Virulence factors and antimicrobial resistance of *Staphylococcus aureus* isolated from the production process of Minas artisanal cheese from the region of Campo das Vertentes, Brazil

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ABSTRACT

Staphylococcus aureus is one of the main pathogens found in cheeses produced with raw milk, including Minas artisanal cheese from Brazil. However, information about *S. aureus* isolated from artisanal cheeses and its sources of production in small-scale dairies is very limited. We aimed to characterize the virulence factors of *S. aureus* isolated from raw milk, endogenous starter culture, Minas artisanal cheese, and cheese handlers from the region of Campo das Vertentes, Minas Gerais, Brazil. We identified the staphylococcal isolates by MALDI-TOF mass spectrometry. We evaluated biofilm production on Congo red agar and polystyrene plates. We used PCR to detect *icaA*, *icaB*, *icaC*, *sea*, *seb*, *sec*, *sed*, *see*, *tsst-1*, *agr*, and *mecA*. We evaluated the expression of staphylococcal toxin genes in PCR-positive staphylococcal isolates using quantitative reverse-transcription PCR, and we evaluated the production of these toxins and their hemolytic activity in vitro. We also evaluated the antimicrobial resistance profile of the staphylococcal isolates. For statistical analysis, we used cluster analysis, χ^2 tests, and correspondence tests. We analyzed 76 staphylococcal isolates. According to PCR, 18.42, 18.42, 2.63, and 77.63% were positive for *sea*, *tsst-1*, *sec*, and *agr*, respectively. We found low expression of staphylococcal toxin genes according to quantitative reverse-transcription PCR, and only 2 staphylococcal isolates produced toxic shock syndrome toxins. A total of 43 staphylococcal isolates (56.58%) had hemolytic activity; 53 were biofilm-forming on Congo red agar (69.73%), and 62 on polystyrene plates (81.58%). None

of the staphylococcal isolates expressed the *mecA* gene, and none presented a multi-drug resistance pattern. The highest resistance was observed for penicillin G (67.11%) in 51 isolates and for tetracycline (27.63%) in 21 isolates. The staphylococcal isolates we evaluated had toxigenic potential, with a higher prevalence of *sea* and *tsst-1*. Biofilm production was the main virulence factor of the studied bacteria. Six clusters were formed whose distribution frequencies differed for hemolytic activity, biofilm formation (qualitative and quantitative analyses), and resistance to penicillin, tetracycline, and erythromycin. These findings emphasize the need for effective measures to prevent staphylococcal food poisoning by limiting *S. aureus* growth and enterotoxin formation throughout the food production chain and the final product.

Key words: *Staphylococcus aureus*, toxins, biofilm, genic expression

INTRODUCTION

Minas artisanal cheese, produced in the Campo das Vertentes region of Minas Gerais is one of the most consumed cheeses in Brazil. The state of Minas Gerais is the largest milk producer in Brazil, producing 25.6% of the national total (Instituto Brasileiro de Geografia e Estatística, 2018), and it is the largest cheese producer in the country. *Staphylococcus aureus* has been described as one of the most common causal agents of food poisoning associated with the consumption of raw milk cheese and is listed as one of the pathogens likely to pose the greatest risk to human health (Simeão do Carmo et al., 2002; Johler et al., 2015; World Health Organization, 2017). The presence of this pathogen in final products is related to several factors, but is usually associated with the use of contaminated raw milk

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and endogenous starter cultures in manufacturing the cheese, as well as with asymptomatic human carriers of *S. aureus* (Castro et al., 2016; Baran et al., 2017; Johler et al., 2018).

Staphylococcus aureus has the potential to cause a variety of diseases in human beings and animals (Boucher et al., 2010; Fey, 2010), depending on the presence or absence of genes that encode for several virulence factors, especially exotoxins such as staphylococcal enterotoxins, toxic shock syndrome toxin (TSST), and hemolysin (Brown et al., 2014). The intake of thermo-stable and preformed staphylococcal enterotoxins may trigger food poisoning, and this is the second- or the third-most common cause of food poisoning around the world. The classic staphylococcal enterotoxins (SEA, SEB, SEC, SED, and SEE) are the toxins most commonly related to cases of food poisoning (Ono et al., 2015).

