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Safety-related properties of staphylococci isolated from food and food environments

M. Marino, F. Frigo, I. Bartolomeoli and M. Maifreni

Department of Food Science, University of Udine, Udine, Italy

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Correspondence
Marilena Marino, Department of Food Science, University of Udine, via Sondrio 2/A, 33100 Udine, Italy.
E-mail: marilena.marino@uniud.it

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Abstract

Aims: To test some safety-related properties within 321 staphylococci strains isolated from food and food environments.
Methods and Results: The isolates were identified as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus pasteuri*, *Staphylococcus sciuri*, *Staphylococcus warneri* and *Staphylococcus xylosus*. Decarboxylase activity was quite common for the various *Staphylococcus* spp., and tyrosine was the most frequently decarboxylated amino acid. The frequency of antibiotic resistance was highest in *Staph. pasteuri* and *Staph. xylosus*. Several of the isolates were tolerant to QAC compounds, and in some cases, QAC tolerance was present in antibiotic-resistant strains. Most of the strains displayed moderate to high adhesion rates to stainless steel and Teflon®. The strains that readily formed biofilms belonged to the species *Staph. aureus*, *Staph. epidermidis* and *Staph. pasteuri*.
Conclusions: An high incidence of some safety hazards was found within the staphylococcal strains of food origin tested in this study. In particular, amino acid decarboxylase activity and biofilm-forming ability were common within strains, and antibiotic resistance and tolerance to QAC-based compounds occurred frequently as well. These characteristics are an important safety concern for food industry.
Significance and Impact of the Study: This work gives a first picture of safety hazards within staphylococcal species isolated from food environments. The presence of disinfectant-resistant staphylococci is a concern because resistance can be genetically transferred between the various *Staphylococcus* species. This could lead an increase and spread of resistant enterotoxic staphylococci and/or pathogenic staphylococci.

Introduction

Staphylococci are ubiquitously distributed in nature and are frequently isolated from food and environmental sources. Several species, such as *Staphylococcus aureus*, *Staphylococcus saprophyticus* and *Staphylococcus epidermidis*, can cause disease in humans (Rodriguez *et al.* 1996). *Staphylococcus aureus* is a foodborne pathogen that is considered one of the world's leading causes of disease outbreak related to food consumption. Contaminated food, such as various meat products, poultry, eggs, dairy, seafood, as well as breads and bakery products, often contains staphylococcal enterotoxins (SE), which often cause food-related illness (Greig *et al.* 2007). Investigations into retail meals have concerned only incidence of *Staph. aureus*, even if other staphylococcal species can occur in food (Mounier *et al.* 2006; Simeoni *et al.* 2008).

Although SE production is the leading cause of food-related disease by staphylococci, other biophysical and biochemical features, such as amino acid decarboxylase activity, adhesion and biofilm formation, and disinfectant and antibiotic resistance can contribute to pathogenicity and should be further studied. While some safety-associated characteristics, such as toxin production and

antibiotic resistance, have been well studied, there are little data available that describe bacterial adhesion, biofilm formation on abiotic surfaces or resistance to chemical disinfectants.

Another important food-related safety concern that is associated with staphylococci is the production of biogenic amines. Biogenic amines are basic organic compounds that can cause food poisoning (Chang *et al.* 2008). The amino acid decarboxylase activity of numerous microbes, such as the enterobacteria, lactic acid bacteria and staphylococci, generates high levels of biogenic amines in contaminated foods. However, the only information that is currently available regarding biogenic amine production by staphylococci is limited to species that were isolated from fermented sausages. Several *Staphylococcus xylosus* strains, as well as *Staphylococcus carnosus*, *Staph. epidermidis*, *Staph. saprophyticus*, *Staphylococcus warneri* and *Staphylococcus piscifermentans*, can decarboxylate amino acids. The amino acids lysine and tyrosine are both typically decarboxylated, while decarboxylation of histidine is rare (de las Rivas *et al.* 2008).

Another important characteristic of bacterial pathogenicity is antibiotic resistance. Over the last several decades, the incidence of antibiotic resistance has continually grown in several bacterial groups, including the staphylococci; this increase in resistance may be attributed to frequent antibiotic administration to livestock for therapeutic or growth purposes and the resulting selective pressure on bacteria. Thus, the food chain represents one possible way that antibiotic-resistant bacteria are transferred (Angulo *et al.* 2004). Genetic resistance is widely present in both coagulase-positive and coagulase-negative staphylococci that have been isolated from various food sources, including meat, milk, dairy products and poultry (Gundogan *et al.* 2005; Simeoni *et al.* 2008). Studies of human clinical staphylococci and food-related staphylococci have shown that there may be an association between tolerance to cleaning disinfectants (especially to the chiefly quaternary ammonium compounds, QACs) and antibiotic resistance. Because genes that encode for disinfectant tolerance reside on plasmids that are commonly transferred among bacteria, widespread disinfectant use can select for antibiotic-resistant staphylococci (Bjorland *et al.* 2005).

