Characterization of coagulase-negative staphylococci from brining baths in Germany

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ABSTRACT

Brining is an important step in cheese making, and using brine baths for this purpose is common practice in German dairies. Time of brining, brine concentration, and composition of the complex and heterogeneous microbiota, including coagulase-negative staphylococci (CNS), contribute to the ripening and taste of cheese. As well as producing staphylococcal enterotoxins, some CNS show antibiotic resistance; therefore, we isolated 52 strains of presumptive CNS from cheese brines from 13 factories in Germany. Species identification by *sodA* gene sequencing revealed that 50 isolates were CNS: 31 *Staphylococcus saprophyticus*, 4 *Staphylococcus carnosus*, 4 *Staphylococcus equorum*, 3 *Staphylococcus sciuri*, 2 *Staphylococcus hominis*, and 2 *Staphylococcus warneri*. One isolate each was identified as *Staphylococcus epidermidis*, *Staphylococcus pasteurii*, *Staphylococcus succinus*, and *Staphylococcus xylosus*. Further subtyping of the *Staph. saprophyticus* isolates to the subspecies level revealed the presence of 6 *Staph. saprophyticus* ssp. *saprophyticus*. Using pulsed-field gel electrophoresis with the identified *Staph. saprophyticus* strains, 12 independent clones were identified, resulting in the exclusion of 18 strains from further testing. In 19 of the remaining 32 CNS isolates, resistance to antibiotics was observed. Resistance was found against oxacillin (17), penicillin (5), and cefoxitin (1). Four isolates expressed resistance to both oxacillin and penicillin. No resistance was found to enrofloxacin, tetracycline, gentamicin, or erythromycin. Then, PCR analysis for antibiotic resistance genes was performed for 22 different genes. Only genes *bla_Z* and *bla_{TEM}* were found in 7 isolates. These isolates were selected for challenge tests with different concentrations of lactic acid and NaCl to examine whether expression of antibiotic resistance was influ-

enced by these stressors. An increase in the minimal inhibitory concentration from 0 to 2.0 µg/mL was seen for trimethoprim/sulfamethoxazole only in one isolate of *Staph. saprophyticus* at an increased lactic acid concentration. Finally, all isolates were tested for genetic determinants (*entA*, *entB*, *entC*, *entD*, and *entE*) of the most common staphylococcal enterotoxins; none of these genes were detected. We found no indication for unacceptable risks originating from the isolated CNS.

Key words: antibiotic resistance, coagulase-negative staphylococci (CNS), brining bath, enterotoxin

INTRODUCTION

Brining baths in cheese factories are used to add salt and other components to freshly pressed and formed cheese for conservation, ripening, and taste. The conditions of the brining process depend on the size, shape, and desired salt content of the cheese being manufactured. The concentration of NaCl in brine varies from 17 to 22%, pH is largely influenced by the pH of the raw cheese (usually from 5.2 to 5.4), and temperature ranges between 13 and 15°C. All parameters depend on the process of cheesemaking and the desired type of cheese. The brining process takes from a few hours to several days. The microbiota in the bath includes lactobacilli, lactococci, enterococci, staphylococci, streptococci, several yeasts and fungi; it contributes to the initiation of ripening and has major influence on taste, shelf life, and safety of the produced cheese (Bockelmann and Hoppe-Seyler, 2001; Sutherland, 2002; Montel et al., 2014). Characteristic of brining baths is their very stable microbial composition (Bockelmann and Hoppe-Seyler, 2001), which ensures constant ripening and maintenance of the cheese produced in that bath. Once such a microbiota is established, the baths may be used for years or decades without changes (Neve et al., 2005; >25 yr of own experience), except for the addition of salt and water to maintain the salt concentration, and for filtration to remove curd particles. Coagulase-negative staphylococci (CNS) are often part of the microbiota because of their high salt tolerance (Bockelmann and Hoppe-Seyler, 2001; Mounier et al.,

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2007). The CNS that are present in brining baths are a potential reservoir for microbiotic transfer because identical clones isolated from brine baths have been found in retail cheeses (Mounier et al., 2006).

The CNS are considered to harbor a variety of genes encoding antibiotic resistance, including *mecA* and *mecC* (IWG-SCC, 2009; Fontes et al., 2013; Chajęcka-Wierzchowska et al., 2015). Besides *mecA* and *mecC*, other *mec* genes, such as *mecB* and *mecD*, have been identified in *Macrococcus caseolyticus* (Baba et al., 2009; Schwendener et al., 2017). It has been shown that bacteria are able to distribute genes to other species of the same genus or to other genera (Alonso et al., 2001; Resch et al., 2008). Especially in industrial settings, cheese output can exceed 100,000 kg/d. If CNS carrying genes that encode antibiotic resistance are present in the brine baths, this might lead to considerable release and distribution of these genes. Sublethal stress may influence the phenotypic expression of antibiotic resistance of bacteria. Stress factors, including pH, salt, or temperature, used in conservation or production of food have been shown to elevate or reduce the expression of antibiotic resistance of *Escherichia coli*, *Salmonella* Typhimurium, *Staphylococcus aureus*, and *Listeria monocytogenes* (McMahon et al., 2007; Al-Nabulsi et al., 2015).

