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Biodiversity and characterization of *Staphylococcus* species isolated from a small manufacturing dairy plant in Portugal

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ABSTRACT

The level and the diversity of the staphylococcal community occurring in the environment and dairy products of a small manufacturing dairy plant were investigated. Species identification was performed using different molecular methods, *viz*. Multiplex-PCR, amplified ribosomal DNA restriction analysis (ARDRA), and *sodA* gene sequencing. The main species encountered corresponded to *Staphylococcus equorum* (41 isolates, 39.0%), *S. saprophyticus* (28 isolates, 26.7%) and *S. epidermidis* (15 isolates, 14.3%). Additionally, low incidence of enterotoxin genes was obtained, with only 9 strains (8.6%) being positive for one or more toxin genes. With regard to antimicrobial resistance, 57.1% of the isolates showed at least resistance against one antibiotic, and 28.6% were multi-resistant, which might accomplish resistance for up to 6 antibiotics simultaneously. These results provided evidence that the presence of *Staphylococcus* species in dairy environment are mostly represented by *S. equorum* and *S. saprophyticus*, and illustrate that carrying antimicrobial resistance genes has become reasonably widespread in cheese and dairy environment.

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1. Introduction

The species of the *Staphylococcus* genus are ubiquitously disseminated in the environment, with a number of species inhabiting specific ecological niches. They are found living naturally on the skin and mucous membranes of warm-blooded animals and humans, which generally imply a commensal or symbiotic relationship with their host. Staphylococci are also isolated from a wide range of foodstuff such as meat, cheese and milk, and from environmental sources such as soil, sand, air and water (Heikens et al., 2005; Kloos and Schleifer, 1986). It was demonstrated that some strains have important technological value, namely, *Staphylococcus xylosus*, *S. carnosus*, and *S. equorum*—all included in the coagulase-negative staphylococci (CNS) group. In particular situations, some species can represent a medical risk, especially if they enter into the host tissue through skin traumas barrier, like inoculation by needles, or implantation of medical devices. Thus, in the last decades, staphylococci have also emerged as important and potential pathogens, mainly in immunocompromised patients, premature newborns, and patients with implanted biomaterials (Heikens et al., 2005). *S. aureus* is a major human pathogen that causes a wide range of diseases. In addition to external lesions and systemic infections, *S. aureus* is also responsible for toxin-mediated diseases, such as toxic shock syndrome (TSS) and staphylococcal food poisoning (SFP) (Blaiotta et al., 2006; Resch et al., 2008). Therefore, they pose a health risk not only for humans, but also as etiological agents of mastitis in veterinary medicine. The exact distinction between clinical importance, pathogenic and/or contaminating isolates is sometimes problematic and complex (Piette and Verschraegen, 2009). However, since staphylococci are widespread, it has become increasingly imperative to exactly identify them at the genus and species level for general public health. Moreover, the high prevalence of staphylococci, mostly from fermented foodstuffs, and their pathogenic potential has been reported, which are both of a major concern in food context (Coton et al., 2010; Even et al., 2010; Irlinger, 2008). Accurate measurement of the impact, sources, transmission mechanisms and control options for the staphylococcal species are also important in the food chain, since control strategies for staphylococci are largely unknown (Zadoks and Watts, 2008).

The aim of the present study was the characterization of the staphylococcal microflora isolated from a small cheese dairy plant. The characterization was based on the staphylococci enumeration from cheese and dairy environment, molecular differentiation of the wild isolates, antibiotic susceptibility profile, detection of 19 kinds of superantigen (SAg), namely, 9 staphylococcal enterotoxins (SEs) (SEA-E, SEG-I and SER) and 9 staphylococcal enterotoxin-like (SEl) (SElJ-Q and SElU) and also the toxic shock syndrome toxin (TSST-1), and on the presence of other virulence factors.