Bacteria can also form biofilms, which are often resistant to sanitizers that are commonly used in plants (Cloete 2003). Staphylococci can form biofilms on various materials and are a common micro-organism that is isolated from food industry surfaces (Shale *et al.* 2005). Biofilm production, which can be phenotypically detected, is a marker of virulence (Jain and Agarwal 2009). Staphylococci biofilm production has been extensively investigated with medical devices and implants, particularly with *Staph. epidermidis* and *Staph. aureus* (O'Gara and Humphreys 2001). However, little information is available regarding staphylococci species in the food environment. Although the results can vary by strain, *Staphylococcus sciuri* and *Staph. xylosus* can form a biofilm on abiotic surfaces and *Staph. carnosus* can adhere to various surfaces (Planchon *et al.* 2007). The ability to adhere to an abiotic surface and form a biofilm may be advantageous for the colonization of food environments and may ultimately cause food poisoning (Brooks and Flint 2008).

Staphylococci are commonly found in catered food, and the goal of this study was to identify and characterize staphylococci species that were isolated from hot and cold meals, catering surfaces and the hands of food workers at several catering companies; additionally, our goal was to study several bacterial characteristics that may influence the food safety, specifically amino acid decarboxylase activity, antibiotic and disinfectant resistance, bacterial adhesion and biofilm formation.

Materials and methods

Isolation and identification of strains

The isolates used in this study were obtained from various hot and cold meals (52 samples), swabs on food contact surfaces (19) and workers' gloved hands (23); all of the samples were collected from various catering establishments in north-east Italy. The food samples were analysed as follows: 25 g of each sample was diluted with 225 ml of Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK) and homogenized for 2 min in a stomacher homogenizer (Lab-Blender 400; PBI, Milan, Italy). Decimal dilutions of the sample suspensions were plated on Baird–Parker Agar (BPA) supplemented with Egg Yolk Tellurite emulsion (Oxoid, UK). Samples were collected from food-related surfaces with sterile swabs that were moistened with a saline solution and rubbed for 20 s over the sample surface. The swab was then placed in 5 ml of saline solution and plated on BPA. Samples from the gloves of the catering staff were obtained by directly placing the glove finger on to a BPA plate for 10 s. The BPA plates were incubated at 37°C for 24 h. Three to five colonies were randomly picked from each sample, and the isolates were Gram stained, examined microscopically and tested for catalase activity. DNA was isolated from the strains using InstaGene Matrix (Bio-Rad Laboratories, Segrate, Italy) according to the manufacturer's recommendations. RAPD profiles were obtained by PCR with the M13 primer according to the protocol of Pinto *et al.* (2005). Each digitalized amplification profile was visually compared with the reference strain profiles of *Staph. aureus* ssp. *aureus* DSMZ 20231^T, *Staph. epidermidis* DSMZ

20044^T, *Staph. saprophyticus* DSMZ 20229^T, *Staph. warneri* DSMZ 20316^T and *Staph. xylosus* DSMZ 20266^T. Species identification was performed via either species-specific PCR (Forsman *et al.* 1997; Martineau *et al.* 1998; Aymerich *et al.* 2003; Morot-Bizot *et al.* 2003, 2004) or 16S rRNA gene sequencing with the P1 and P4 primers (Klijn *et al.* 1991). The sequencing samples were analysed with the BLAST algorithm (Altschul *et al.* 1997) to identify the species, and only strains that had a unique RAPD profile were further characterized.

Detection of amino acid decarboxylative ability

Amino acid decarboxylase activity towards histidine, tyrosine, ornithine and lysine was qualitatively assessed by the method described by Bover-Cid and Holzapfel (1999). Briefly, after three overnight growth cycles in Nutrient Broth (Oxoid, Milan, Italy) that contained 0·1% of each precursor amino acid, each culture was streaked in duplicate on to differential plates with or without the various amino acids. The plates were incubated at 37°C for 48–72 h. A colour change from yellow to purple indicated a positive reaction, i.e. that the respective amino acid decarboxylase was present.

Antibiotic susceptibility testing

Resistance towards eight antibiotics was assessed for each strain with the disc diffusion method (NCCLS 2003) and bacterial growth on Müller–Hinton agar plates (Oxoid, Italy). The antibiotics tested were erythromycin (15 μg), methicillin (5 μg), tetracycline (30 μg), vancomycin (30 μg), ampicillin (10 μg), gentamicin (10 μg), rifampicin (5 μg) and penicillin G (10 IU); all of the antibiotics were from Oxoid, Italy.

Tolerance to disinfectants

Tolerance towards eight disinfectants was tested for each strain by using the microdilution broth method in Müller–Hinton broth to determine the MIC according to the NCCLS standard (Anonymous 1999). The lowest concentration of an antimicrobial agent that totally inhibited the bacterial growth after 24 h of incubation at 37°C was considered as the MIC. The disinfectants tested were alkyl-benzyl-dimethyl ammonium chloride (ADBAC), dimethyl-didecil ammonium chloride (DDAC), QAC-1 (a commercial product containing 0·2% benzalconium chloride), QAC-2 (1·2% DDAC), OX-1 (5–15% citric acid and 15–30% hydrogen peroxide), OX-2 (15–30% acetic acid, 5–15% peracetic acid and 5–15% hydrogen peroxide), CL-1 (>30% sodium dichloroisocyanurate dihydrate) and CL-2 (15% sodium hypochlorite active chlorine). The appropriate concentrations of each solution were freshly prepared before each experiment. For each disinfectant, a sterile 1% stock solution was prepared, except in the case of CL-2, which was diluted to 5%.

