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DIVERSITY AND CHARACTERIZATION OF *STAPHYLOCOCCUS* SPP. IN FOOD AND DAIRY PRODUCTS: A FOODSTUFF SAFETY ASSESSMENT

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ABSTRACT

The aim of the current work was to study the diversity of *Staphylococcus* spp. in different types of food products that are available to consumers, to assess its safety from a public health standpoint. A total of twenty food samples (raw milk, white cheese, raw minced meat, beef salami and salted fish) were purchased from the markets. Detection of Staphylococci was performed by selective plating on mannitol salt agar and Staphylococcus medium 110. Identification of presumptive isolates was achieved by phenotypical traits; API Staph System and 16S rRNA sequencing. Raw milk was found to have more diverse species of coagulase-negative staphylococci as well as MRSA. Coagulase-negative *Staphylococcus* species detected in cheese, beef salami and salted fish are the species that are usually involved in the ripening or fermentation process. In conclusion, we found low safety hazards associated with coagulase-negative *Staphylococcus* species isolated from foodstuffs, the only exception is the ready-to-drink raw milk and raw minced meat which was found to be in low microbiological quality and its consumption must be avoided.

Keywords: Food, Dairy, Safety assessment, *Staphylococcus*, PCR, 16S rRNA

INTRODUCTION

The genus *Staphylococcus* is a gram positive bacteria; that belongs to phylum Firmicutes. The spherical cells of this genus are usually arranged in grape like irregular clusters. The genus *Staphylococcus* comprised of at least 40 species that are common inhabitants of skin and respiratory tract of humans and warm-blooded animals, few species are also found in soil and aquatic environments as a part of natural microbial flora (**Abulreesh 2011**, **Bhattacharya *et al.*, 2014**).
Some members of the genus *Staphylococcus*, such as *Staphylococcus aureus*, are capable of causing various infections to human, ranging from mild food poisoning to life-threatening diseases (**Abulreesh and Organji 2011**; **Abulreesh *et al.*, 2017**), other species such as *Staphylococcus epidermidis* has been recognized as an emerging pathogen causing a number of infections, particularly in immunocompromized patients (**Otto, 2009**). *Staphylococcus aureus* is considered as an important food poisoning causative agent and its presence in foodstuffs indicates poor hygiene practice (**Abulreesh, 2011**, **2014; Abulreesh and Organji, 2011**). Other staphylococcal species are recognized for their positive role in the food industry, particularly fermented food products, i.e. *Staphylococcus saprophyticus* (**Samelis *et al.*, 1998**) and *Staphylococcus scuiri* as starter culture in the production of fermented meat (**Sanchez Mainar *et al.*, 2016; Laranjo *et al.*, 2017**). Given that foodstuffs can be easily contaminated with pathogenic staphylococci (e.g. *S. aureus*) and since that antibiotic resistance genes and other virulence determinants can be easily transferred between pathogenic and non-pathogenic staphylococcal species (e.g. *S. scuiri*) that are used in starter cultures, from a public health stand point this necessitates the assessment of food products for the presence and characteristics of staphylococcal species found in foodstuffs.
The routine identification/differentiation/characterization of staphylococcal species is mainly based on a number of phenotypical traits; one important distinguishing feature of the staphylococci is the coagulase test, which is used mainly to differentiate *S. aureus* from other coagulase-negative staphylococci such as *S. epidermidis* (**Forbes, 2009**). Other important phenotypical traits that are used for routine identification/differentiation of staphylococcal spices are production of pigments (staphyloxanthin); blood hemolysis on blood agar plates and the production of staphylococcal heat-stable nuclease that can cleave both of the DNA and RNA (**Forbes, 2009**). Commercial kits for wide range of biochemical tests are also used routinely for identification/differentiation of staphylococcal species, the API Staph System, produced by bioMerieux is a reliable fast method for identifying 23 species of gram-positive cocci, including the clinically important staphylococcal species. The API Staph System includes 20 tests that are used for the differentiation of *Staphylococcus* sp., *Kocuria* and *Micrococcus* species (**Sampimon *et al.*, 2009**). To perform more rapid and accurate identification, several molecular methods were described for the differentiation of staphylococcal species, restriction fragment length polymorphism (RFLP); Pulsed-Field Gel Electrophoresis (PFGE); amplified fragment length polymorphism (AFLP); random amplification of polymorphic DNA (RAPD PCR) and 16S rRNA sequencing haven been successfully used to identify and differentiate between various staphylococcal species (**Takahasi *et al.*, 1999**; **Ghebremedhin *et al.*, 2008**; **Forbes, 2009**; **Morandi *et al.*, 2009**; **Hu *et al.*, 2013**).
The aim of the current work is to investigate the diversity and characterization of *Staphylococcus* species in different foodstuffs and to provide a safety assessment of these products from a public health standpoint.

MATERIAL AND METHODS

Samples collection

A total of 20 food samples were collected from supermarkets and local farms. These samples comprised of white semi-solid cheese (5), raw bovine milk (5), raw minced meat (5), beef salami (3) and ready-to-eat salted fish (sardine) (2). Raw milk (unpasteurized) samples were purchased from local farms in a ready-to-drink disposable screw-top plastic bottle. Minced meat, cheese, beef salami and salted fish were purchased from local supermarkets. All samples were transferred to the laboratory on ice, away from direct sunlight. Microbiological analysis began as the same day of sampling.