An additional undesired property of some CNS is the ability to produce staphylococcal enterotoxins. With regard to milk and milk products, genes encoding staphylococcal enterotoxins have been shown for CNS (species not given) from soft cheese (Fontes et al., 2013), for *Staph. capitis*, *Staph. equorum*, *Staph. lentus*, *Staph. simulans*, and *Staph. xylosus* from goat milk, whey, and cheese (Vernozy-Rozand et al., 1996b); for *Staph. chromogenes*, *Staph. hyicus*, *Staph. warneri*, and *Staph. xylosus* isolated from raw cow milk (Oliveira et al., 2010); as well as for *Staph. piscifermentans*, *Staph. saprophyticus*, *Staph. sciuri*, and *Staph. xylosus* from Minas frescal cheese (fresh cheese, cow milk; Nunes et al., 2016); and for *Staph. epidermidis*, *Staph. hyicus*, and *Staph. warneri* of dairy origin (Rodrigues et al., 2017).

The objective of this study was to characterize CNS isolated from brining baths of different cheese factories in Germany in terms of virulence factors and potential antibiotic resistance, and to test whether antibiotic resistance is influenced by sublethal stress.

MATERIALS AND METHODS

Brine Bath Samples and Isolation of Staphylococci

Brine bath samples from 13 German dairies were provided by Horst Neve (Department of Microbiology and

Biotechnology, Max Rubner-Institut, Kiel, Germany). For isolation of staphylococci, 5 mL of brine fluid was centrifuged at $14,000 \times g$ at 4°C for 10 min. The pellet was washed twice with 5 mL of 0.85% NaCl (wt/vol), applying the same centrifugation conditions. The final pellet was resuspended in 5 mL of NaCl-peptone [0.85% NaCl, 0.1% (wt/vol) tryptone; Oxoid, Wesel, Germany]. Of this suspension, $2 \times 50 \mu\text{L}$ and $2 \times 100 \mu\text{L}$ aliquots were surface spread onto Baird-Parker agar (Baird-Parker agar base supplemented with 5% (vol/vol) egg yolk tellurite emulsion; both from Oxoid), and incubated for 48 h at 37°C. Typical colonies (one of each type) were streaked onto blood agar (Columbia blood agar base supplemented with 5% (vol/vol) defibrinated sheep blood; both from Oxoid), and incubated for 24 h at 37°C. All black or gray colonies with or without a zone of egg yolk precipitation or clearance were regarded as typical on Baird-Parker agar.

Identification of Species

Isolates from blood agar were identified to the genus level by Gram stain, catalase test, and subculture onto furazolidone agar (von Rheinbaben and Hadlok 1981). As phenotypic tests for species identification, hemolysis, coagulase test with coagulase rabbit plasma with EDTA (Becton Dickinson, Heidelberg, Germany), and API 32 ID STAPH (bioMérieux, Nürtingen, Germany) were applied.

For genotypic identification of species, amplification and partial sequencing of the *sodA* gene was applied, following the method described by Poyart et al. (2001). For amplification, primers *sodAd1* and *sodAd2* were used. The PCR products were purified following the GeneJET gel extraction protocol (Thermo Fisher Scientific, Carlsbad, CA). The amplicons were cloned into the pJET1.2/blunt vector following the protocol provided by Thermo Scientific. Sanger sequencing (Sanger and Coulson, 1975) was carried out at LGC Genomics GmbH (Berlin, Germany). As proposed by Poyart et al. (2001), the obtained sequences were trimmed for the ~429-bp fragment within the *sodA* gene with the dDNAis Max software (Hitachi Software Engineering Co. Ltd., Tokyo, Japan) and analyzed with BLAST (Zhang et al., 2000). The species were determined according to the maximum score.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was applied to type isolates of *Staph. saprophyticus*. For PFGE, the protocol supplied by the Centers for Disease Control PulseNet was used with slight modifications (CDC, 2015). Modifications were as follows: 1.5 mL of lysis

buffer was used instead of 3 mL, and 5 μ L of lysostaphin solution was used instead of 4 μ L. Electrophoresis was performed after restriction with *Sma*I (Fermentas, St. Leon-Rot, Germany) in a Chef-DR III system (Bio-Rad Laboratories GmbH, Munich, Germany). Running conditions were 16.5 h at 14°C, angle 120°, 6 V/cm, and pulse time 5/40 s. The gels were digitally photographed in an UV-transilluminator (Gel Doc 2000, Bio-Rad Laboratories GmbH). Cluster analysis was performed with GelComparII, version 6.50 (Applied Maths, Sint-Martens-Latem, Belgium) using the following settings: 1% optimization, 1.2% position tolerance. The similarity between band positions was determined by Dice similarity coefficient. For construction of dendrograms, the unweighted pair group method using arithmetic averages (UPGMA) algorithm was selected. For control, *Staph. saprophyticus* DSM 20229 was used.

Antimicrobial Susceptibility Testing

For screening of antibiotic susceptibility, the disk diffusion test as described by the Clinical and Laboratory Standards Institute (CLSI, 2008), was applied. For practical reasons, only one substance of each main group of antibiotics was used. Disks and loads were used as follows: cefoxitin (30 μ g), enrofloxacin (5 μ g),

erythromycin (15 μ g), gentamicin (10 μ g), oxacillin (1 μ g), penicillin G (10 U), and tetracycline (30 μ g) (all from Oxoid). For evaluation, breakpoints as proposed by CLSI (2008) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019) were used.

In addition, MIC were determined utilizing the microdilution method provided by CLSI (2008). The MIC for all strains were determined for oxacillin and the respective antibiotics for all strains showing genetic determinants of antibiotic resistance. Again, the breakpoints as provided by CLSI (2008) and EUCAST (2019) were applied for evaluation.