Adhesion to stainless steel and Teflon® surfaces

The adhesion tests were performed according to Bore and Langsrud (2005) on 50 × 25 × 1 mm coupons of stainless steel (AISI 304, no. 4 finish) or Teflon® (PTFE). Briefly, the sterile coupons were placed vertically into 50-ml tubes that contained 30 ml of Tryptone Soya Broth (Oxoid, Italy); the tubes were inoculated with 1% of an overnight culture and were incubated statically at 37°C for 24 h. At the end of incubation time, the CFU ml^{-1} of the suspension was calculated on Standard Plate Count Agar plate (Oxoid, Italy). The coupons were then transferred to 30 ml of MRD (Oxoid, Italy) and gently shaken at room temperature for 15 min. After transferring the coupons to 30 ml of fresh MRD, the samples were sonicated for 15 min in a Branson 5200 ultrasound bath (Branson Ultrasonics Corp., Danbury, USA). The CFU ml^{-1} was then measured on PCA plates (Plate Count Agar; Oxoid, Milan, Italy). The degree of adhesion was calculated as follows:

$$\%\ \text{attachment} = \frac{\text{cells attached}}{\text{cells in the growth medium} + \text{cells attached}}$$

Biofilm-forming ability

The ability of the strains to form a biofilm was investigated in flat-bottom 96-well polystyrene microtitre plates according to the protocol of Stepanović *et al.* (2000). For each strain, four wells of a microtitre plate were filled with 200 μl of bacterial suspension in Tryptone Soya Broth (Oxoid, Italy) supplemented with 1% glucose, and the plates were incubated aerobically at 37°C for 24 h under static conditions. Then, the content of each well was aspirated, and each well was washed three times with 250 μl of sterile physiological saline. The attached bacteria were fixed with methanol for 15 min, and then the plates were emptied and left to dry. The plates were stained for 5 min with 200 μl of crystal per well. Excess stain was rinsed off with tap water, and after the plates were air-dried, the dye bound to biofilm was resolubilized with 160 μl of glacial acetic acid. The OD of each well was measured at 570 nm by using a Sunrise Microplate Reader (Tecan, Männedorf, Switzerland).

Results

Three hundred and twenty-one strains were isolated from hot and cold meals, swabs and workers' hands from

Table 1 *Staphylococcus* species isolated from food, food contact surfaces and hands of food care workers

Species	Total	Food	Workers	Surfaces
Staphylococcus aureus	86	24	50	12
Staphylococcus epidermidis	78	29	25	24
Staphylococcus saprophyticus	71	37	27	7
Staphylococcus pasteuri	36	27	6	3
Staphylococcus sciuri	25	18	0	7
Staphylococcus warneri	14	10	0	4
Staphylococcus xylosus	11	11	0	0
Total	321	156	108	57

foodservice settings. The most predominant species present in the isolates was *Staph. aureus*, which accounted for 26·8% of strains (Table 1). This species was present in the food (15·4% of isolates), on the food contact surfaces (21·1%) and on the gloves of the catering staff (46·3%). Of the 321 isolates, 24·3, 22·1 and 11·2% of strains were *Staph. epidermidis, Staph. saprophyticus* and *Staphylococcus pasteuri*, respectively. The remaining minority of samples were *Staph. sciuri*, *Staph. warneri* and *Staph. xylosus*. All of these species were isolated from both the food and the work surfaces.

Decarboxylase activity was quite common for the *Staphylococcus* spp. (Table 2), and over 75% of the isolated strains could decarboxylate at least one amino acid, and tyrosine was the most frequently decarboxylated amino acid. The ability to decarboxylate two to four amino acids was present in 104 of 321 staphylococcal isolates. *Staphylococcus aureus* strains were able to decarboxylate only one amino acid; tyrosine was the most frequently decarboxylated amino acid, and histidine was the second most common. Also *Staph. epidermidis* and *Staph. pasteuri* decarboxylated only one amino acid, while the ability to decarboxylate two or more amino acids was common within *Staph. saprophyticus*, *Staph. sciuri*, *Staph. warneri* and *Staph. xylosus*. Most *Staph. xylosus* isolates (10 of 11 strains) decarboxylated all four amino acids tested.

The frequency of antibiotic resistance among the isolates is listed in Table 3. All of the isolates were susceptible to rifampicin and vancomycin, while there was high resistance to penicillin G and ampicillin. Resistance to tetracycline and erythromycin was observed for >20% of the isolates, particularly for the CNS. Only a small number of *Staph. aureus*, *Staph. epidermidis*, *Staph. pasteuri* and *Staph. sciuri* strains were resistant to gentamicin. Methicillin resistance was detected for only seven *Staph. aureus* and *Staph. epidermidis* strains that were isolated from the food handlers. The frequency of antibiotic resistance varied within the species and was higher for the *Staph. pasteuri* and *Staph. xylosus* isolates (Table 4). *Staphylococcus aureus* had the lowest number of resistant strains compared to all of the isolated species, while there was a large number of antibiotic-resistant *Staph. pasteuri* strains. The *Staph. warneri*, *Staph. xylosus*, *Staph. aureus*, *Staph. saprophyticus* and *Staph. sciuri* strains were resistant to as many as four antibiotics, whereas the *Staph. epidermidis* and *Staph. pasteuri* strains were resistant to as many as five antibiotics.