Detection of *Staphylococcus* species in foodstuffs

Two solid media were used for the detection of *Staphylococcus* species from food and dairy samples, these media were mannitol salt agar (Oxoid, Basingstoke, UK) and Staphylococcus 110 medium (Oxoid). The raw milk samples were serially diluted and one mL of dilutions 10^{-4} and 10^{-5} were plated onto agar plates of the two media used. For each minced meat; beef salami and salted fish ,

a sample of 10 g was weighted and dispersed aseptically in 90 mL of sterile deionized water and homogenized in sterile polyethylene bags using stomacher 400 circular (Seward, Worthing, UK) for 2 min. Each preparation was serially diluted, one mL of 10^{-4} and 10^{-5} of each dilution was plated onto isolation media. All determinations were made in duplicates. Plates were incubated aerobically at 34 °C, all plates were observed after 24 h and scored after 48 h (**Abulreesh and Organji, 2011**).

Phenotypical traits

All presumptive staphylococcal isolates were examined morphologically by Gram's staining technique and also for their ability to produce clumping factor by using the Staphaurex latex agglutination test kit (Oxoid); observed for blood hemolytic activity on blood agar plates (Oxoid); production of catalase; oxidase; observed for their ability to produce deoxyribonuclease on DNase agar (Oxoid); observation of pigment production on blood agar and nutrient agar (Oxoid) (**Abulreesh and Organji, 2011; Abulreesh, 2014**; **Abulreesh *et al.*, 2017**). All presumptive staphylococcal isolates were identified using the API Staph (bioMerieux, Marcy l'Etoile, France), according to manufacturer's instruction (**Sampimon *et al.*, 2009**).

Determination of decarboxylase activity

Detection of decarboxylase activity for all presumptive staphylococci isolates was determined on decarboxylase screening medium containing lysine and ornithin as described by **Bermudez *et al.* (2012)**, agar plates incubated for 2-5 days at 37 °C under aerobic conditions. The appearance of purple color around the colonies was an indication of the production of biogenic amines from precursor amino acid.

16S rRNA identification

Presumptive staphylococcal isolates were subjected to PCR amplification of the 16S rRNA genes (**Assaeedi *et al.*, 2011**), followed by sequencing as molecular confirmatory test. Genomic DNA was isolated according to **Wang *et al.* (2001)**, using primers (GF: 5'-AGTTTGACTCTGGCTCAG-'3) and (GR: 5'-TACGGCTACCTTGTTACGACTT-'3) (**Elmenofy *et al.*, 2014**) (Bioneer, Korea). The PCR reaction mixture (50 µL total volume) contained 200 µM of each dNTP, 0.5 µM primers, 10 mM tris HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 2.5 U *Taq* polymerase (ABgene, Surry, UK) and 100 ng of template DNA. Amplification of DNA using primers GF and GR; was performed at the following temperature cycle: denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 2 min, final extension at 72 °C for 7 min. A total volume of 20 µL of PCR products were analyzed by 1% agarose gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5 mg mL^{-1}) staining and UV transillumination. Sequencing of PCR products was performed by Bioneer Corp., (Korea), following the procedure described by **Sanger *et al.* (1977)**.

Sequence analysis

The 16S rRNA sequences were initially analyzed by using BLAST. The sequence from the isolates and sequences of strains belonging to the same phylogenetic group and other representatives of the *Staphylococcus*, *Micrococcus* and *Kocuria* species (retrieved from the NCBI database) were aligned using the Clustal X software **(Thompson *et al.*, 1997)**. The resulting trees were displayed with Tree View **(Saitou and Nei (1987)**. The phylogenetic reconstruction was done using the neighbor joining method (**Page, 1996**) using *Acinetobacter calcoaceticus* as an outgroup.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was carried out employing the disk diffusion method (Kirby-Bauer technique), according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (**EUCAST, 2014**). Three antibiotics were purchased from Mast Diagnostics (Bootle, UK); oxacillin (1.0 µg $disk^{-1}$); vancomycin (30 µg $disk^{-1}$) and novobicin (5.0 µg $disk^{-1}$). All antimicrobial susceptibility testing were performed by preparing a 0.5 McFarland standard suspension of each isolate and lawn culture was done on Mueller-Hinton agar (Oxoid), plates were incubated aerobically at 34 °C for 18 h (**Abulreesh and Organji, 2011**; **Abulreesh *et al.*, 2017**).

Control strain

Staphylococcus aureus ATCC 29213 and NCTC 12989 were used as a positive control strains throughout the study.

RESULTS AND DISCUSSION

Diversity of staphylococci in foodstuffs

A total of 50 presumptive isolates were recovered from all tested samples that were morphologically spherical and gave positive reaction to Gram's staining technique. Presumptive staphylococcal isolates were detected based on colonial features on both mannitol salt agar (coagulase-positive staphylococci produce colonies surrounded by bright yellow zones, whereas coagulase-negative staphylococci produce colonies with reddish purple zones) and Staphylococcus 110 medium (the formation of orange colonies indicates coagulase-positive staphylococci, whilst, non coagulase-positive species form white colonies) (**Bridson, 1998**). The origin of each isolate and their phenotypical characteristics (i.e. production of coagulase, catalse and DNAse; type of hemolysis; and antibiotic susceptibility), biogenic amines production and 16S rRNA identification are listed in **Table 1**.

Based on the API Staph System, 11 staphylococcal species were identified (28 isolates out of 50 presumptive isolates); *S. aureus* (4), *S. epidermidis* (3), *S. saprophyticus* (9), *S. sciuri* (4), *S. lentus* (1), *S. xylosus* (1), *S. warneri* (1), *S. hominis* (3), *S. caprae* (1), *S. simulans* (1), *S. equorum* (1), while API Staph system was unable to identify 22 isolates, 16S rRNA sequencing identify those isolates as one *Staphylococcus aureus* (only 92 % homology; Fig. 2), *Micrococcus luteus* (3 isolates, n = 50), *Kocuria rhizophila* (2 isolates, n = 50), *Macrococcus caseolyticus* (7 isolates) and other species (e.g. *lactoccocus* spp, (8 isolates). *Lactococcus* spp. that does not belong to staphylococci and related genera will not be further discussed in this study.