Genetic Determinants for Antibiotic Resistance, and Production of Enterotoxin and Coagulase

Reference strains, primers, and PCR conditions for all PCR reactions applied for detection of genetic determinants of antibiotic resistance, enterotoxin production, and coagulase are listed in Tables 1 and 2.

The DNA extraction from samples was performed as follows: Isolates were incubated on blood agar at 36°C for 24 h. Single colonies were picked and incubated in 5 mL of the medium showing the best growth [brain heart infusion broth (BHI), Todd-Hewitt broth

Table 1. Bacterial strains used as controls in PCR for detection of enterotoxin, antibiotic resistance, and coagulase genes

Species	Strain no.	Origin	Target
<i>Staphylococcus aureus</i>	722	FRI ¹	<i>entA</i>
<i>Staph. aureus</i>	243	FRI	<i>entB</i>
<i>Staph. aureus</i>	137	FRI	<i>entC₁</i>
<i>Staph. aureus</i>	1151m	FRI	<i>entD</i>
<i>Staph. aureus</i>	326	FRI	<i>entE</i>
<i>Enterococcus faecium</i>	FAIR-E 25	MBT ²	<i>ermB</i> ; <i>msrA/B</i>
<i>Ent. faecium</i>	FAIR-E 151	MBT	<i>ermB</i> ; <i>msrA/B</i>
<i>Ent. faecium</i>	FAIR-E 225	MBT	<i>tet</i> (M)
<i>Staph. aureus</i>	11822	DSM ³	<i>mecA</i> , <i>coa</i>
<i>Staph. aureus</i>	BAA-2312	ATCC ⁴	<i>mecC</i>
<i>Enterococcus faecalis</i>	V 583	EUURL-AR ⁵	<i>vanB</i>
<i>Staph. aureus</i>	pT181-like	EUURL-AR	<i>tet</i> (K)
<i>Staph. aureus</i>	pSTS9-like	EUURL-AR	<i>tet</i> (L)
<i>Staph. aureus</i>	E 19771 tpA	EUURL-AR	<i>blaZ</i>
<i>Staph. aureus</i>	1206::Tn554	EUURL-AR	<i>ermA</i>
<i>Bacillus subtilis</i>	B.3HU104/pE194	EUURL-AR	<i>ermC</i>
<i>Staphylococcus hyicus</i>	7711211-1	EUURL-AR	<i>cfr</i>
<i>Escherichia coli</i>	31 Holland	EUURL-AR	<i>bla_{TEM}</i>
<i>E. coli</i>	TG1/pUC18	EUURL-AR	<i>bla_{FOX}</i>
<i>E. coli</i>	J53/pMG247	EUURL-AR	<i>bla_{DHA}</i>
<i>E. coli</i>	SR225	EUURL-AR	<i>aac</i> (3'')-IV
<i>Klebsiella pneumoniae</i>	23823	EUURL-AR	<i>aac</i> (3'')-II
<i>E. coli</i>	K2	EUURL-AR	<i>ant</i> (2'')-I
<i>Staphylococcus saprophyticus</i>	20229	DSM	Negative control

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³German Collection of Microorganisms and Cell Cultures (Leibniz Institute, Braunschweig, Germany).

⁴American Type Culture Collection (Manassas, VA).

⁵European Union Reference Laboratory-Antimicrobial Resistance (Lyngby, Denmark).

Table 2. List of primers and annealing temperatures used for the PCR in this study