The staphylococci isolates were tested for resistance to QAC-based and oxidative disinfectants. Figure 1 shows box plots that represent the distribution of the MIC values for each microbial species. The median MIC values for ADBAC and DDAC ranged between 2·44–4·88 and 0·61–2·44 μg ml^{-1}, respectively. Of the 321 isolates, 54

Table 2 Amino acid decarboxylase activity* of the isolates

Species	his	tyr	lys	orn	his–tyr	his–orn	lys–tyr	lys–orn	tyr–orn	his–lys–tyr	his–tyr–orn	lys–tyr–orn	his–lys–tyr–orn
Staphylococcus aureus (*n* = 86)	12	31	7	3									
Staphylococcus epidermidis (*n* = 78)	9	33	2	9									
Staphylococcus saprophyticus (*n* = 71)	3				8	5	9	12	25				
Staphylococcus pasteuri (*n* = 36)		9		20									
Staphylococcus sciuri (*n* = 25)						1	1			5	2	13	
Staphylococcus warneri (*n* = 14)										1		9	3
Staphylococcus xylosus (*n* = 11)													10
Total	24	73	9	32	8	6	10	12	25	6	2	22	13

his, histidine; tyr, tyrosine; lys, lysine; orn, ornithine.
*No. of positive strains.

Table 3 Antibiotic resistance of the *Staphylococcus* strains

Antibiotic	Total	(%)	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus saprophyticus*	*Staphylococcus pasteuri*	*Staphylococcus sciuri*	*Staphylococcus warneri*	*Staphylococcus xylosus*
Tetracycline	65	20·2*	10·5	2·6	28·2	44·4	60·0		45·5
Ampicillin	150	46·7	26·7	41·0	47·9	72·2	72·0	50·0	63·6
Penicillin G	159	49·5	31·4	43·6	47·9	80·6	72·0	50·0	81·8
Erythromycin	70	21·8	12·8	5·1	19·7	72·2	52·0	28·6	
Methicillin	7	2·2	3·5	6·4					
Gentamicin	19	5·9	4·7	9·0		22·2	12·0		

*Percentage of resistant strains.

Table 4 Incidence of *Staphylococcus* spp. strains resistant to multiple antibiotics

Species	R strains (%)	No. strains resistant to no. antibiotics				
		1	2	3	4	5
Staphylococcus aureus	40·7	7	17	8	3	
Staphylococcus epidermidis	51·3	8	25	5		2
Staphylococcus saprophyticus	54·9	7	13	5	16	
Staphylococcus pasteuri	83·3	1	8	6	14	1
Staphylococcus sciuri	72·0			5	13	
Staphylococcus warneri	42·9		2	4		
Staphylococcus xylosus	81·8	2	2	5		

strains had MIC values that were above 4·88 μg ml^{-1} for ADBAC. *Staphylococcus aureus* was the most tolerant species, although tolerance was also seen for the *Staph. epidermidis*, *Staph. pasteuri*, *Staph. saprophyticus* and *Staph. sciuri* isolates; four *Staph. pasteuri* and four *Staph. saprophyticus* strains had an MIC for ADBAC of 19·53 μg ml^{-1}. There were no strains that were resistant to DDAC, as they could only grow below a DDAC concentration of 2·44 μg ml^{-1}. The median values for QAC-1, which is a commercial product that contains 0·2% benzalkonium chloride (BC), ranged between 625 and 1250 μg ml^{-1}, and *Staph. epidermidis*, *Staph. pasteuri* and *Staph. saprophyticus* were the most resistant species. Approximately 30% of tested strains were resistant to QAC-1. Similar to DDAC, all of the staphylococci strains that were tested were sensitive to QAC-2, which is a commercial product that contains 1·2% DDAC.

The most frequent MIC value for OX-1, which is a commercial mixture of hydrogen peroxide and citric acid, was 156·25 ppm. All of the staphylococcal strains that were tested were sensitive to OX-2, and the majority of these isolates had an MIC value of 78·13 ppm. The most frequent MIC value for CL-1 was 625 ppm, and some *Staph. aureus* and *Staph. pasteuri* strains had MICs as high as 2500 ppm. The MICs for CL-2 were significantly higher. Most of the strains had an MIC of 5000 ppm, while a few strains had values as high as 10 000 ppm.

A majority of the strains had moderate adhesion (<20%) to stainless steel, while there was a relatively higher rate of adhesion to the Teflon® surface. There was much variability between the tested strains for their ability to adhere to the Teflon, and 12·5% of the strains had an adhesion ability that ranged between 20 and 40% (Table 5). Only a small amount (19·9%) of the strains showed moderate to strong biofilm formation on the polystyrene microtitre plates. The species that most readily formed a biofilm were *Staph. aureus*, *Staph. epidermidis* and *Staph. pasteuri*.

Discussion

Staphylococci are typically found in both fermented and nonfermented animal and plant food environments. Previous studies have mainly focused on the incidence and the characterization of *Staph. aureus* in foods and on the role played by species as *Staph. xylosus* and *Staph. carnosus* in proteolysis, lipolysis and flavour formation in fermented sausages. Currently, there is little information available regarding the incidence of CNS in food and the associated environments, as well as on the bacterial properties of the staphylococcal species that affect food safety.

