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Is staphylococci population from milk of healthy goats safe?

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ABSTRACT

The aim of this study was to assess the species and the genetic diversity of the staphylococci population in raw milk from healthy goats. Isolates representative of all genotypes were screened for their potential pathogenicity by the occurrence of some relevant safety-related properties, such as antibiotic resistance, presence of virulence factor genes, biofilm formation ability and biogenic amine production. A total of 314 staphylococci were isolated, and randomly amplified polymorphic DNA-PCR analysis displayed 48 genotypes. Isolates were identified as belonging to *S. epidermidis* (87.5%), *S. caprae* (6.2%), *S. aureus* (4.2%) and *S. simulans* (2.1%) species.
The antibiotic resistance varied strongly with strains, with *S. epidermidis* and *S. aureus* strains showing resistance to more number of antibiotics. A high occurrence of strains harbouring hemolysin genes was also found in both species. On the contrary, none of the strains assayed harboured enterotoxin or amino acid decarboxylase genes, and, although a moderate or high biofilm formation was observed in 29% of the strains, they did not harbour *ica*A or *ica*D genes. This study gives a first and extensive picture of safety-related properties within *Staphylococcus* species isolated from milk of healthy goats, displaying that these species can act as a reservoir for spreading genes related to safety.

1. Introduction

Staphylococci are ubiquitously distributed bacteria, living naturally on the skin and in mucous membranes of warm-blooded animals and humans, but they have also been isolated from a wide range of foodstuffs, such as fermented meat, milk and cheese (Irlinger, 2008; Soares et al., 2011) and from environmental sources, such as soil, sand, air and water (Kloos and Scheleifer, 1986). *Staphylococcus* (*S.*) species can be coagulase-negative (CNS) or coagulase-positive (CPS), and, between these CPS, *S. aureus* is the most relevant since its presence has been associated with food intoxication and human and animal infections; therefore, its occurrence in foods has been extensively analysed (Song et al., 2015; Spanu et al., 2013). On the contrary, the presence of some CNS, such as *S. xylosus*, *S. carnosus* and *S. equorum,* in fermented foods has been reported to be interesting since they may contribute positively to the development of flavour and aroma (Irlinger, 2008) as a consequence of the production of extracellular enzymes. Therefore, they have been used as starter cultures for meat fermentations (Rantsiou et al., 2005). However, some of these species can also be potentially pathogenic because of their enterotoxigenic capacity (Zell et al., 2008), multidrug-resistance (Resch et al., 2008) and aminobiogenic capacity (Landeta et al., 2007). Furthermore, some authors (Irlinger, 2008; Novakova et al., 2006) have reported nosocomial infections caused by CNS in patients with depressed immune systems. These facts display the relevance of determining the presence of CNS in foods and of analysing certain safety aspects in these isolates in order to confirm their "Qualified Presumption of Safety" as recommended by the European Food Safety Authority (EFSA, 2004). The detection of transmissible antibiotic resistance markers in strains from raw food is regarded as an issue of great concern because the strains can act to spread these markers through the dairy food chain (Viridis et al., 2010).

Analyses of technological and safety-related properties in the staphylococcal community from foods, including milk and milk products, have been reported (Essid et al., 2007; Landeta et al., 2013; Ruaro et al., 2013; Viridis et al., 2010).

The aims of this study were to determine the species and the genetic diversity of the staphylococcal population in raw milk from healthy goats. Isolates representative of all genotypes were assessed for their potential pathogenicity by the occurrence of some relevant safety-related properties, such as antibiotic resistance, presence of virulence factor genes, biofilm formation capacity and biogenic amine production. Both phenotypic and molecular methods were implemented in this study.