Gene	Primer name	Primer sequence ¹	Annealing temperature (°C)	Reference
Virulence factors				
<i>coa</i>	staph-coa1	ACCACAAGGTACTAGATCAACG	60	Fontes et al. (2013)
	staph-coa2	TGCTTTTCGATTGTTTCGATGC		
<i>entA</i>	staph-sea1	GCAGGGAACAGCTTTAGGC	59	
	staph-sea2	GTTCTGTAGAAGTATGAAACA		
<i>entB</i>	staph-seb1	ACATGTAATTTTGATATTTCGCACTG	60	
	staph-seb2	TCGAGGCATCATGTCATACCA		
<i>entC</i>	staph-sec1	CTTGTATGTATGGAGGAATACAA	51	
	staph-sec2	TGCAGGCATCATATCATACCA		
<i>entD</i>	staph-sed1	GTGGTGAATAGATAGGACTGC	60	
	staph-sed2	ATATGAAGGTGCTCTGTGG		
<i>entE</i>	staph-see1	AGGTTTTTTCACAGGTCAATCC	60	Rall et al. (2008)
	staph-see2	CTTTTTTTTCTTCGGTCAATC		
Species identification				
<i>sodA</i>	sodAd1	CCITAYICITAYGAYGCIYTIGARCC	37	Poyart et al. (2001)
	sodAd2	ARRTARTAIGCRTGYTCCCAIACRTC		
Antibiotic resistance				
<i>tet</i> (M)	tet(M) fw	GTGGACAAAGGTACAACGAG	60	Liu et al. (2012)
	tet(M) rv	CGGTAAAGTTTCGTCACACAC		
<i>tet</i> (K)	tet(K) fw	TTATGGTGGTTGTAGCTAGAAA	52	Gevers et al. (2003)
	tet(K) rv	AAAGGGTTAGAACTCTTGAAA		
<i>tet</i> (L)	tet(L) fw	GTMGTTGCGCGCTATATTCC	60	
	tet(L) rv	GTGAAMGRWAGCCCACCTAA		
<i>msrA/B</i>	msrA/B fw	GCAAATGGTGTAGGTAAGACAACCT	51	
	msrA/B rv	ATCATGTGATGTAAACAAAAT		
<i>ermB</i>	ermB fw	CATTTAACGACGAAACTGGC	52	
	ermB rv	GGAACATCTGTGGTATGGCG		
<i>ermA</i>	ermA fw	TATCTTATCGTTGAGAAGGGATT	53	Liu et al. (2012)
	ermA rv	CTACACTTGGCTTAGGATGAAA		
<i>ermC</i>	ermC fw	CTTGTTGATCACGATAATTTCC	55	
	ermC rv	ATCTTTTAGCAAACCCGATTTC		
<i>vanB</i>	vanB-P1	TTGCATGGACAAATCACTGG	52	Stinear et al. (2001)
	vanB-P2	GCTCGTTTTCTGTATGGATG		
<i>cfr</i>	cfr fw	TGAAGTATAAAGCAGGTTGGGAGTCA	58	Kehrenberg and Schwart (2006)
	cfr rv	ACCATATAATTGACCACAAGCAGC		
<i>mecA</i>	mecA-for	AAAATCGATGGTAAAGGTTGGC	60	Liu et al. (2012)
	mecA-rev	AGTTCTGCAGTACCGGATTTGC		
<i>mecC</i>	mecC fw	GAAAAAAGGCTTAGAACGCCTC	50	Stegger et al. (2012)
	mecC rv	GAAGATCTTTTCCGTTTTTCAGC		
<i>bla_{TEM}</i>	bla _{TEM} fw	GCGGAACCCCTATTTG	55	Olesen et al. (2004)
	bla _{TEM} rv	ACCAATGCTTAATCAGTGAG		
<i>bla_{CTX-M}</i>	bla _{CTX-M} fw	ATGTGCAGYACCAGTAARGTKATGGC	60	Hasman et al. (2005); Hendriksen et al. (2009)
	bla _{CTX-M} rv	TGGGTTRAARTARGTSACCAGAAYSAGCGG		
<i>bla_{DHA}</i>	bla _{DHA} fw	CTGATGAAAAAATCGTTATC	60	Yan et al. (2002)
	bla _{DHA} rv	ATTCAGTGCACCTCAAAATA		
<i>bla_{FOX}</i>	bla _{FOX} fw	CGAGCAGACSCGTGTTTCGAG	50	D'Andrea et al. (2006)
	bla _{FOX} rv	TTGGCCAGCATGACGATG		
<i>bla_Z</i>	bla _Z fw	CAAAGATGATATAGTTGCTTATTCTCC	50	Kaase et al. (2008)
	bla _Z rv	TGCTTGACCACTTTTATCAGC		
<i>aac</i> (3'')-IV	aac3-IV fw	GTTACACCGGACCTTGGA	60	Guerra et al. (2001)
	aac3-IV rv	AACGGCATTGAGCGTCAG		
<i>ant</i> (2'')-I	ant2''-I fw	ATCTGCCGCTCTGGAT	55	Akers et al. (2010)
	ant2''-I rv	CGAGCCTGTAGGACT		
<i>aac</i> (3'')-II	aac3-II fw	TGAAACGCTGACGGAGCCTC	60	Vliegenthart et al. (1989)
	aac3-II rv	GTGGAACAGGTAGCACTGAG		
<i>aac</i> (6')-aph(2')	aac-aph fw	TTGGGAAGATGAAGTTTTTAGA	53	Liu et al. (2012)
	aac-aph rv	CCTTTACTCCAATAATTTGGCT		

¹R = A and G; W = A and T; Y = C and T; S = G and C; M = A and C; K = G and T; I = A, C, G, and T

(**THB**), or tryptone soy broth (**TSB**); all from Oxoid], at 36°C for 24 h on a shaker (100 rpm). From each culture, 2 to 4 mL was centrifuged at 5,000 × *g* for 10

min. The supernatant was discarded and the cells were stored at −20°C. Cell-wall lysis was accomplished by adding 20 µL of lysostaphin (5 mg/mL; Sigma-Aldrich

Chemie GmbH, Munich, Germany) and 180 μ L of 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1.2% Triton (TET; AppliChem GmbH, Darmstadt, Germany) to the pellet. The mixture was incubated overnight at 37°C. The DNA was purified using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany). Departing from the protocol provided with the kit, 20 μ L of proteinase K and 200 μ L of buffer AL (both from the kit) were added to the mixture and incubated at 56°C for 30 min. Another incubation step at 95°C for 15 min was added. The next steps were carried out according to the protocol (QIAamp DNA Mini and Blood Mini Handbook 02/2015, p. 34–35, step 6–11; <https://www.qiagen.com/ch/resources/download.aspx?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en>). The concentration of DNA was measured with a NanoPhotometer (Implen GmbH, Munich, Germany). For the reference strains obtained from EURL-AR (Table 1), boiling lysates were used as positive controls for PCR as recommended. These were produced by picking 1 to 3 colonies grown on blood agar and dissolving them in TET solution. Afterward, the tubes were boiled for 10 min, centrifuged for 5 min at $20,000 \times g$, diluted 1:10 in TET solution, and stored at -20°C . The DNA of all other reference strains (Table 1) was purified as mentioned before.