In this study, we isolated 321 strains from RTE (ready-to-eat) foods, food-processing surfaces and food workers. The majority of the strains were identified as *Staph. aureus*. The high frequency of this pathogen in RTE foods represents a potential health hazard to consumers and indicates improper handling and cross-contamination (Chen *et al.* 2001). *Staphylococcus aureus* was also frequently found on the food preparation surfaces and the gloves of the workers, indicating that cross-contamination can also occur through these mediums, which could significantly influence the spread of food-related disease. An inadequately cleaned surface can contaminate any food that it comes into contact with, which can increase the microbial load of the food and decrease its shelf-life (Moore *et al.* 2001). *Staphylococcus aureus* was the most frequent species that was isolated from the catering workers; the incidence of *Staph. aureus* found in this study is

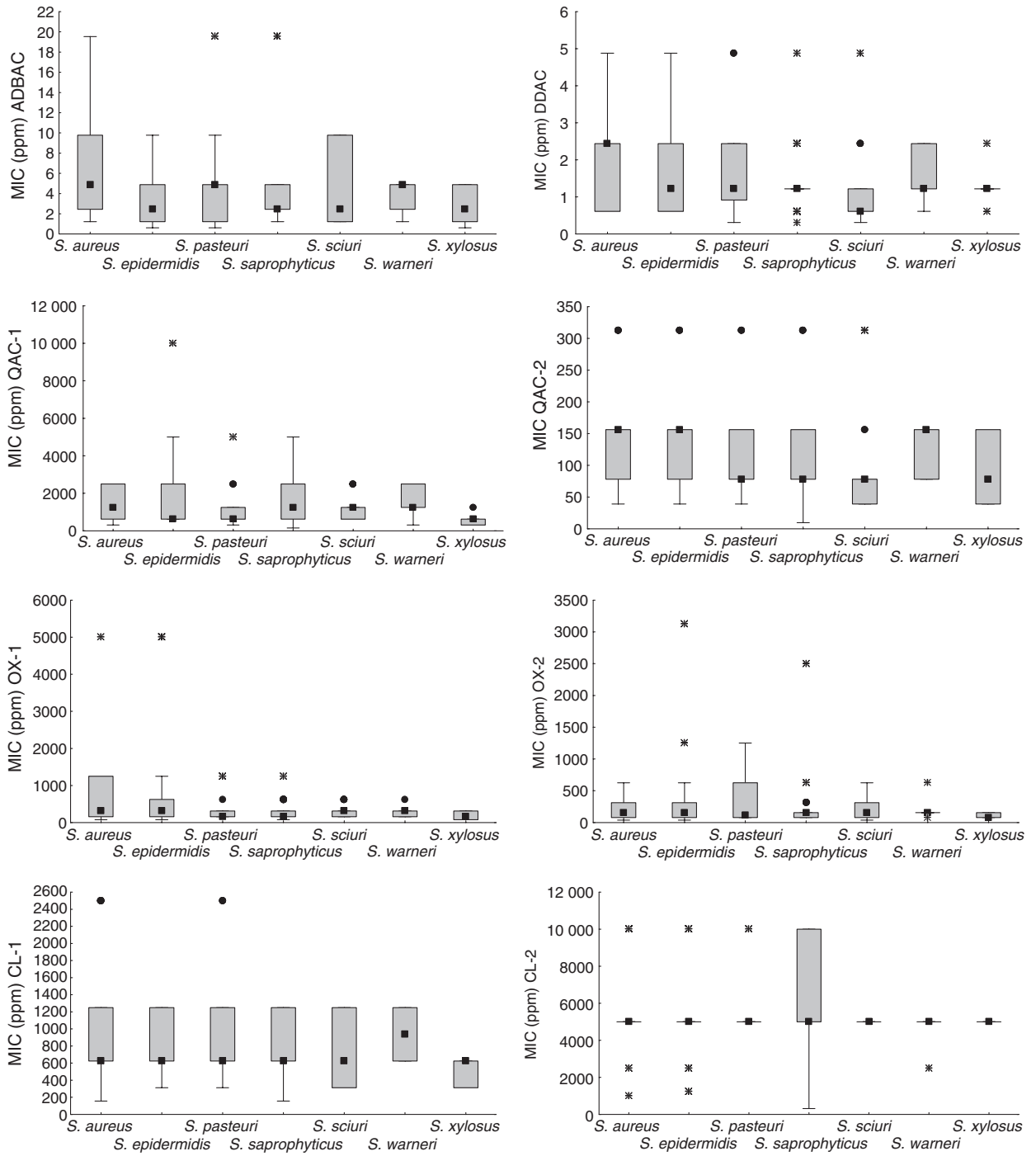

Figure 1 Disinfectant tolerance of the *Staphylococcus* strains. Each box plot represents the 25th and 75th percentiles (bottom and top of the box), the 5th and 95th percentiles (whiskers below and above box), the median (solid square), the outliers (solid dots) and the extremes (asterisks).

much higher than data from previously published reports where CNS were more prevalent than *Staph. aureus* on the hands of restaurant workers (Udo *et al.* 1999). This discrepancy may reflect differences in the distribution of staphylococcal flora on different body sites, as well as different distribution among populations that live in various geographical regions. The presence of *Staph. aureus* on the gloves of the catering staff emphasizes the possibility that food handlers play a major role in spreading foodborne disease.