2. Materials and methods

2.1. Sampling procedure and microorganism isolation

Samples were collected from a manufacturing cheese plant located at "Herdade dos Esquerdos". This unit is located in the center-east

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(Monforte) area of Portugal. The samples were collected at different stages of cheese manufacture, considering sampling from different sources, *viz.* cheese maker hands, milk from the cooling (milking room) and storage (cheese plant) tanks, cheese making vat, rectangular stainless steel table (where the curd is pressed and moulded), moulds, ripening chambers (ground, wall, and shelves), support and brush of the washing machine, packaging machine, and soft cheese (Prado) throughout the ripening period. Additionally, samples were also taken from cheeses with visual defects, mainly, soft (Prado) and semi-soft categories (Nisa and Merendeira de Nisa), with 30 days of maturation. The batches were manufactured from raw ewe's milk, without the addition of starter culture and using traditional methods.

Surfaces and equipments were sampled during processing and after the cleaning and disinfection procedure following the International Organization for Standardization (ISO 18593:2004). After processing, the unit was cleaned and decontaminated with an alkaline chlorinated (4.5%, v/v) solution. The surfaces were sampled by swabbing a surface of 100 cm^2. The microorganisms were enumerated by spread plating in Baird Parker medium (BPM, Lab M, Lancashire, UK) at 37 °C for 48 h. For each sampling point, 5 to 10 colonies on plates of BPM were randomly selected by using a Harrison disc (Harrigan and McCance, 1976) and plated on Brain Heart Infusion agar (Difco, Detroit, USA). The strains were preliminary screened by Gram staining and catalase test and stored at −80 °C in Nutrient broth (Biokar Diagnostics, Beauvais, France) containing 30% of sterile glycerol until further analysis. A total of 119 isolates were obtained for further characterization.

2.2. DNA preparation

DNA template was isolated by the guanidine–isothiocyanate extraction method as described by Cuny and Witte (1996).

2.3. Reference strains

The type strains used in this study were: *S. lentus* LMG 19109, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 14990, *S. capitis* subsp. *capitis* ATCC 27840, *S. hominis* subsp. *hominis* ATCC 27844 *S. lugdunensis* ATCC 43809, *S. haemolyticus* ATCC 29970, *S. chromogenes* LMG 19102, *S. caprae* LMG 19123, *S. xylosus* ATCC 29971, *S. warneri* ATCC 27836, *S. hyicus* LMG 19101, *S. saprophyticus* subsp. *saprohpyticus* ATCC 15305, *S. sciuri* subsp. *sciuri* ATCC 29062, *S. simulans* ATCC 11631, *S. equorum* ATCC 43958, *Micrococcus luteus* ATCC 10240, and *M. lylae* ATCC 27569.

In addition, as positive controls for SAg genes, detection from the type strains of *S. aureus* were used as follows: R5371/00 (SEA, SEG, SEH, SEI, SElM, SElN, SElO, SElU and TSST-1), R5460/00 (SEB, SEG, SEH, SElM, SElN, SElO, SElU and TSST-1), FRI 472 (SED, SEG, SElJ, SElM, SElN, SElO and SER), 3169 (SEC, SED, SElJ, SElL, SElO, SER and TSST-1), and FRI 913 (SEA, SEC, SEE, SElL, SElK, SElQ and TSST-1) obtained by Prof. A. Løvseth (Section of Feed and Food Microbiology, National Veterinary Institute, Ullevålsveien, Oslo, Norway).

2.4. Staphylococcus identification

2.4.1. Multiplex-PCR

The total collection of the isolates obtained was submitted to Multiplex-PCR in two separate PCR runs. Amplifications were performed form each isolate with the primers Tstag422/Tstag765 (Martineau et al., 2001), Sap1/Sap2, STAA-AuI/STAA-AuII, STAE-EpF/STAE-EpR, STAS-SiI/STAS-SiII, STAH-HyI/STAH-HyII and STAC-ChrI/STAC-ChrII (Forsman et al., 1997) allowing the identification of the *Staphylococcus* genus in the two mixes and of the *S. saprophyticus*, *S. aureus* and *S. epidermidis* species in mix 1, and *S. simulans*, *S. hyicus* and *S. chromogenes* species in mix 2, respectively.