Sample and reference DNA were diluted to 1 to 20 ng/ μ L before the PCR reactions were performed. The PCR were performed on a Labcycler (Sensoquest, Göttingen, Germany) in a final volume of 20 μ L containing 1 μ L of DNA as the template, 0.3 μ M each primer (Table 2, TIB MolBiol GmbH, Berlin, Germany), 0.4 μ M dNTPs, 1 U of HOT FIREPol DNA Polymerase (Solis BioDyne, Tartu, Estonia), $1\times$ reaction buffer B [0.8 M Tris-HCl, 0.2 M $(\text{NH}_4)_2\text{SO}_4$, 0.2% wt/vol Tween-20], and 2.5 mM MgCl_2 (all Solis BioDyne).

Challenge Tests

To test whether sublethal acidic and osmotic stress would change antibiotic susceptibility, 7 isolates that showed antibiotic resistance were chosen, and the tests were performed according to McMahon et al. (2007). Briefly, approximately 10^6 cfu of each isolate was inoculated in 10 mL of brain heart infusion broth (BHI) adjusted with lactic acid to different pH values (5.0, 7.0, 6.5, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0) or adjusted with NaCl to different salt concentrations (0, 3, 4, 4.5, 5, 5.5, 6, 10, 12, 13, 13, 15, 18%). For each strain, the sublethal stress level was defined as the pH or NaCl concentration that reduced growth at least by 75% as measured by optical density at 600 nm (OD_{600}) after overnight incubation at 37°C.

For MIC testing at sublethal stress, BHI prepared as described above was used to inoculate commercially prepared microtiter plates containing 25 different antibiotics (Micronaut-S MVZ Saar GP2, Merlin Diagnostica, Bornheim, Germany). Antibiotics and loads per well are listed in Table 3. Testing was performed 3 times for each isolate.

RESULTS AND DISCUSSION

Brine samples from 13 cheesemaking facilities were examined for the presence of CNS. In total, 52 isolates were obtained and further characterized utilizing phenotypic and genotypic tests. A group of selected strains showing antibiotic resistance were used for challenge tests.

Identification of Species

All strains were gram-positive cocci with a positive catalase reaction. As expected for staphylococci, they did not grow on furazolidone agar containing 20 μ g/mL of the antibiotic, whereas micrococci would (von Rheinbaben and Hadlok 1981).

Partial sequencing of the *sodA* gene resulted in BLAST identifications between 99 and 100%. Two strains were identified as *Macrococcus caseolyticus*. Ten *Staphylococcus* species were identified (no. of isolates): *Staph. saprophyticus* (31), *Staph. carnosus* (4), *Staph. equorum* (4), *Staph. sciuri* (3), *Staph. hominis* (2), *Staph. warneri* (2), *Staph. epidermidis* (1), *Staph. pasteurii* (1), *Staph. succinus* (1), and *Staph. xylosus* (1). This is in good agreement with findings of Coton et al. (2010), who mostly detected *Staph. equorum*, *Staph. xylosus*, *Staph. epidermidis*, and *Staph. saprophyticus* on cheese; and with Organji et al. (2018), who found *Staph. equorum*, *Staph. epidermidis*, and *Staph. caprae* on cheese. Mounier et al. (2006) detected several CNS (*Staph. epidermidis*, *Staph. equorum*, *Staph. pasteurii*, *Staph. saprophyticus*) in the environment, in brine baths, and on cheese in a dairy that produced smear-ripened cheese. Furthermore, they found identical clones of *Staph. saprophyticus* in brine baths and on the respective cheeses.

Phenotypic testing using API 32ID STAPH showed congruent results to *sodA* sequencing in 20 cases. For 2 strains, no API profile could be obtained. Compared with *sodA* sequencing, 3 isolates were identified as *Staph. xylosus* instead of *Staph. equorum*. Four strains identified by sequencing as *Staph. carnosus* showed profiles of *Staph. saprophyticus* (2), *Staph. kloosii* (1), and *Staph. xylosus* (1). Further mismatches (*sodA* vs. API) were *Staph. succinus* versus *Staph. xylosus*, *Staph.*

Table 3. Antibiotics and respective loads per well onto commercially prepared microtiter plates for MIC testing

Substance	Abbreviation	Loads per well ($\mu\text{g/mL}$)
Ampicillin	AMP	8, 2
Ampicillin/sulbactame	AMS	8/4
Cefacolin	CEZ	8
Cefoxitin	COX	4
Ceftriaxone	CRO	2, 1, 0.5
Imipenem	IMP	4, 0.5, 0.125
Meropenem	MER	4, 0.5, 0.25
Oxacillin	OXA	2, 0.25
Penicillin	PEN	2, 0.125, 0.0625
Piperacillin	PIP	8
Piperacillin/tazobactam	PIT	8/4
Tetracycline	TET	4, 2
Tigecyclin	TGC	0.5
Linezolid	LIZ	4, 2
Erythromycin	ERY	0.5, 0.25
Daptomycin	DPT	4, 1
Clindamycin	CLI	0.5, 0.25
Vancomycin	VAN	4, 2, 1
Trimethoprim/sulfamethoxazole	T/S	2/38, 0.5/9.5
Ciprofloxacin	CIP	2, 1
Levofloxacin	LEV	2, 1
Rifampicin	RAM	1
Gentamicin	GEN	8, 4
Tobramycin	TOB	4
Fosfomycin	FOS	32

pasteurii versus *Staph. warneri*, and *Staph. hominis* versus *Staph. haemolyticus* (Table 4). Similar observations regarding differing results of species identification of the 2 methods were reported by Poyart et al. (2001), supporting a greater discriminative power of *sodA* sequencing compared with phenotypic testing.