Biofilm production represents another important virulence factor of *S. aureus*; it contributes to colonization of the agent in cellular tissues and abiotic surfaces, and it creates a protective barrier for the bacteria to actions of the host immune system and of antimicrobial agents (Waters et al., 2016). Bacterial biofilms grow by proliferation and production of an extracellular matrix. One of the extracellular polymeric substances of the staphylococcal biofilm is the polysaccharide intercellular adhesin (PIA), which is a poly-*N*-acetyl glucosamine (Joo and Otto, 2012). Its biosynthesis is performed by genes located in the operon *ica* (cluster of genes *ica* ADBC), and studies have demonstrated a relationship between the presence of operon *ica* and the formation of biofilm in foodborne *S. aureus* (Cramton et al., 1999; Melo et al., 2016).

Another serious public health problem associated with *S. aureus* in food is its resistance to antimicrobials, because it triggers serious infections with limited therapeutic options (Ibed and Hamim, 2014). Methicillin-resistant *S. aureus* (MRSA) is a leading example of bacterial resistance in the world (Ibed and Hamim, 2014) and is characterized by acquisition of the *mecA* gene, which is located on the chromosomal cassette *mec* (SCCmec; Basset et al., 2013). For many years, MRSA staphylococcal isolates have been recognized as pathogens unique to hospitals. However, studies have documented the spread of MRSA in foods (Costa et al., 2015; Jamali et al., 2015).

The expression of some virulence factors of *S. aureus*, including SE, TSST, hemolysin α , and genes involved in biofilm formation is controlled by the accessory gene regulator (**agr**). The *agr* was identified as a quorum-sensing system in *S. aureus* and is encoded by the locus (*agr*), which controls expression of the RNA molecule (RNAIII; Morrison and Dunman, 2011). Once this

molecule reaches a critical concentration according to cell density, the expression of the virulence factors is initialized (Nielsen et al., 2014).

The objective of this study was to investigate the in vitro production of virulence factors and the antimicrobial resistance profile of staphylococcal isolates from raw milk, endogenous starter culture, Minas artisanal cheese, and cheese handlers from Campo das Vertentes, Brazil.

MATERIALS AND METHODS

Sampling (Staphylococcal Isolates)

The study was carried out with 76 staphylococcal isolates from raw milk ($n = 14$), endogenous starter cultures ($n = 5$), Minas artisanal cheese ($n = 42$), and cheese handlers ($n = 15$) from 9 rural properties located in Campo das Vertentes, Minas Gerais, Brazil. Minas artisanal cheeses are made in 7 officially recognized regions throughout the state. They are a traditional product, made on small farms, using raw milk, rennet, and endogenous starter culture, which is the whey collected from previously produced cheeses. The collected samples were stored in brain heart infusion (BHI) broth (Difco, Kansas City, MO) with added with glycerol (20%) and frozen at -20°C .

Phenotypic Characterization and Identification of Isolates by MALDI-TOF MS

Staphylococcal isolates were cultured in BHI agar (Difco), and the plates were incubated under aerobiosis at 37°C for 18 to 24 h. The colonies were confirmed by Gram stain to determine the morphology of bacterial cells by catalase (Carter, 1998), coagulase, and DNase tests, according to Silva et al. (2010); and by fermentation of mannitol, according to Bergey and Holt (1994). Identification of bacteria was confirmed by proteomic analysis using MALDI-TOF MS. The spectra were acquired using the FlexFlex MicroFlex LT mass spectrometer (Bruker Daltonics, Billerica, MA) and recorded in positive linear mode within a mass range of 2,000 to 20,000 Da. The identification criteria were those recommended by the manufacturer (Assis et al., 2017).

