i>	<i>sea, seb, sec, sed, see, selk, selr, selu</i>	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	3.6; 2.2; 1.8; 0.7; 1.0; 0.1; 0.1; 1.0	0.71 ± 0.08
<i>S. sciuri</i>	KT955004(98)	4	<i>sea, seb</i>	—	—	—	0.12 ± 0.01
	KT955009(98)	3	<i>sea, seb, sec</i>	<i>seb</i>	33 ± 0.8	3.2	0.26 ± 0.01
	KT955006(98)	3	<i>sea, seb, sec, sed, seh, sei</i>	<i>sea, sed</i>	33.16 ± 1.8; 19 ± 0.4	9.2	0.36 ± 0.02

¹Values are displayed as the means ± SD of assays performed in duplicate.²The presence of enterotoxin genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selk*, *selm*, *seln*, *selo*, *selq*, *selr*, *selu*, and *tstH1* was tested by PCR using specific sets of primers.³mRNA transcripts for *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *selk*, *selm*, *seln*, *selo*, *selq*, *selr*, *selu*, *tstH1* All enterotoxins genes were evaluated in triplicate by real time RT-PCR tests.⁴mRNA 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 *selo*, *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. piscifermentans* 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 *seb*, 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*, *seg*, *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 JX114799.1 = *Staphylococcus* spp. JX114799.1; lanes KT955005 = *Staphylococcus saprophyticus* KT955005; lane KT955008 = *Staphylococcus xylosus* KT955008; lane KT955007 = *S. saprophyticus* KT955007. (D) Lane M 100 bp = DNA ladder plus; lane KT955013 = *S. saprophyticus* KT955013; lane KT955012 = *S. xylosus* KT955012. (E) Lane M 100 bp = DNA ladder plus; lane KT955010 = *S. saprophyticus* KT955010. (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 sulfamethoprim.

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, *S. piscifermentans* KT955006, *S. saprophyticus bovis* KT955013, and *S. xylosus* KT955012 showed MIC values of 1.0 mg/mL. *Staphylococcus xylosus* KT955012 and *S. 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 *S. 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

CNS strain	Antimicrobial agent resistance ¹	Multiple antimicrobial resistance (MAR) index ²
<i>Staphylococcus</i> spp.	SXT, CIP, NEO, GEN, CLO, VAN, CLI, TET, CPM, OXA, PEN, CFO, ERI, LZD	0.93
<i>S. saprophyticus</i> KT955005	SXT, CIP, NEO, GEN, CLO, VAN, CLI, RIF, CPM, OXA, PEN, CFO, ERI, LZD	0.93
<i>S. saprophyticus</i> KT955007	CIP, NEO, GEN, CLO, VAN, OXA, PEN, CFO	0.53
<i>S. sciuri</i> KT955004	OXA, PEN, TET, ERI	0.26
<i>S. sciuri</i> KT955009	SXT, VAN, OXA, PEN	0.26
<i>S. saprophyticus</i> KT955013	CLO, VAN, OXA, PEN, CFO, ERI	0.40
<i>S. piscifermentans</i> KT955006	SXT, CLO, VAN, CLI, TET, RIF, OXA, PEN	0.53
<i>S. xylosus</i> KT955011	SXT, CIP, NEO, CLO, VAN, CLI, TET, RIF, CPM, OXA, PEN, CFO, ERI, LZD	0.93
<i>S. xylosus</i> KT955008	SXT, CIP, NEO, CFO	0.26
<i>S. xylosus</i> KT955012	CIP, NEO, VAN, CLI, TET, RIF, CPM, OXA, PEN, CFO, ERI, LZD	0.86
<i>S. saprophyticus</i> KT955010	SXT, CIP, NEO, GEN, CLO, VAN, CLI, RIF, OXA, PEN, CFO	0.73

¹SXT = sulfamethoprim; CIP = ciprofloxacin; NEO = neomycin; GEN = gentamicin; CLO = chloramphenicol; CLI = clindamycin; VAN = vancomycin; TET = tetracycline; RIF = rifampicin; CPM = cefepime; 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 to. *Staphylococcus aureus* strains ATCC WB81 (*sea*), ATCC 13563 (*sed*), ATCC 27664 (*see*) with MAR index = 0.5 and *S. aureus* strains ATCC14458 (*seb*), ATCCWB72 (*sec*) 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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