After excluding 18 isolates of *Staph. saprophyticus* after testing with PFGE (see below), 32 isolates were tested further: 25 did not show hemolysis on blood agar, 3 exhibited α -hemolysis, and 4 β -hemolysis. All were coagulase negative according to phenotypic and genotypic tests.

As multiple strains of *Staph. saprophyticus* were isolated from different brine baths, 30 isolates were typed by PFGE to examine clonal relatedness. Twelve pulso-types were identified, which resulted in the exclusion of 18 strains from further testing (data not shown). *Staphylococcus saprophyticus* ssp. *saprophyticus* is a biosafety level 2 organism and should not be present in brine baths, whereas subspecies *bovis* is classified as a level 1 organism. According to Hájek et al. (1996) and the respective UK standard (Anonymous, 2014), the subtypes can be differentiated by phenotypic tests for nitrate reductase, pyrrolidonyl arylamidase, and D-ribose fermentation; *Staph. saprophyticus* ssp. *saprophyticus* is negative for all of these tests. Negative reactions in all tests were obtained with 6 of the isolates, indicating that these belong to the subspecies *saprophyticus*, whereas the remaining isolates showed

positive reactions with some of the tests and were left untyped (subspecies *bovis* should show positive results in all tests). As *sodA* sequencing did not help to identify the subtypes and *rpoB* sequencing is likewise not a useful additional test (Mellmann et al., 2006), an attempt to further subtype the questionable strains will be done by whole-genome sequencing.

A frequent occurrence of *Staph. saprophyticus* in cheese was reported by Nunes et al. (2016; 4 isolates from 10 samples of Minas frescal cheese), by Kürekci (2016; 6 isolates from 17 different cheese types in Turkey), and others (Vernozy-Rozand et al., 1996a; Irlinger et al., 1997; Delbès and Montel, 2005; Mounier et al., 2006). Typing to the subspecies level was not performed by any of the working groups cited above. The occurrence of *Staph. saprophyticus* ssp. *saprophyticus* in brine, as we have shown here, does not necessarily imply that these bacteria are transferred to cheese, although that is most likely (for review, see Irlinger et al., 2015). In fact, such a transfer has already been demonstrated (Mounier et al., 2006). Further experiments will reveal whether the clones we isolated from brine can be found on respective retail cheeses. As *Staph. saprophyticus* (without differentiation to the subspecies level) is the second most frequent causative organism of uncomplicated urinary tract infection (UTI) in women and can cause UTI in males of all ages (Raz et al., 2005), the occurrence of this organism in brine raises concerns regarding the safety of cheese. However, although

Table 4. Characterization of CNS isolates from brine baths in Germany

Factory	Strain no. ¹	sodA sequencing	API 32 ID STAPH ²	MIC-OXA ³		Disk diffusion assay ⁴							PCR (gene)
				µg/mL	R/S	OXA	CEF	PEN	ENR	TET	GEN	ERY	
1	101a	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.500	R	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	104a	<i>Staph. xylosus</i>	<i>Staph. xylosus</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	104b	<i>Staph. succinus</i>	<i>Staph. xylosus</i>	0.250	S	S	R	S	S	S	S	S	<i>bla</i> _{TEM}
	104g	<i>Staph. sciuri</i>	<i>Staph. sciuri</i>	0.500	R	R	R	S	S	S	S	S	<i>bla</i> _{TEM}
2	105a	<i>Staph. pasteurii</i>	<i>Staph. warneri</i>	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	105b	<i>Staph. sciuri</i>	<i>Staph. sciuri</i>	0.125	S	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	105c	<i>Staph. equorum</i>	<i>Staph. xylosus</i>	0.125	S	S	S	S	S	S	S	I	<i>bla</i> _{TEM}
	106a	<i>Staph. hominis</i>	<i>Staph. hominis</i>	0.060	S	S	S	S	S	S	S	S	<i>bla</i> _Z , <i>bla</i> _{TEM}
3	106b	<i>Staph. hominis</i>	<i>Staph. haemolyticus</i>	0.125	S	S	S	R	S	S	S	S	<i>bla</i> _Z , <i>bla</i> _{TEM}
	110d	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _Z , <i>bla</i> _{TEM}
	111d	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _Z , <i>bla</i> _{TEM}
	111h	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.250	S	R	S	S	S	S	S	S	<i>bla</i> _Z , <i>bla</i> _{TEM}
4	112a(BA)	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	112a(BP)	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	112g	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	114a	<i>Staph. equorum</i>	<i>Staph. xylosus</i>	0.250	S	R	S	S	S	S	S	I	<i>bla</i> _{TEM}
5	115b	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	116a	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	1.000	R	R	S	R	S	S	S	S	<i>bla</i> _{TEM}
	161	<i>Staph. carnosus</i>	<i>Staph. saprophyticus</i>	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	163	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	1.000	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
7	117a	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	0.250	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	118a	<i>Staph. carnosus</i>	<i>Staph. saprophyticus</i>	0.060	S	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	118b	<i>Staph. sciuri</i>	<i>Staph. kloosii</i>	0.060	S	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	119a	<i>Staph. epidermidis</i>	<i>Staph. sciuri</i>	0.060	S	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
9	119b	<i>Staph. warneri</i>	<i>Staph. epidermidis</i>	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	120c	<i>Staph. saprophyticus</i>	<i>Staph. warneri</i>	0.500	R	R	S	S	S	S	S	S	<i>bla</i> _{TEM}
	120i	<i>Staph. saprophyticus</i>	<i>Staph. saprophyticus</i>	1.000	R	R	S	R	S	S	S	S	<i>bla</i> _{TEM}
	121b	<i>Staph. carnosus</i>	<i>Staph. saprophyticus</i>	0.500	R	R	S	R	S	S	S	S	<i>bla</i> _{TEM}
11	125a	<i>Staph. carnosus</i>	<i>Staph. saprophyticus</i>	0.125	S	S	S	R	S	S	S	S	<i>bla</i> _{TEM}
	125d	<i>Staph. equorum</i>	<i>Staph. xylosus</i>	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	126a	<i>Staph. warneri</i>	No profile	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
	126b	<i>Staph. equorum</i>	No profile	0.125	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
Σ	32		<i>Staph. xylosus</i>	0.060	S	S	S	S	S	S	S	S	<i>bla</i> _{TEM}
			Σ resistant (R)		12	15	1	5					