Detection of Virulence Genes by Conventional PCR

Bacterial DNA was extracted according to the method described by Pitcher et al. (1989). Then, genomic DNA was used in PCR amplification reactions to detect the following genes: classical enterotoxin-related (*sea*, *seb*, *sec*, *sed*, and *see*), toxin from toxic shock syndrome

Table 1. Target and primer (F, forward; R, reverse) concentrations used for quantitative reverse-transcription PCR

RNA target	Primer sequence (5'-3')	Primer (μmol/L)	Efficiency (%)	Amplicon size (bp)	Reference
<i>sea</i>	F: CCTTTGGAAACGGTTAAAACG R: TCTGAACCTTCCCATCAAAAAC	1.0	90.60	127	Becker et al., 1998
<i>tsst-1</i>	F: TTCACTATTTGTAAAAGTGTGACACCCACT R: TACTAATGAATTTTTTTATCGTAAGCCCTT	1.0	91.29	180	Jarraud et al., 2002
<i>sec</i>	F: AATAAACCGGTTGATTCTAAAAGTGTGAA R: ATCAAAATCGGATTAACATTATCCATTC	0.3	90.60	80	Klotz et al., 2003
16S	F: CNACGCGAAGAACCCTTANC R: CGACAGCCATGCANACCT	0.3	93.25	98	Huse et al., 2008

(*tsst-1*), biofilm formation (*icaA*, *icaB*, and *icaC*), quorum-sensing agr system (*agr*) and methicillin resistance (*mecA*) according to the methodologies described in the literature (Becker et al., 1998; Reinoso et al., 2004; Dias-Souza et al., 2013; Breves et al., 2015). Amplification reactions were performed in a final volume of 25 μL, using 12.5 μL of Pré Mix 2× (Phoneutria, Belo Horizonte, Brazil) and 20 ng of DNA.

Expression of Genes Encoding Production of Staphylococcal Enterotoxins and TSST

The total RNA of the staphylococcal isolates carrying toxigenic genes in the conventional PCR was obtained from a bacterial colony newly cultured in BHI broth (Difco) and incubated at 37°C for 20 h under mechanical agitation (180 rpm), using the RNeasy Mini Kit (Qiagen, Valencia, CA). The quality and quantity of RNA samples were evaluated by agarose gel electrophoresis, and also by spectrophotometric analysis using the NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Waltham, MA). The samples were treated with Turbo DNase (Ambion, Austin, TX) for cDNA synthesis, which was performed using 1 μg of the total RNA sample and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's guidelines (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_041053.pdf). Quantitative reverse-transcription PCR (qRT-PCR) was performed using the 7500 Real-Time PCR system (Applied Biosystems) and the analyses were evaluated using Sequence Detection software (version 2.4; Applied Biosystems). The qRT-PCR was performed as described by Sandes et al. (2017). Briefly, we used SYBR green PCR Master Mix 2x (Applied Biosystems) and specific primers for the *sea*, *sec*, and *tsst-1* genes. The conserved 16S rRNA gene was used as a reference for standardization. The efficiency of each pair of primers was calculated using the standard serial dilution curve as described in the Applied Biosystems protocol (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_041053.pdf). The ex-

perimental approach was optimized by adjusting the primer concentrations for efficiency and specificity (Table 1). Amplification reactions were performed in a final volume of 20 μL, using 10 μL of Master Mix SYBR green and 50 ng of cDNA. The thermal cycle consisted of initial stages at 50°C for 2 min and 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. After amplification, we performed a dissociation curve analysis (95°C for 15 s, 60°C for 15 s and 95°C for 15 s) to verify the specificity of the generated amplicons. Analyses of qRT-PCR for all toxins were performed in triplicate. Expression levels of enterotoxins producing staphylococcal strains (FRI361, N315, and 36/2014) were used as calibration data. The results are presented in graphs with the relative mRNA level for each toxin obtained by the relative derivative quantification method described by Hellemans et al. (2007).