As confirmed by 16S rRNA, the presumptive staphylococcal isolates were belonging to seven clusters out of the 11 currently classified staphylococcal clusters; these clusters are *S. aureus* group (*S. aureus*); *S. epidermidis group* (*S. epidermidis* and *S. caprae*); *S. saprophyticus* group (*S. saprophyticus*; *S. eqourum* and *S. xylosus*); *S. haemolyticus* group (*S. hominis*); *S. simulans* group (*S. simulans*); *S. warneri* group (*S. warneri*) and *S. sciuri* (*S. sciuri* and *S. lentus*) (**Fig. 1**), altogether those seven clusters represent three major staphylococcal groups based on the ability of the species to produce the staphylococcal clumping factor; coagulase-positive staphylococci (*S. aureus*); coagulase-negative staphylococci (*S. caprae*; *S. epidermidis*; *S. hominis*; *S. simulans* and *S. warneri*) and coagulase-negative novobicin-resistant staphylococci (*S. equorum*; *S. saprophyticus*; *S. lentus*; *S. sciuri* and *S. xylosus*) (**Fig 1**; **Table 1**).

The diversity of staphylococcal species in food and dairy samples are listed in **Table 2**. Raw milk was found to have more species than any other samples, with seven different staphylococcal species that belong to all three groups (Coagualse-positive; coagulase-negative and coagulase-negative novobicin-resistant staphylococci). Milk was also found to be contaminated with other non-staphylococcal Gram-positive cocci (i.e. *M. lutues* and *K. rhizophila*), the total number of isolates that were recovered from milk were 18 isolates belonging to eight taxa (**Table 2**). Cheese and beef salami came second to milk in terms of species diversity, with four species each. However, coagulase-positive *S. aureus* were present in cheese samples and absent in beef salami, while *Ma. caseolyticus* was more prevalent in beef salami. On the other hand both cheese and beef salami had a diversity of coagulase-negative staphylococci, yet different genera where found in each sample type (**Table 2**). Minced meat and ready-to-eat salted fish contained less species diversity with only three species found in each sample type (**Table 2**). Notably *S. saprophyticus* (3 isolates) were found in minced meat and salted fish, as well as *Ma. caseolyticus* (4 isolates). Two isolates of *S. aureus* were found in minced meat, while *S. lentus* was detected in salted fish (**Table 2**).

The staphylococci are a diverse group of Gram-positive, spherical bacteria that are wide spread in various ecological niches. Members of the staphylococci are of great public health importance as being causative agents of diverse infections in human and warm blooded animals and/or being recognized for their technological role in food production industry, particularly flavor and aroma production in fermented foodstuff (**Irlinger, 2008**). In this study we found that ready-to-drink raw milk that is sold at farms is highly contaminated with various staphylococcal species, 13 strains that belong to seven different species, most of which belong to coagulase-negative staphylococci, with *S. saprophyticus* being the predominant species. Similar observations where noted elsewhere, where diverse coagulase-negative staphylococci were found in raw cow milk in the United States (**Sawant *et al.*, 2009**) and Morocco (**Bendahou *et al.*, 2008**). It was observed that *S. epidermidis*, *S. simulans*, *S. hominis* and *S. xylosis*, are commonly detected in raw milk elsewhere (**Bendahou *et al.*, 2008**; **Sawant *et al.*, 2009**). The source of staphylococci in raw milk is likely from the animals themselves as *S. aureus* is major cause of subclinical, clinical and chronic mastitis (**Aqib *et al.*, 2017**). Although coagulase-negative staphylococci are considered as mildly pathogenic species, they are frequently isolated from raw milk and there is growing evidence that coagulase-negative staphylococci are significant cause of subclinical mastitis (**Bendahou *et al.*, 2008**; **Sawant *et al.*, 2009**).

The presence of *Staphylococcus* species in cheese may not always indicates contamination; in fact some coagulase-negative *Staphylococcus* species play an important role in cheese production as they are involved at the early stages of ripening process (**Irlinger, 2008**). In this study we tested only one type of cheese,

white semisolid cheese and surprisingly, we noted low diversity of staphylococci in the tested samples in comparison with other studies (**Coton *et al.*, 2010**). Only three coagulase-negative species were detected, *S. epidermidis*; *S. caprae* and *S. equorum*, these species were frequently detected in cheese as they are involved in ripening process, particularly *S. equorum* (**Irlinger, 2008**; **Coton *et al.*, 2010**). Notably we detected *S. aureus* in cheese samples and their presence only indicate contamination/lack of hygiene practices as *S. aureus* play no role in cheese technology (**Abulreesh and Organji, 2011**).