¹Strain no. from Max Rubner-Institute (Kiel, Germany).
²Identified to species level using the API 32 ID STAPH (bioMérieux, Nürtingen, Germany).
³MIC-OXA = MIC of oxacillin; R = resistant; S = sensitive.
⁴OXA = oxacillin, CEF = cefoxitin, PEN = penicillin, ENR = enrofloxacin, TET = tetracycline, GEN = gentamicin, ERY = erythromycin, I = intermediate.

Staph. saprophyticus is a common bacterium found on skin of humans and animals and on other food besides from cheese (Hovelius and Mardh, 1984; Hedman et al., 1990), to date there is no proven case (by clonal identification) of food-borne UTI reported. Coagulase-negative staphylococci as starter cultures, in general, must not harbor virulence factors (Irlinger 2008). It would be interesting to evaluate the pathogenic potential of the *Staph. saprophyticus* ssp. *saprophyticus* clones and other species (*Staph. carnosus*, *Staph. sciuri*, *Staph. warneri*) isolated from brine in this study.

Antimicrobial Susceptibility Testing

All isolates were tested for antimicrobial susceptibility by applying phenotypic and genetic tests (Table 4). For screening purposes, we used the disk diffusion test for representatives of the 5 main groups of antibiotics (penicillin, enrofloxacin, tetracycline, gentamicin, and erythromycin). Five of 32 strains showed resistance to penicillin and 2 strains showed intermediate resistance to erythromycin. In addition, to screen for methicillin resistance, disk diffusion assays with cefoxitin and oxacillin and MIC tests for oxacillin were utilized. Only one strain displayed resistance to cefoxitin, whereas, for oxacillin, 15 isolates showed resistance in the disk diffusion test and 12 with the MIC test. The MIC values for resistant strains were close to the respective breakpoints, which indicates low-level resistance (Table 4). The same was true for the zone diameters of disk diffusion tests (data not shown). In 6 cases, results for the disk diffusion and MIC tests for oxacillin were not consistent. Five isolates showed resistance by disk diffusion assay and a negative MIC test, and one showed the opposite. Differences in test results are well known for both tests (for review, see Jorgensen and Ferraro, 2009). None of the strains resistant to oxacillin by MIC or disk diffusion assay or resistant to cefoxitin showed a positive PCR result for *mecA* or *mecC* genes. This confirms a study showing that methicillin-resistant *M. caseolyticus* isolated from bulk tank milk contained neither *mecA* nor *mecC* (MacFadyen et al., 2018). In this case, methicillin resistance was encoded mostly by the *mecD* gene, which was originally isolated from mastitic milk (Schwendener et al., 2017). A further source of methicillin resistance is the *mecB* gene, which has been naturally transferred from *M. caseolyticus* to *Staph. aureus* (Becker et al., 2018). Further investigations will reveal which gene encodes for the methicillin resistance observed in the present study.

To evaluate the phenotypic tests, we applied breakpoint tables provided by CLSI (2008) and EUCAST (2019). Regarding MIC of oxacillin, both tables rec-

ommended the same breakpoint (0.5 µg/mL). For disk diffusion, the test zone diameters for breakpoint estimation provided by EUCAST for most of the antibiotics were larger than those provided by CLSI. In these cases, we used the CLSI breakpoints. For cefoxitin, the same breakpoints were given by both organizations, and for oxacillin and enrofloxacin, no breakpoints were provided by EUCAST.

In total, 20 PCR tests were applied to testing for genes encoding antibiotic insensibility. The *bla*_{TEM} gene was detected in 6 strains and *bla*_{TEM} and *bla*_Z in one strain. For these strains, the MIC for penicillin were determined. All were sensitive to this antibiotic, although one strain of *Staph. succinus* and one strain of *Staph. hominis* were resistant to penicillin in the disk diffusion assay. This is similar to the results obtained for oxacillin.

Interestingly, most strains displaying phenotypic or genetic resistance were from factory 2, which unlike all other factories produced cheese exclusively from raw milk (Table 4). This may also explain the diversity of species present in the brine bath of this dairy.

In contrast to reports from other authors on CNS from different cheese types in Turkey (Kürekci, 2016), Minas frescal cheese, or from cheese processing environments from Brazil (Nunes et al., 2016; Rodrigues et al., 2017) only 4 strains showed resistance to both oxacillin and penicillin. No further multiresistant strains were detected in this study.