2. Materials and methods

2.1. Sampling and bacterial isolation

Goat milk samples were obtained from an intensive livestock farm located southwest of the city of Toledo, Spain. This farm had a barn with around 1200 Murciano-Granadina breed goats. The animals were in good health and were fed at a flat rate of 0.8–1.0 kg/d with a

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commercial concentrate consisting of barley, wheat bran, soy hull meal, sunflower seed extract, molasses, calcium carbonate, wheat flour, carob flour, toasted soy extract, corn gluten feed and sodium chloride with 13.5% protein, 10,000 IU/kg vitamin A, 2500 IU/kg vitamin D3 and an adequate mineral mixture. Alfalfa (0.5 kg) and grass silage (0.5 kg) were also added to this concentrate. No antibiotics were administered. The goats were allocated indoor space with free access to an open yard.

A total of 80 milk samples were taken from healthy goats. Before sampling, nipples were cleaned as described by Jiménez et al. (2013). Samples were taken by manual expression using sterile gloves and recovered in a sterile tube. The first drops (~1 mL) were discarded. The samples were maintained at 4 °C while being transported to the laboratory and analysed within 4 h.

The samples were serially diluted in sterile saline solution, plated in duplicate on Baird-Parker Agar (BPA, Scharlab, Barcelona, Spain) and incubated aerobically at 37 °C for 48 h. Counts were expressed as colony forming units (cfu) per millilitre of milk. From countable plates between three and six colonies were randomly picked and purified by sub-culturing onto Tryptic Soy Agar (TSA, Scharlab). Pure cultures were Gram stained and tested for catalase production before to be grown in Triptic Soy Broth (TSB, Scharlab) and stored at −80 °C with 20% (v/v) glycerol (Panreac, Barcelona, Spain).

2.2. Genotyping of isolates by randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis

Genomic DNA was obtained from well-developed single colonies on TSA following the procedure described by Rodas et al. (2003). To genotype isolates, RAPD-PCR analysis using the M13 primer (5′-GAGGGTGGCGGTTCT-3′; Integrated DNA Technologies, Inc., Coralville, USA) was performed following the procedure described by Ruiz et al. (2008). A reproducibility study to determine the minimum percentage of similarity (r) necessary for strain discrimination was also carried out as described by those authors.

RAPD-PCR gels were photographed with a KODAK DC290 Zoom Digital Camera (Eastman Kodak Company, Rochester, New York, USA). The patterns were normalised and further processed using GelCompar version 4.0 analysis software (Applied Maths, Kortrijk, Belgium). Isolates were grouped using the Pearson product–moment correlation coefficient and cluster analysis by unweighted pair group method with arithmetic average (UPGMA).

2.3. Identification of isolates

Representative isolates from clusters obtained in the numerical analysis of RAPD-PCR patterns were identified at the species level by multiplex PCR based on the *tuf* gene (Martineau et al., 2001). Primers *tuf-g* (5′-GGTGTACCAGCATTAGT-3′), *tuf-a* (5′-TTCAGTATGTGGTGTAA-3′) and *tuf-e* (5′-TTCGTGCATACCGATGA-3′) (Fisher Scientific) and conditions reported by Jiménez et al. (2008) were used. The primer pairs *tuf-g*/*tuf-e* and *tuf-g*/*tuf-a* result in a 370 bp *S. epidermidis* species-specific fragment and a 530 bp *S. aureus* species-specific fragment, respectively. The reference strains used in this study were *S. epidermidis* 232^T and *S. aureus* 86^T from the Spanish Type Culture Collection (CECT).

Isolates not identified using multiplex PCR were analysed by MALDI-TOF mass spectrometry by Probisearch SL (Fundación Parque Científico de Madrid, Spain).

2.4. Antibiotic resistance assays

Antibiotic resistance was assayed using both phenotypic and molecular analysis. In phenotypic analysis, resistance to 11 antimicrobials was determined using antibiotic discs (Bio-Rad, Mares-la-Coquette, France). Pharmacological classes and specific antibiotics employed in this study were β-lactams (penicillin: 10 μg per disc, ampicillin: 10 μg per disc), glycopeptides (vancomycin: 30 μg per disc), tetracycline (30 μg per disc), aminoglycosides (streptomycin: 10 μg per disc, gentamicin: 10 μg per disc), chloramphenicol (30 μg per disc), macrolides (erythromycin: 15 μg per disc), quinolones (ciprofloxacin: 5 μg per disc), lincosamides (clindamycin: 2 μg per disc) and nitrofurans (nitrofurantoin: 300 μg per disc).