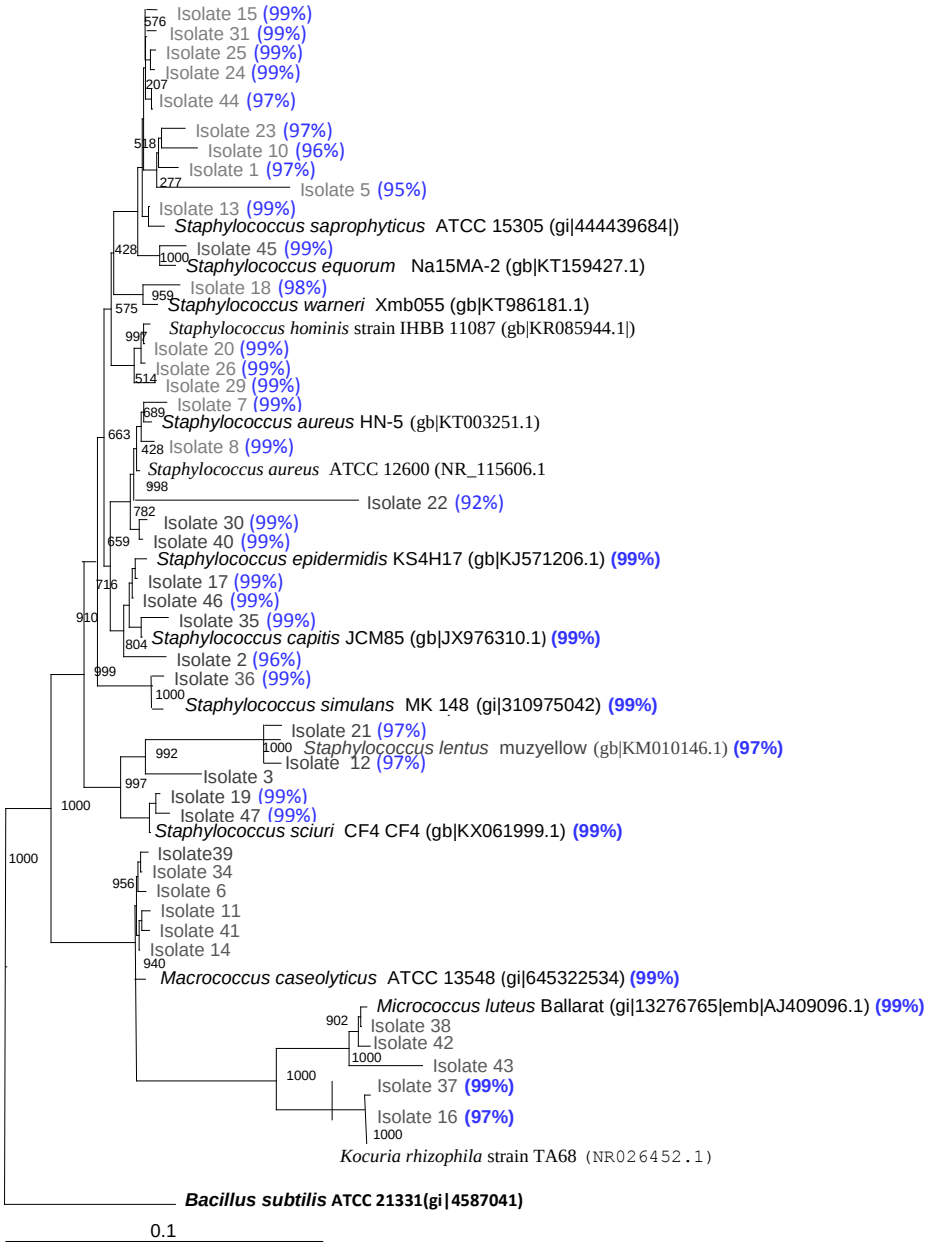

Figure 1 Neighbor-joining tree showing the phylogenetic position of *Staphylococcus*, *Micrococcus*, *Macrococcus* and *Kocuria* species isolated from different foodstuffss and related reference strains based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0. 1 substitution per nucleotide position.

The diversity of *Staphylococcus* species in beef salami, minced meat and salted fish was very low. *Staphylococcus saprophyticus* was frequently detected in all three sample types, other coagulase-negative species encountered were *S. hominis* and *S. sciuri* (beef salami), and *S. lentus* (salted fish). *Staphylococcus saprophyticus* plays a role in the fermentation process of meat such as sausages and salami; therefore they are commonly recovered from fermented meat products (**Coton *et al.*, 2010**; **Even *et al.*, 2010**; **Leroy *et al.*, 2010**; **Genis and Tuncer, 2017**). The incidence of *S. saprophyticus* and *S. lentus* in salted and smoked ready-to-eat fish was reported elsewhere (**Chajecka-Wierzchowska *et al.*, 2015**).

One presumptive isolate that was unidentifiable by API Staph system, yet phenotypically bearing resemblance to *S. aureus*, that is isolate (S22). Molecular identification of this isolate revealed similarity of 92% to *S. aureus*. However, when isolate (S22) was separated in a new phylogenetic dendogram (**Fig 2**), the isolate was distinguished in separate cluster. This result may suggest that isolate (S22) is new genus; however more taxonomic studies such as DNA-DNA hybridization and fatty acid profile are required to validate this assumption.

Molecular identification showed that seven presumptive isolates that were unidentifiable by API Staph system belong to the genus *Macrococcus*, particularly *Ma. caseolyticus* (**Fig.3**). *Macrococcus* spp was formerly classified as *Staphylococcus* spp., closely related genera Gram. Other Gram-positive cocci that were not identifiable by API Staph system and identified by 16S rRNA sequencing include *Micrococcus luteus* (3 isolates) and *Kocuria rhizophila* (2 isolates).

In this study we noted the presence of other Gram-positive cocci that are closely related to *Staphylococcus* species, these other genera were *Micrococcus luteus*, and *Kocuria rhizophila* which were found in raw milk, and *Macrococcu caseolyticus* which was detected in beef salami; minced meat and salted fish. *Micrococcus luteus* and *Kocuria rhizophila* are both actinobacteria, members of the family Micrococcaceae (**Kovacs *et al.*, 1999**; **Young *et al.*, 2010**) and very closely related to the genus *Staphylococcus*, their presence in raw milk is probably due to contamination during milking or bottling as they are common inhabitants of the soil. *Macrococcus caseolytics* (formerly known as *Staphylococcus caseolyticus*) was proposed as new species to be separated from the genus *Staphylococcus* based on phylogenetic analysis, DNA-DNA hybridization, 16S rRNA sequence, morphological and phenotypical characteristics (**Kloos *et al.*, 1998**). *Macrococcus caseolyticus* was detected in beef salami and salted fish, its presence may be explained by the fact that *Ma.*

caseolyticus plays a role in the fermentation process and has been commonly detected in fermented meat products in Italy and Turkey (**Blaiotta *et al.*, 2004**; **Genis and Tuncer, 2017**).