Challenge Tests

Based on the experimental setup of McMahon et al. (2007), 7 selected isolates were challenged in a first step with sublethal concentrations of lactic acid and salt. In these isolates, genes encoding β-lactamase (*bla*_{TEM} or *bla*_Z) were found and phenotypic resistance against β-lactam antibiotics (OXA and PEN) were detected (strains 101a, 104b, 104g, 105a, 105c, 106c, 112g; Table 4). Utilizing BHI with different concentrations of lactic acid or NaCl, we determined the optimum growth conditions and reduction of growth by 25%, measured as reduction in OD₆₀₀, and by 75% (sublethal stress level according to McMahon et al., 2007) (Table 5). Twenty-five antibiotics or combinations of antibiotics were used to challenge the selected strains with sublethal stress. Only 3 strains showed altered antibiotic resistance, all during challenge with acid (Table 6). *Staphylococcus hominis* (106b) showed slightly decreased resistance to penicillin, as did one strain of *Staph. saprophyticus* (101a). Another *Staph. saprophyticus* (112g) isolate displayed increased resistance to trimethoprim/sulfamethoxazole at a low level. For erythromycin and

Table 5. Determination of sublethal stress levels for selected isolates of CNS in brain heart infusion broth at different pH or NaCl concentrations after overnight incubation

Species	Strain no.	pH value			NaCl concentration (%)		
		Growth optimum	>25% reduction ¹	>75% reduction	Growth optimum	>25% reduction	>75% reduction
<i>Staph. saprophyticus</i>	101a	7.5	7.0	6.5	4.5	10.0	13.0
<i>Staph. succinus</i>	104b	6.5	6.0	5.5	6.0	10.0	15.0
<i>Staph. sciuri</i>	104g	7.5	6.0	5.5	0	4.5	13.0
<i>Staph. pasteurii</i>	105a	7.5	6.0	5.5	4.5	12.0	15.0
<i>Staph. equorum</i>	105c	7.5	6.5	6.0	5.0	6.0	10.0
<i>Staph. hominis</i>	106b	6.5	6.5	5.0	5.0	10.0	13.0
<i>Staph. saprophyticus</i>	112g	7.5	6.5	6.0	3.0	10.0	12.0
<i>Staph. aureus</i>	ATCC 29213	7.5	5.5	5.0	4.0	10.0	15.0

¹Reduction in optical density at 600 nm.

oxacillin (both used in the challenge tests), McMahon et al. (2007) reported strong effects during challenge of 4 isolates of *Staph. aureus* with NaCl and acid. The MIC for erythromycin increased up to 3 times under challenge with NaCl and acid. For oxacillin, the MIC decreased up to 3-fold under the challenged conditions except for a 3-fold increase for one isolate during challenge with NaCl. The obvious higher stability of the selected isolates in this study against a challenge with salt and acid may be due to their adaptation to brine baths for many years or even decades.

Genetic Determinants for Enterotoxin Production

All isolates were tested for genetic determinants of the most common staphylococcal enterotoxins *entA*, *entB*, *entC*, *entD*, and *entE*. None of these genes were detected. Similar results were obtained by Rodrigues et al. (2017) with CNS from dairies. None of the abovementioned enterotoxin genes were found in *Staph. epidermidis*, *Staph. hyicus*, or *Staph. warneri*; however, some rarer staphylococcal enterotoxin genes were present in those isolates. In contrast, gene and enterotoxin expression for all abovementioned common staphylococcal enterotoxins (and several others) was demonstrated for *Staph. piscifermentans*, *Staph. saprophyticus*, *Staph. sciuri*, and *Staph. xylosus* (Nunes et al., 2016). Expression and genetic determinants for SEE were detected in *Staph. capitis*, *Staph. equorum*, *Staph. lentus*, *Staph. simulans*, and *Staph. xylosus* (Vernozy-Rozand et al., 1996b). Production of SEC and SED by *Staph. chromogenes* in skim milk was reported by Oliveira et al. (2010). With regard to these findings, further testing of the brine bath isolates may be worthwhile.

CONCLUSIONS

The microbiota (including CNS) of brine baths contributes to cheesemaking in an essential way and should not be of concern for food safety. For the group of CNS investigated in this study, we showed that genes encoding common enterotoxins were not present. Few genes encoding for antibiotic resistance were detected in a small number of isolates, whereas a considerable number expressed antibiotic resistance at a low level. Some of the isolated species could be of concern in terms of biosafety, especially the relatively high numbers of *Staph. saprophyticus* ssp. *saprophyticus* detected. Based on these results, no indication of unacceptable risk originating from the isolated CNS was found. For a final assessment of the isolates in terms of food safety, further investigations, especially whole-genome sequencing, should be applied.

Table 6. Influence of sublethal stress on antibiotic resistance of CNS from brine baths

Species	Strain no.	Substance ¹	Stressor	MIC ² (µg/mL)		
				Growth optimum	>25% stress level	>75% stress level
<i>Staph. saprophyticus</i>	101a	T/S	pH	0	0	2.0
<i>Staph. hominis</i>	106b	PEN	pH	2.0	0.125	0.125
<i>Staph. saprophyticus</i>	112g	PEN	pH	0.125	0	0

¹T/S = trimethoprim/sulfamethoxazole; PEN = penicillin.

²Median of 3 repeats.

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