Table 5 Adhesion and biofilm formation of the *Staphylococcus* strains

	Attachment stainless steel/Teflon				Biofilm-forming ability			
Species	<20	20 < *n* < 40	40 < *n* < 60	<60	Absent	Weak	Moderate	Strong
Staphylococcus aureus	86/83	0/3	0/0	0/0	7	58	15	6
Staphylococcus epidermidis	71/61	7/14	0/1	0/2	4	59	9	6
Staphylococcus saprophyticus	67/52	4/13	0/4	0/2	2	28	6	0
Staphylococcus pasteuri	36/30	0/2	0/4	0/0	4	51	8	8
Staphylococcus sciuri	23/17	2/4	0/4	0/0	3	19	3	0
Staphylococcus warneri	14/10	0/4	0/0	0/0	1	12	1	0
Staphylococcus xylosus	11/11	0/0	0/0	0/0	0	9	2	0
Total	308/264	13/40	0/13	0/4	21	236	44	20

Of the total 321 isolates identified in this study, 24·3 and 22·1% of the strains were *Staph. epidermidis* and *Staph. saprophyticus*; these CNS can be pathogenic because they produce enterotoxins, are resistant to several antibiotics and can form a biofilm (Gazzola and Cocconcelli 2008; Simeoni *et al.* 2008); 11·2% of isolates were identified as *Staph. pasteuri*, which is a species that is widely distributed in food and the environment, as well as on handling and cleaning tools that are used to prepare RTE foods (Christison *et al.* 2007; Simeoni *et al.* 2008).

Staphylococcus sciuri, *Staph. warneri* and *Staph. xylosus* are nonpathogenic species that are frequently found in fermented meats (i.e. fermented sausages), where their presence is usually considered as safe. However, several CNS isolated from food can carry enterotoxin genes, such as SED, SEC and TSST-1 (Rodriguez *et al.* 1996; Udo *et al.* 1999). In this study, these species were isolated from both the food and work surfaces, suggesting that cross-contamination can occur and is therefore a safety concern.

Decarboxylase activity was quite common in the *Staphylococcus* spp. that were isolated in this study, and >75% of the isolated strains could decarboxylate at least one amino acid, with tyrosine being the most frequent substrate. The ability of the staphylococci to decarboxylate amino acids has been well documented, but this information is limited to species isolated from fermented sausages (Drosinos *et al.* 2007; Landeta *et al.* 2007). Little information is currently available regarding the pathogenic potential of species frequently isolated from food, such as *Staph. epidermidis* and *Staph. saprophyticus*, to produce biogenic amines. Additionally, there are almost no data describing the amino acid decarboxylase activity of *Staph. aureus* and *Staph. pasteuri*. In this study, over 60% of the *Staph. aureus* strains showed decarboxylase activity. Positive strains were able to decarboxylase only one amino acid; tyrosine was the most common substrate, followed by histidine. The ability to decarboxylate only one amino acid was common also within *Staph. epidermidis* and *Staph. pasteuri*, while the other species isolated within this study could decarboxylate up to two (*Staph. saprophyticus*), three (*Staph. sciuri*) and four amino acids (*Staph. warneri* and *Staph. xylosus*). Interestingly, the ability to decarboxylate more than one amino acid at the same time was widespread mainly within species frequently found in fermented meat, where free amino acids are usually detected during the fermentation process (Simonová *et al.* 2006). Indeed, the presence of high amounts of free amino acids in fermented meats could act as a selective pressure and favour the growth of decarboxylative species well adapted to the substrate. It should be noted that if high concentrations of biogenic amines can be present mainly in fermented foods, the potential role of amino acid decarboxylative flora in nonfermented food should not be underestimated. In fact, high levels of biogenic amines have been detected in fish, fruit, juices, vegetables, meat and milk (Önal 2007). Moreover, the results of this study clearly show that staphylococcal strains potentially capable to produce biogenic amines could be widespread in environments where nonfermented foods are processed.

All of the isolates that were obtained in this study were susceptible to rifampicin and vancomycin, which agrees with previously published data for staphylococci isolated from food (Acco *et al.* 2003; Kastner *et al.* 2006). Resistance to rifampicin or vancomycin is often found in clinical strains (Srinivasan *et al.* 2002) because these antibiotics are commonly prescribed to treat human disease. Recently, vancomycin-resistant *Staph. epidermidis* strains were isolated from cured pork meat (Simeoni *et al.* 2008). A majority of the isolates were resistant to penicillin G and ampicillin. Penicillin resistance typically predicts susceptibility to other β-lactamase antimicrobial agents (e.g. ampicillin); indeed, resistance to these antibiotics was similar among all of the species that were isolated in this study. Resistance to penicillin and ampicillin was widespread in accordance with natural resistance for