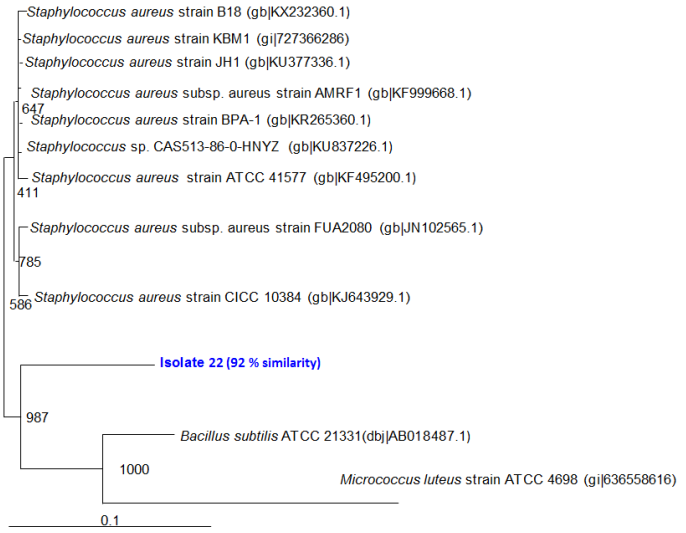

Figure 2 Neighbor-joining tree showing the phylogenetic position of **Isolate 22**, *Staphylococcus* species and reference strains based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0. 1 substitution per nucleotide position.

Safety assessment of Staphylococci in foodstuffs

Most of the staphylococci isolated and identified in this study were not exhibiting resistance to antibiotics (methicillin and vancomycin), with exception of all *S. aureus* that were isolated from milk, cheese and minced meat were methicillin resistant (MRSA). In addition to the novobicin-resistant coagulase-negative staphylococci that were isolated from all samples type (*S. saprophyticus*, *S. eqourum*, *S. xylosus*, *S. scuiri* and *S. lentus*). We noted two *S. epidermidis* isolates with resistance to methicillin, one from cheese and one from raw milk (MRSE). It was notable that all *S. scuiri* isolated from beef salami, *S. simulans* and *S. warneri* recovered from milk and *S. eqourum* isolated from cheese showed delayed hemolytic activities on sheep blood agar plates. Detection of biogenic amines production from both lysine and ornithin was observed in all *S. saprophyticus* recovered from all types of samples, *S. epidermidis* isolates were also positive for decarboxylase activity on lysine and orinithin, while one isolates of *S. aureus* and *S. warniri* from milk and one *S. eqourum* from cheese were found to be positive for biogenic amines production from lysine (**Tables 1 & 2**).