In Vitro Biofilm Production

For qualitative evaluation of biofilm production, the Congo red agar (CRA) assay was performed, as described by Pedroso et al. (2016) and according to the method described by Arciola et al. (2002). On CRA, biofilm-producing staphylococcal isolates form black colonies, and isolates that do not produce biofilm form red colonies. Quantitative biofilm production was evaluated in sterile, flat-bottom inert polystyrene microplates containing 96 wells, as described by Pedroso et al. (2016) and according to Dias-Souza et al. (2013). A medium without inoculum was used as the negative control, and *Staphylococcus epidermidis* ATCC 12228 was used as a reference sample. After incubation of the plates for 20 h at 37°C, absorbance was measured using an ELISA reader at 620 nm (Amaral et al., 2005). Both assays were performed in triplicate.

In Vitro Production of Toxins

Detection of classical enterotoxins (SEA, SEB, SEC, SED) and TSST was performed using the optimum

sensitive plate (**OSP**) technique (Robbins et al., 1974). The staphylococcal isolates were grown in 5 mL of BHI broth and incubated at 37°C for 24 h. After incubation, Petri dishes containing 20 mL of BHI agar with 1% yeast extract added were coated with dialysis membrane plates. Then, 0.5 mL of the culture was poured onto the membrane surface and the plates were incubated at 37°C for 24 h. The membranes were then washed twice with a total of 2.5 mL of phosphate buffer (20 mM, pH 7.4): 1.5 mL in the first wash and 1.0 mL in the second. The washed cultures were transferred to a tube and centrifuged at $10,000 \times g$ for 10 min at 4°C. Supernatants were collected, and 20 µL of thimerosal were added to them. The supernatants were then used to verify the production of TSST and the classical enterotoxins by the reaction between the standard antiserum specific to each enterotoxin and the culture supernatant. Positive controls for the toxins (pure toxins) were used to check the activity of the standard antiserum. The toxins used as standards and the specific antiserum were kindly provided by the Laboratory of Staphylococcal Enterotoxins, FUNED (Belo Horizonte, Minas Gerais, Brazil).

In Vitro Production of Hemolysin

The hemolytic activity of the staphylococcal isolates was evaluated by spreading the microorganisms on base agar medium (Difco) supplemented with 5% equine blood and incubating them at 37°C for 18 to 24 h.

Antimicrobial Resistance Test

All staphylococcal isolates were tested for antimicrobial resistance by disk diffusion assay, according to the CLSI (2015) guidelines. The following antimicrobials and their respective concentrations were used: sulfamethoxazole-trimethoprim (25 µg), oxacillin (1 µg), cefoxitin (30 µg), vancomycin (30 µg), erythromycin (15 µg), penicillin G (10 U), chloramphenicol (30 µg), gentamicin (10 µg) and tetracycline (30 µg).

Hierarchical Cluster Analysis, χ^2 Test, and Correspondence Analysis

A hierarchical cluster analysis was performed as described by Pedroso et al. (2016). Briefly, we used SPSS (version 15.0, SPSS Inc., Chicago, IL) to identify groups of staphylococcal isolates with 14 different characteristics: phenotypic (DNase and mannitol fermentation capacity), toxin production by the PCR (SEA, SEC, TSST) and OSP method, gene detection (*agr*), hemolytic activity, biofilm formation (qualitative and quantitative), and antimicrobial resistance (oxacillin, erythromycin, penicillin, and tetracycline). We did

not use other evaluation characteristics because they showed only 1 response type. The Euclidian distance between each pair of observations was used as an instrument to measure similarity between groups, and the Ward hierarchical clustering method was used to group similar staphylococcal isolates (Hair et al., 2003; Pedroso et al., 2016, 2018).

A hierarchical cluster analysis was conducted as described by Torres Filho et al. (2018). Briefly, this analysis involves 3 separate phases: the first phase divides the total sample into smaller subgroups; the second phase verifies whether the groups are significantly different and theoretically significant; the third phase generates the cluster profiles, describing the characteristics of each in terms of the 14 characteristics described above (Jain et al., 1999; Hair et al., 2003). Selection of the number of clusters was performed according to Hair et al. (2003): examinations were conducted to the point at which the error variances were reduced as passing from a smaller to a larger number of clusters. We applied the χ^2 test to evaluate the frequency distributions of the 14 characteristics and the clusters at a significance level of 5% (Hair et al., 2003; Pedroso et al., 2016, 2018).