β-lactams of staphylococcus induced by exposure to penicillins (Werckenthin *et al.* 2001). In fact, several staphylococci from several food sources showed an high incidence of resistance to these two β-lactams (Acco *et al.* 2003; Gundogan *et al.* 2005; Moon *et al.* 2007; Pereira *et al.* 2009). Penicillin resistance is plasmidic, and therefore, it spreads out very quickly to several other strains. Unlikely, methicillin resistance is chromosomic, and therefore, its diffusion is slower than the former (Pesavento *et al.* 2007). In this study, methicillin resistance was only detected for *Staph. aureus* and *Staph. epidermidis*. In fact, isolation of methicillin-resistant staphylococci (MRSA) from food is rare (Pesavento *et al.* 2007; Sawant *et al.* 2009). The only MRSA that were identified in this study were isolated from the food handlers. This finding is significant because food workers that carry MRSA have been identified to be a major cause of community-acquired foodborne outbreaks (Kluytmans *et al.* 1995; Jones *et al.* 2002). Moreover, the presence of methicillin-resistant *Staph. epidermidis* on the workers may lead to severe infections in immunocompromised hosts (Walther and Perreten 2007). Therefore, the spread of MRSA by food or food handlers should be prevented.

Resistance to tetracycline and erythromycin was observed for >20% of the isolates, especially for the CNS. The *Staph. aureus* strains were generally resistant to penicillin, neomycin and gentamicin, whereas the CNS were additionally resistant to tetracycline, chloramphenicol, erythromycin and lincomycin (Perreten *et al.* 1998). Only a small number of the *Staph. aureus*, *Staph. epidermidis*, *Staph. pasteuri* and *Staph. sciuri* strains were resistant to gentamicin. Faria *et al.* (2009) previously reported that there is a low incidence of reduced susceptibility (intermediary or resistance phenotype) for CNS (mainly *Staph. pasteuri* and *Staph. saprophyticus*) that were isolated from waste and drinking water.

The frequency of antibiotic resistance varied between the species and was the highest in *Staph. pasteuri* and *Staph. xylosus*. *Staphylococcus aureus* had the lowest frequency of resistant strains, accordingly to Werckenthin *et al.* (2001) who previously showed that CNS were phenotypically less susceptible to antimicrobial agents than coagulase-positive staphylococci. In contrast, Simeoni *et al.* (2008) reported that there was a similar pattern of antibiotic resistance genes between the CNS and *Staph. aureus* isolates. These discrepancies between phenotypic resistance and the presence of a specific gene are not infrequent, as the antibiotic resistances may constitute an intrinsic character, or it may be the result of possible unknown resistance genes (Perreten *et al.* 1998; Hummel *et al.* 2007). Various frequencies of antibiotics resistance, ranging from 20% to 90%, have been reported for *Staph. aureus* and CNS from different sources (Gundogan *et al.* 2005; Kastner *et al.* 2006). In this study, a high incidence (83·3%) of antibiotic resistance was found for *Staph. pasteuri*. This species appears to be ubiquitous, as it is commonly found in food-associated and aquatic environments; previous studies have shown that this species has a high frequency of resistance (Simeoni *et al.* 2008; Faria *et al.* 2009; Savini *et al.* 2009). Other CNS species that are widely distributed in food and the environment, such as *Staph. xylosus* and *Staph. sciuri*, had a high frequency of antibiotic resistance in our study. A high frequency of strains that were resistant to multiple antibiotics (i.e. 4 or 5) was observed for *Staph. warneri*, *Staph. xylosus*, *Staph. aureus*, *Staph. saprophyticus*, *Staph. sciuri*, *Staph. epidermidis* and *Staph. pasteuri*. Several researchers have previously hypothesized that the presence of antimicrobial substances places environmental pressure on the bacteria and thus selects for resistant phenotypes (McMahon *et al.* 2007; Hawkey 2008; Faria *et al.* 2009). Antibiotic resistance in CNS and *Staph. aureus* may cause health problems in humans because these species can cause nosocomial and bloodstream infections.

In this study, staphylococci were tested for their tolerance to QAC-based and oxidative sanitizers. Most strains had MIC values for ADBAC and DDAC that were within the standard range for commercial QACs (i.e. 0·5–5·0 μg ml^{-1}) (Petrocci 1983). However, 16·8% of the strains had MIC values that were >4·88 μg ml^{-1} for ADBAC and are defined as tolerant (Bjorland *et al.* 2003). Besides the staphylococci, QAC tolerance is present in many microbial groups, including the lactic acid bacteria, enterobacteria, *Listeria monocytogenes* and *Pseudomonas* species (Sidhu *et al.* 2002). *Staphylococcus aureus* was the most tolerant species, although other species were tolerant as well. Four *Staph. pasteuri* and four *Staph. saprophyticus* isolates had an MIC of 19·53 μg ml^{-1}. Tolerance to DDAC lacked within strains that grew until a concentration of 2·44 μg ml^{-1}. The different behaviour of the strains towards the two QAC compounds (ADBAC and DDAC) can be attributed to the different mechanisms of antimicrobial action. Indeed, the ADBAC molecules form a single monolayer that covers the *Staph. aureus* cells at the end of primary uptake, whereas the DDAC forms a double monolayer (Ioannou *et al.* 2007).