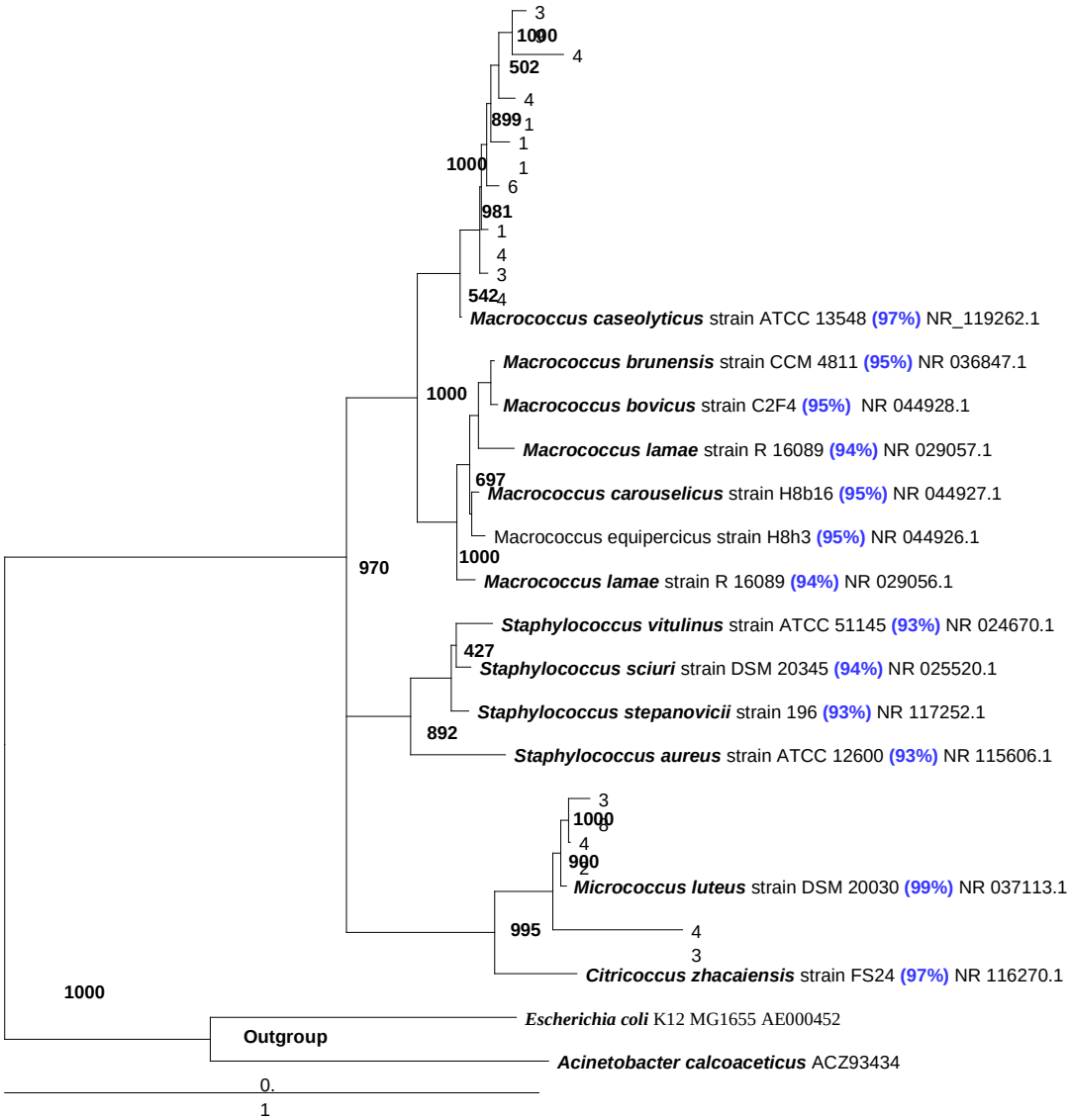

Figure 3 Neighbor-joining tree showing the phylogenetic position of *Macrococcus caseolyticus* and *Micrococcus luteus*, and reference strains based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0. 1 substitution per nucleotide position.

Table 1 Phenotypical traits of *Staphylococcus, Micrococcus* and *Macrococcus* spp. isolated from foodstuffs

Isolate	16S rRNA ID	Type of sample	hemolysis	Decaroxylase	Oxidase	Catalase	Coagulase	Antibiotic Susceptibility Test			DNAas	Pigments
								Van	Oxa	Novo		
1S	*S. saprophyticus*	Raw milk	–	+	-	+	-	20 (S)	22 (S)	10 (R)	–	+
2S	*S. epidermidis*	Raw milk	-	+	-	+	–	22 (S)	10 (R)	33 (S)	–	–
3S	*S. sciuri*	*Beef salami*	–	+	+	+	–	22 (S)	34 (S)	40 (R)	-	+
4S	*Ma. caseolyticus*	Salted fish	–	-	+	+	-	20 (S)	33 (S)	37 (S)	–	-
5S	*S. saprophyticus*	Salted fish	-	-	-	+	–	25 (S)	38 (S)	11 (R)	-	-
6S	*Ma. caseolyticus*	*Minced meat*	–	-		+	+	18 (S)	40 (S)	19 (S)	-	+
7S	*S. aureus*	Raw milk	+	+	-	+	+	20 (S)	0 (R)	21 (S)	+	+
8S	*S. aureus*	Cheese	+	-	-	+	+	18 (S)	0 (R)	22 (S)	+	+
10S	*S. saprophyticus*	Raw milk	-	+	-	+	-	28 (S)	25 (S)	11 (R)	–	+
11S	*Ma. caseolyticus*	Salted fish	–	-	+	+	–	18 (S)	27 (S)	18 (S)	–	-
12S	*S. lentus*	Salted fish	–	-	+	+	–	18,(S)	33 (S)	24 (S)	-	(+)*
13S	*S. xylosus*	Raw milk	+	+	-	+	-	20 (S)	33 (S)	10 (R)	–	+
14S	*Ma. caseolyticus*	Salted fish	–	-	+	+	–	18 (S)	40 (S)	24 (S)	–	-
15S	*S. saprophyticus*	Raw milk	–	+	-	+	–	20 (S)	42 (S)	10 (R)	-	–
16S	*K. rhizophila*	Raw milk	–	-	-	+	–	21 (S)	33 (S)	29 (S)	–	+
17S	*S. epidermidis*	Raw milk	–	+	-	+	–	22 (S)	35 (S)	40 (S)	-	–
18S	*S. warneri*	Raw milk	(+)*	+	-	+	–	20 (S)	30 (S)	39 (S)	–	–
19S	*S. sciuri*	Beef salami	(+)*	+	+	+	–	16 (S)	32 (S)	12 (R)	–	–
20S	*S. hominis*	*Beef salami*	-	-	-	+	–	23 (S)	30 (S)	27 (S)	-	–
21S	*S. sciuri*	Beef salami	–	+	+	+	–	27 (S)	40 (S)	11 (R)	–	-
22S	*S. aureus* (92%)†	Cheese	+	-	-	+	+	20 (S)	0 (R)	23 (S)	+	+
23S	*S. saprophyticus*	*Raw milk*	-	+	-	+	–	20 (S)	22 (S)	10 (R)	–	–
24S	*S. saprophyticus*	*Minced meat*	–	+	-	+	-	20 (S)	33 (S)	10 (R)	–	–
25S	*S. saprophyticus*	*Minced meat*	–	+	-	+	-	19 (S)	25 (S)	10 (R)	–	+
26S	*S. hominis*	Raw milk	–	-	-	+	–	23 (S)	30 (S)	36 (S)	–	–
29S	*S. hominis*	Raw milk	–	-	-	+	–	30 (S)	30 (S)	40 (S)	-	–
30S	*S. aureus*	Minced meat	+	-	-	+	+	15 (S)	0 (R)	29 (S)	+	+
31S	*S. saprophyticus*	Raw milk	–	+	-	-	-	23 (S)	22 (S)	11 (R)	–	–
33S	*S. sprophyticus*	*Beef salami*	–	+	-	-	-	21 (S)	40 (S)	11 (R)	–	+
34S	*Ma. caseolyticus*	*Beef salami*	–	-	+	+	-	13 (S)	44 (S)	20 (S)	-	-
35S	*S. caprae*	Cheese	-	-	-	+	–	14 (S)	33 (S)	26 (S)	-	–
36S	*S. simulans*	Raw milk	(+)*	-	-	+	–	15 (S)	35 (S)	31 (S)	-	–
37S	*K. rhizophila*	Raw milk	–	-	-	+	–	18 (S)	22 (S)	46 (S)	-	+
38S	*M. luteus*	Raw milk	–	-	+	+	–	12 (S)	25 (S)	15 (S)	-	+
39S	*Ma. caseolyticus*	Beef salami	–	-	+	+	-	18 (S)	27 (S)	25 (S)	-	–
40S	*S. aureus*	Mined meat	+	-	-	+	+	16 (S)	0 (R)	26 (S)	+	+
41S	*Ma. caseolyticus*	Beef salami	-	-	+	+	-	15 (S)	44 (S)	26 (S)	-	–
42S	*M. luteus*	Raw milk	-	-	+	+	-	22 (S)	33 (S)	40 (S)	-	+
43S	*M. luteus*	Raw milk	-	-	+	+	-	18 (S)	35 (S)	37 (S)	-	+
44S	*S. saprophyticus*	Raw milk	–	+	-	-	-	26 S	20 S	10 R	–	–
45S	*S. equorum*	Cheese	(+)*	+	-	+	-	20 (S)	30 (S)	11 (R)	–	–
46S	*S. epidermidis*	Cheese	–	+	-	+	-	19 (S)	9 (R)	37 (S)	-	–
47S	*S. sciuri*	Beef salami	–	+	+	+	-	27 (S)	22 (S)	12 (R)	–	–