Correspondence analysis was conducted as described by Pedroso et al. (2016). Briefly, it was carried out using SAS (version 9.2; SAS Institute Inc., Cary, NC), at a significance level of 5%, to visualize the relationship between the 6 significant characteristics by χ^2 test, hemolytic activity, biofilm formation (qualitative and quantitative), and antimicrobial resistance (erythromycin, penicillin and tetracycline), and the clusters. Using this exploratory and descriptive technique, these characteristics and clusters were spatially represented, providing a perceptual map (Hair et al., 2003; Pedroso et al., 2016, 2018).

RESULTS AND DISCUSSION

Phenotypic Characterization and Species Identification

All staphylococcal isolates were confirmed as gram-positive cocci, catalase positive, and coagulase and DNase positive, except for 2 samples that were DNase negative. A total of 68 staphylococcal isolates (89.47%) were able to ferment mannitol; this is an important characteristic of *S. aureus*, although some staphylococcal isolates may show a negative result on this test because of the late fermentation of mannitol (Paiva, 1991). Negative results in mannitol fermentation tests and DNase tests were also reported by Kateete et al. (2010) when analyzing clinical staphylococcal isolates. Presumptive staphylococcal isolates were confirmed at the species level by proteomic analysis using MALDI-

TOF MS, in which the 76 studied samples presented a score $\geq 2,000$, validating identification of the species as *S. aureus*.

Biofilm Production

We analyzed in vitro biofilm production on CRA and in polystyrene plates. In the first test, 53 staphylococcal isolates (69.73%) were characterized as biofilm-forming. In the polystyrene plates, the staphylococcal isolates were classified according to the relationship between the optical density of the control and the optical density of the isolates: nonadherent (14 staphylococcal isolates = 18.42%), poorly adherent (56 staphylococcal isolates = 73.68%), and moderately adherent (6 staphylococcal isolates = 7.90%). We found no strongly adherent staphylococcal isolates in the present study.

Biofilm production is an important virulence factor of *S. aureus*, associated with its survival in food-processing environments and its persistence in biomaterials used as invasive medical devices (Becker et al., 2014). Although biofilm formation is widely studied under clinical conditions — for example, in implants and medical materials — few studies have reported the production of biofilm in foods (Santos et al., 2014; Di Ciccio et al., 2015; Cruzado-Bravo et al., 2019). A biofilm can be defined as a community structure of bacterial cells protected by a polysaccharide or protein matrix, produced by the microorganism itself, and adhering to both biotic and abiotic surfaces (Simões et al., 2010). Biofilm formation by *Staphylococcus* spp. represents an evolutionary advantage to the microorganism, because bacterial cells are resistant to adverse environmental conditions such as antimicrobial and sanitizing agents, desiccation, and UV radiation (Cha et al., 2013).

The structuring of a biofilm is a process that involves 4 main phases: adhesion, aggregation, maturation, and dispersion. These phases involve events that are physiologically different from each other and require specific virulence factors (Figueiredo et al., 2017). In the aggregation phase, the bacteria start to secrete substances that are responsible for the maintenance of adhesion and the layer that surrounds the biofilm. In *S. aureus*, the major molecule responsible for intercellular adhesion is PIA (Mack et al., 1996), a partially deacetylated polymer of *N*-acetylglucosamine which, together with other polymers such as teichoic acids, proteins, and extracellular DNA (eDNA), constitute the major part of the extracellular matrix of biofilm-forming *S. aureus* (Sugimoto et al., 2018). Biosynthesis of PIA is performed by genes contained in the *ica* locus, which comprise an *N*-acetylglucosamine transferase (*icaA* and *icaD*), a PIA deacetylase (*icaB*), a PIA exporter (*icaC*), and a regulatory gene (*icaR*; Gerke et al., 1998; Vuong

et al., 2004). According to Grinholc et al. (2007), all staphylococcal isolates harboring the *icaA* gene also harbor *icaD*. Thus, detection of 1 of these genes can be used to determine whether the staphylococcal isolate is a potential biofilm producer.