Approximately 30% of tested strains were QAC-1 tolerant, as strains that have MIC values >4 μg ml^{-1} for BC are considered as tolerant (Bjorland *et al.* 2003). The BC tolerance of the staphylococci likely contributes to survival in a food-processing environment. Moreover, staphylococci tolerance to BC is concerning because tolerance is associated with an increase in resistance to β-lactams of food-related staphylococci and methicillin-resistant *Staph. aureus* (Sidhu *et al.* 2001). All of the staphylococci strains that were tested were sensitive to

QAC-2, which is a commercial product that contains DDAC. If the concentration of DDAC that is present within QAC-2 is considered, then the MICs values are within the range quoted for commercial QACs. Several QAC-tolerant strains showed phenotypic resistance to erythromycin, tetracycline, ampicillin and penicillin. Cross-resistance to QACs and various antibiotics has already been reported for various bacterial species, including *Staphylococcus* spp. isolated from the food industry, *Pseudomonas aeruginosa* and *Escherichia coli*. Such cross-resistance seemed to be mediated by nonspecific multidrug efflux pumps encoded by various *qac* genes located frequently on plasmids and that can be highly mobile within and between different bacterial species (Heir *et al.* 1999; Bjorland *et al.* 2007). This may lead to the survival, growth and spread of enterotoxic staphylococci and/or staphylococci that may be clinically significant. Besides, as QAC-based compounds are used for numerous industrial purposes, long-term exposure of the environment to QACs might expose microbial communities to subinhibitory concentrations causing emergence of more resistant clones with changes in their susceptibility also to other antimicrobial agents (McBain *et al.* 2002).

The effect of peracids (mixtures of hydrogen peroxide and organic acids) towards the staphylococci was also studied. These new hydrogen peroxide formulations represent a way to stabilize and accelerate the biological activity of unformulated H_2O_2, which has weak and slow microbicidal activity. The staphylococci strains tested in this study were not tolerant to OX-1 (a commercial mixture of hydrogen peroxide and citric acid), as the recommended concentration of percitric acid is between 0·5 and 2% (Bal *et al.* 2006). To date, no information is available regarding the activity of percitric acid towards vegetative bacterial forms. OX-2, which is a mixture of peracetic acid and hydrogen peroxide, is a strong oxidizing agent that is effective against bacteria, yeast, fungi and bacterial spores; this disinfectant is currently widely used by the food and beverage industries (Stanga 2010). All of the staphylococcal strains that were tested were sensitive to OX-2. The MIC values were significantly lower than those reported by Vessoni Penna *et al.* (2001), who reported a peracetic acid MIC of 4620 μg ml^{-1} for *Staph. aureus*. The efficacy of peracetic acid against staphylococci is a concern because this microbial group can form biofilms on several surfaces. Another concern is that gene expression of exotoxins, which can cause food poisoning and toxic shock syndrome, may be stimulated by peracetic acid and hydrogen peroxide (Chang *et al.* 2006).

In this study, staphylococcal strains were evaluated with two commercial products that release free chlorine in the form of hypochlorous acid: sodium dichloroisocyanurate dihydrate (CL-1) and sodium hypochlorite (CL-2). Chlorination is an effective disinfecting process for food and food-processing environments which has been widely used since the 19th century; however, excessive use (hyperchlorination) can cause several environmental and human health problems. The MIC values for CL-1 were approximately 625 ppm, while the MICs for CL-2 were significantly higher. Only a few strains had values that are consistent with the recommended concentration for commercial preparation. High tolerance of *Staph. aureus* to chlorine has been associated with the ability of the bacteria to attach to surfaces and produce slime (Bolton *et al.* 1988).

The staphylococci strains were tested for their ability to adhere to stainless steel and Teflon® surfaces, which are materials that are widely used in food-processing environments. Biofilm formation was also measured by determining adhesion to polystyrene microtitre plates. It is well known that the ability of a micro-organism to quickly attach to surfaces is critical for survival during cleaning processes (Bore and Langsrud 2005). A majority of the strains showed moderate adhesion to stainless steel, while a relatively higher rate of attachment was observed for the Teflon® surface. Adhesion, which is the first step in biofilm formation, is mainly governed by physiochemical interactions between the material surface and bacterial surface (Di Bonaventura *et al.* 2008); in staphylococci, the bacterial surface characteristics are associated with the strain rather than the species (Planchon *et al.* 2007). In this study, 19·9% of the *Staph. aureus*, *Staph. epidermidis* and *Staph. pasteuri* strains had moderate to strong biofilm formation on the polystyrene microtitre plates, according to the criteria of Stepanović *et al.* (2000). While numerous species, including *Staph. aureus* and *Staph. epidermidis*, are known to form biofilms in food environments, to our knowledge, this is the first report that describes biofilm formation by *Staph. pasteuri*. Bacterial attachment to various surfaces poses a serious risk for the food industry. Moreover, biofilm production has been recently reported to be a marker of pathogenic potential for the staphylococci and multidrug-resistant staphylococci (Jain and Agarwal 2009).

In conclusion, the results of this study clearly show that staphylococci contamination of foods and food environment is a concern that should be continually monitored. Bacterial characteristics that influence the food safety, such as biogenic amine production, antibiotic and sanitizer resistance, and biofilm formation are quite common within this microbial genus, and these traits may influence bacterial survival and contamination of food-processing environments. In particular, a relatively high occurrence of QAC-tolerant strains has been evidenced within staphylococcal isolates, and in several cases, this feature was present in antibiotic-resistant strains. As the

selection of disinfectant-tolerant strains can be linked to the spread in the environment of strains resistant to clinically important antimicrobial agents, such characteristic should be regarded as a food safety concern.

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