Legend: (+)* = Delayed reaction; (92 %)† = this isolate had 92 % homology with *S. aureus*; S = Susceptible; R = Resistant, Oxa = oxacillin; Van = vancomycin; Novo = novobicin

Table 2 Diversity of *Staphylococcus*, *Micrococcus*, *Macrococcus* and *Kocuria* species in foodstuffs

Sample type (n)	Cos-Positive *Staphylococcus* (n)	Cos-Negative *Staphylococcus*		*Micrococcus* (n)	*Macrococcus* (n)	*Kocuria* (n)
		Novobicin-susceptible (n)	Novobicin-resistant (n)			
Cheese (5)	*S. aureus* (2)‡	*S. epidermidis* (1)‡*⊗ *S. caprae* (1)	*S. equorum* (1)†*			
Milk (5)	*S. aureus* (1)‡*	*S. epidermidis* (2)‡*⊗ *S. simulans* (1)† *S. warneri* (1)†* *S. hominis* (2)	*S. saprophyticus* (5)*⊗ *S. xylosus* (1)⊗	*M. luteus* (3)		*K. rhizophila* (2)
Beef salami (3)		*S. hominis* (1)	*S. saprophyticus* (1)*⊗ *S. sciuri* (4)†*⊗		*Ma. caseolyticus* (3)	
Minced meat (5)	*S. aureus* (2)‡		*S. saprophyticus* (2)*⊗		*Ma. caseolyticus* (1)	
Salted fish (2)			*S. saprophyticus* (1)*⊗ *S. lentus* (1)		*Ma. caseolyticus* (3)	

Legend: ‡ - Methicillin-resistant, † - delayed hemolysis activity on sheep blood agar plates, * - biogenic amines producers from lysine, ⊗ - Biogenic amines producers from ornithin, n = number of samples and isolates

Food safety is a major concern to consumers; public health authorities and the food industry. There have been a number of regulations concerning the safety assessment of microorganisms in food; that is based on taxonomy; pathogenecity; familiarity and end use (**Talon and Leroy, 2011**). In this study we assessed the safety of different types of foodstuffs that are ready for consumption for the presence of *Staphylococcus* species based on those criteria, except we did not perform any pathogenecity testing on the isolates we detected from all tested samples.

Ready-to-drink raw milk is widely available in farms, we previously found that ready-to-drink raw milk is heavily contaminated with toxigenic *Bacillus cereus* (**Organji *et al.*, 2015**) and *Staphylococcus* sp. (**Abulreesh and Organji, 2011**), although we did not further identify the staphylococci isolates beyond their coagulase activity, we noted high prevalence of resistant strains to more than one antimicrobial agent. We further confirm the low microbiological quality of ready-to-drink raw milk; that is heavily contaminated with diverse species of both coagulase-positive and negative staphylococci. Both *S. aureus* and *S. epidermidis* were methicillin resistant and biogenic amines producers, *S. simulans* and *S. warneri* were both exhibiting delayed hemolytic activity on sheep blood agar, in addition to the presence of biogenic amines-producing *S. saprophyticus* and *S. xylosus*. The contamination of raw milk with these species indicates either mastitic animals or lack of hygiene practice, with the presence of *S. aureus* that is associated with food poisoning (**Aqib *et al.*, 2017**), and biogenic amines-producing coagulase-negative staphylococci, some studies showed that coagulase-negative staphylococci in milk were capable of producing classical enterotoxins (**Irlinger, 2008**), the consumption of raw milk must be avoided and its selling should be banned.

The low diversity of *Staphylococcus* sp. in cheese may suggest good hygiene practice and/or the use of pasteurized milk, which reflects low health risks. Both of *Staphylococcus equorum* and *S. caprae* are involved in the ripening process during cheese production (**Irlinger, 2008**), while *S. epidermidis* is commonly isolated from cheese (**Coton *et al.*, 2010**). Despite the presence of a very low number of MRSA and MRSE with decarboxylase activity, we assume low health risks are associated with the consumption of cheese samples we examined in this study.