Previously, a 1226-bp fragment was amplified with St210 (5′-CCCGGAGGAAGAGAAAGAA-3′) and St1436 (5′-GGAATATCAACCTGT-TATCCATCG-3′) universal primers from the 23S rRNA gene and used as PCR internal control for assessing the DNA quality. Multiplex-PCR reactions were performed in a final volume of 10 μL according to the following conditions: 50 ng of the template DNA, 5 μL of 10× Multiplex Master Mix (QIAGEN), and 1 μL of primer mixture 1 or 2 as appropriated (0.5 μM of each primer). PCR amplifications were performed in a DNA thermal cycler Techne TC-512 (Cambridge, UK). Amplification cycles included an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s, extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The amplified PCR products were resolved by electrophoresis in a 2% agarose (Invitrogen, Paisley, UK) gel at 150 V for 60 min and visualized by ethidium bromide staining.

2.4.2. ARDRA

The 56 *Staphylococcus* spp. isolates unidentified by the Multiplex-PCR were submitted to ARDRA analysis. Two universal primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1436R (5′-GGAATATCAACCTGT-TATCCATCG-3′) were used to amplify a 3.5 kb fragment containing the 16S rRNA gene, 16S–23S intergenic spacer region, and about 1436 bp of the 23S rRNA gene. Amplification reactions were performed in a total volume of 15 μL containing: 100 ng of DNA solution, 0.75 U TaKaRa (TaKaRa Bio, Shiga, Japan), 7.5 μL 2× GC buffer I, 2.5 mM of each dNTP, 0.2 μM of each primer and adjusted to the final volume with water. The PCR mixtures were denatured (3 min at 95 °C) and then subjected to 30 cycles of amplification (30 s at 94 °C, 6 min at 57 °C, and 5 min at 72 °C) plus one additional cycle at 72 °C for 10 min. Amplification products were analysed on 1% (w/v) agarose gel. For restriction digests, 6–12 μL of each amplicon was digested with 5 U of *Vsp*I, *Pvu*II, *Ssp*I, *Xmn*I, *Eco*RV and *Hind*III (Fermentas, York, UK) endonucleases for 12 h at 37 °C followed by enzyme inactivation at 80 °C for 20 min. Restriction fragment patterns were separated by electrophoresis on 1% agarose gel at 150 V for 2 h and visualized by ethidium bromide staining.

2.4.3. sodA amplification and sequencing

Complementary identification of one representative isolate previously identified by Multiplex or ARDRA and the 11 unidentified isolates was performed by *sodA* gene sequencing as described by Poyart et al. (2001). PCR amplifications were performed in a final volume of 50 μL containing 300 ng of DNA template, 0.7 μM each primer, and 25 μL NZYTaq 2× Green Master Mix (NZYTech). The PCR mixtures were denatured (3 min at 95 °C) and then subjected to 30 cycles of amplification (40 s at 95 °C, 80 s at 40 °C, and 60 s at 72 °C) plus a final chain elongation cycle at 72 °C for 5 min. PCR products were resolved by electrophoresis on a 1% agarose gel stained by ethidium bromide, and purified by using the ExoSAP-IT (USB) and directly sequenced with the *d*1 and *d*2 primers. Sequence comparisons against international databanks were performed using BLAST (Altschul et al., 1990).

The combination of these methods allowed the identification of nine species (Table 1), reflecting the complex microflora of these cheese dairy plant.

2.5. Multiplex-PCR for detection of SAg genes

The primers used to detect the SAg genes and Multiplex-PCR reactions were prepared as described by Hwang et al. (2007).

2.6. Antibiotic susceptibility test

Antimicrobial susceptibility testing were performed by the agar dilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 2002) using a multipoint inoculator MastScan (SCAN 400, 2003). The minimum inhibitory concentrations (MICs) of the following antimicrobial agents and dilution range (in μg/mL) were determined: ampicillin (0.125–16), erythromycin (0.125–32), gentamycin (0.125–64), penicillin G (0.125–16), neomycin (0.25–16), tetracycline (0.125–16), cephalexin (0.25–32), and oxacillin

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