Cells from overnight cultures in Tryptic Soy Broth (TSB, Scharlab) were recovered by centrifugation (10,000 ×*g*, 5 min, 4 °C) and suspended in saline solution until $OD_{600} = 0.5$. Mueller-Hinton agar (Scharlab) plates were seeded with this suspension and, five min later, the antibiotic discs were placed onto the surface of the agar.

After incubation at 37 °C for 24 h, the diameter of inhibition halos around the discs was measured. Strains were classified as sensitive, intermediate or resistant according to the breakpoints recommended by the Clinical and Laboratory Standards Institute (2012). Analyses were carried out in duplicate.

In addition, PCR amplifications of well-known structural genes associated with resistance to β-lactams (*bla*TEM, *oxa*, *mec*A), glycopeptides (*van*A), tetracyclines (*tet*M), aminoglycosides (*aac6′/aph2″*), macrolides (*erm*A, *erm*B, *erm*C, *mef*A, *msr*A), and quinolones (*gyr*A, *grl*A) were performed using conditions described elsewhere (Aminov et al., 2001; Choi et al., 2003; Lina et al., 1999; Murakami et al., 1991; Roberts et al., 1999; Saha et al., 2008; Schmitz et al., 1998; Vahaboglu et al., 1998; Zhao et al., 2001). Primers and corresponding PCR annealing temperatures are listed in Table 1.

A PCR using primers TStaG422 (5′-GGCCGTGTTGAACGTGGTCAAATCA-3′) and TStag765 (5′-TIACCATTTCAGTACCTTCTGGTAA-3′) (Martineau et al., 2001) targeting the *tuf* gene, which is present at all staphylococcal species, was used as a positive control.

2.5. PCR detection of virulence and amino acid decarboxylase genes

Multiplex PCR reactions with the primers listed in Table 1 and conditions described by Even et al. (2010) were carried out to detect the presence of genes involved in the expression of enterotoxin A (*sea*), enterotoxin B (*seb*), enterotoxin C (*sec*), enterotoxin D (*sed*), enterotoxin E (*see*), enterotoxin J (*selj*), enterotoxin R (*selr*), a hemolysin-like protein, exotoxin-like 9 (*set9*), hemolysin (SE_0613), hemolysin III (SE_1760 and *hlyIII*), toxic shock syndrome toxin-1 (*tst1*) and delta hemolysin (*hld*). The *tuf* gene was used as a positive control.

For simultaneous detection of tyrosine decarboxylase (*tdc*), histidine decarboxylase (*hdc*) and ornithine decarboxylase (*odc*) genes, multiplex PCR reactions were performed using the conditions described by Coton et al. (2010) and the primers listed in Table 1. The detection of the lysine decarboxylase (*ldc*) gene was performed using the conditions described by De las Rivas et al. (2006) and the primers listed in Table 1. The *tuf* gene was used as a positive control.

2.6. Enterotoxin production

Enterotoxin A (SEA), B (SEB), C (SEC), D (SED) and E (SEE) were detected by a sandwich enzyme immunoassay using the Ridascreen SET (r-biopharm AG, Darmstadt, Germany) kit. The strains were cultured aerobically in 10 mL of Brain Heart Infusion Broth (BHIB, Scharlab) at 37 °C for 72 h. Supernatants were collected by centrifugation (4000 ×*g*, 10 min, 10 °C) and filtered through sterile 0.22 μm filters. The assay was performed following the manufacturer's instructions. Strains were assayed in duplicate.

2.7. Biofilm formation

Biofilm formation was determined as described by Asai et al. (2015). Briefly, sterile 96-well polystyrene microtiter plates were filled with 200 μL of TSB and inoculated with 5% (v/v) of an overnight grown culture. The microtiter plates were incubated at 37 °C for 24 h, after which the cultures were aspirated with a pipette and discarded. The adherent biofilm in each well was gently washed thrice with sterile