Coagulase-negative staphylococci play an important role in fermented meat technology (**Talon and Leroy, 2011**), therefore the *S. hominis*, *S. saprophyticus* and *S. sciuri* that were found in beef salami samples were all commonly isolated in different types of fermented meat products as they are involve in the production process (**Coton *et al.*, 2010**; **Even *et al.*, 2010**; **Talon and Leroy, 2011**). In this respect probably no health risks are associated with all staphylococci detected in beef salami samples. On the other hand, the species found in raw minced meat, most likely represent contamination due to lack of hygiene practice or cross contamination during cutting or handling of meat. Two MRSA isolates and *S. aprophyticus* with decarboxylase activity were detected in raw minced meat, other studies highlighted the diversity of *Staphylococcus* species in raw meat at retail shops (**Goja *et al.*, 2013**), particularly enterotoxin-producing MRSA (**Carrel *et al.*, 2017**) and suggested the need for good hygiene practice during handling and processing of raw meat at retail shops and processing plants.

Various *Staphylococcus* species were frequently detected in salted and fermented fish, *S. xylosus*; *S. saprophyticus*; *S. warneri* and *S. epidermidis* among the frequently isolated species (**Um and Lee, 1996**; **Gassem, 2017**), in this study only two coagulase-negative novobicin-resistance species were detected in salted sardines samples, *S. saprophyticus* and *S. lentus*, and these species were also detected in salted and/or fermented fish elsewhere (**Um and Lee, 1996**; **Gassem, 2017**), probably coagulase-negative staphylococci play a role in the flavor production in salted and/or fermented fish due to their ability to tolerate high concentration of salts.

There have been various reports describing human infections related to the coagulase-negative *Staphylococcus* species that we detected in all types of foodstuffs tested in this study, particularly the species that are involved in food technology; *S. epidermidis*; *S. equorum*; *S. caprae*; *S. saprophyticus*; *S. xylosus*; *S. simulans*; *S. warneri*; *S., hominis*; and *S. sciuri* (**Novakova *et al.*, 2006**; **Irlinger, 2008**; **Piette and Verschraegen, 2009**; **Coimbra *et al.*, 2011**; **Mallet *et al.*, 2011**; **Dansey *et al.*, 2015**; **Lee *et al.*, 2015**). However, none of these diseases were related to the consumption of food, but all were nosocomial infections, furthermore, some of these isolates that were detected in food were genetically diverse and distinct from isolates of nosocomial, and mastitis origin suggesting different genetic clusters, thus no link between isolates of food origin and human infections (**Talon and Leroy, 2011**; **Lee *et al.*, 2015**).

Antibiotic resistant staphylococci frequently found in foodstuffs, particularly food of animal origin are major concern to public health authorities. Generally, coagulase-negative *Staphylococcus* species are reservoir for antibiotic resistance genes; the species that are commonly found on the skin of animals may contaminate foodstuffs such as milk or meat and consequently may be detected in fermented products made from contaminated raw foodstuffs (**Irlinger, 2008**). Furthermore, given that coagulase-negative staphylococci act as reservoir for resistance gene, it would possible that such resistance genes could be transferred from staphylococci of animal origin to *Staphylococcus* species that are implicated in human infections (**Irlinger, 2008**). There have been various reports describing the incidence of multidrug resistant coagulase-negative staphylococci in milk and other dairy products (**Sawant *et al.*, 2009**; **Even *et al.*, 2010**; **Coton *et al.*, 2010**), raw meat (**Ou *et al.*, 2017**), fermented meat (**Even *et al.*, 2010**; **Coton *et al.*, 2010**; **Genis and Tuncer, 2016**), and slated/fermented seafood (**Chajecka-Wierzchowska *et al.*, 2015**). It is worth mentioning that in this study we found that all coagulase negative staphylococci were susceptible to oxacillin, and vancomycin, including novobicin-resistant coagulase-negative spices. The only exception was *S. epidemidis* isolates that were detected in milk and cheese were found to be resistant to oxacillin (MRSE), and the detection of MRSA, which further confirm the unsuitability of ready-to-drink raw milk for consumption due to its low microbiological quality.

Biogenic amines are nitrogen compounds that are formed as a result of decaroxylase activity on a precursor amino acid. In addition to its unfavorable effects on flavor, biogenic amines in foodstuffs are associated with a number of health issues such as allergic responses, migraines, gastric and intestinal problems, particularly when its formed in excessive quantities in foodstuffs (**Bermudez *et al.*, 2012**). In this study we found that all isolates belonging to *S. aureus*, *S. saprophyticus*, *S. sciuri*, *S. xyloses*, *S. equorum*, *S. warneri* and *S. epidemidis* were biogenic amines producers. These results are in agreement with other studies that reported decarboxylase activities of staphylococci found in foodstuffs (**Bermudez *et al.*, 2012**; **Genis and Tuncer, 2016**). It is believed that biogenic amines content in food in concentration above 120 mg / kg is toxicologically relevant (**Pachlova *et al.*, 2016**), and given the low number of biogenic amines-producing staphylococci detected in all food samples in this study we assume that the levels of biogenic amines produced by this relatively low number of species is probably lower than (120 mg / kg) that could constitute human health risks.

In this study we also detected other Gram-positive cocci in all tested samples, *Macrococcus caseolyticus* was detected in all samples except raw milk and cheese, while *Micrococcus luteus* and *Kocuria rhizophila* were only detected in raw milk. There have been other studies reporting the incidence of *Ma. caseolyticus* in fermented meat (**Blaiotta *et al.*, 2004**; **Genis and Tuncer, 2017**). Possibly, *Ma. caseolyticus* plays a role in the fermentation process among the coagulase-negative consortia of fermented meat and fish products. On the other hand, the detection of soil inhabitant actinobacteria *M. luteus* and *K. rhizophila* in raw milk further confirm the low microbiological quality of raw milk.

CONCLUSION

In conclusion, we found low safety hazards associated with coagulase-negative Staphylococcus species isolated from foodstuffs (cheese; beef salami; salted fish), the only exception is the ready-to-drink raw milk which was in low microbiological quality and its consumption must be avoided.

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