We did not detect *icaA*, *icaB*, or *icaC* in any of the staphylococcal isolates. The absence of these genes in the biofilm-forming staphylococcal isolates in the present study suggests the participation of other genes in biofilm formation. The literature describes the formation of biofilm in staphylococcal isolates by independent mechanisms of PIA (Foster et al., 2014). One of these mechanisms involves the action of a surface protein bound to the cell wall of *S. aureus* called biofilm-associated protein (Bap). Bap was first described in staphylococcal isolates from cases of mastitis, and may play a role in bovine mammary gland infection (Speziale et al., 2014). Mutagenesis by transposon, followed by identification of the inactivated gene, showed that the Bap protein is involved in the formation of biofilm produced by staphylococcal isolate V329 from bovine mastitis (Cucarella et al., 2001). The C surface protein of *S. aureus* (SasC) represents another protein factor involved in cell aggregation and biofilm formation. The expression of SasC mediates the formation of bacterial aggregates, increased adhesion to polystyrene, and biofilm formation in *S. aureus* (Schroeder et al., 2009).

Detection of Genes Encoding Staphylococcal Toxins

We also evaluated the staphylococcal isolates for the presence of toxin-encoding genes using PCR, and 26 (34.21%) carried at least 1 of the genes. We observed that the genes with the highest occurrence were *sea* and *tsst-1* (14 isolates = 18.42%), followed by *sec* (2 isolates = 2.63%). One isolate had the genes *sea*, *sec*, and *tsst-1*, and 2 other staphylococcal isolates (1 from cheese and 1 from raw milk) had *tsst-1*. We did not detect *seb*, *sed*, or *see* in any of the isolates included in the present study.

The enterotoxin SEA has been described as the main enterotoxin involved in food poisoning (Hennekinne et al., 2012; Clarisse et al., 2013), followed by SED, SEB, and SEC (Hu and Nakane, 2014), but SEE has also been shown to be responsible for staphylococcal food poisoning (Guidi et al., 2018). In France, for example, the first reported foodborne outbreak caused by SEE was described in only 2009, and it involved fresh cheese made from raw milk (Ostyn et al., 2010).

Of the total staphylococcal isolates carrying the *sea* gene, 7 (50%) were obtained from Minas artisanal cheese, 4 (28.57%) from raw milk, and 3 (21.43%) from endogenous starter cultures. Of staphylococcal isolates carrying the *sec* gene, 1 was isolated from cheese and

the other from raw milk. Of staphylococcal isolates that harbored *tsst-1*, 6 (42.86%) were obtained from cheese, 2 (14.29%) from raw milk, 1 (7.14%) from an endogenous starter culture, and 5 (35.71%) from handlers. The high occurrence of *tsst-1* in isolates obtained from handlers is worrisome, because *S. aureus* can be found in the skin, gastrointestinal tract, and nostrils of food handlers, and these sites become possible distribution reservoirs of pathogenic *S. aureus* (Verhoeven et al., 2014). Estimates of the prevalence of staphylococcal isolates with *tsst-1* in healthy participants ranged from 13 to 25%, indicating a high disease potential, although the incidence of cases has been relatively low (1–4/100,000; Gaventa et al., 1989; Parsonnet et al., 2005; Mégevand et al., 2010; Lamy et al., 2012).

Expression of Genes Encoding Staphylococcal Toxins

Figure 1 shows that of the 30 genes detected by the PCR assay, 25 had their relative expression confirmed. We observed an absence of expression for 4 *sea* genes, present in 3 isolates from cheese and 1 from raw milk, and for *tsst-1* present in an isolate from raw milk.

We compared the expression of the toxin genes from each staphylococcal isolate with the expression of the toxin genes from the positive control samples to calculate relative quantification, considering that the normalized relative expression rate for each toxin gene is equal to 1. Figure 1 shows that only 1 *tsst-1* gene belonging to *S. aureus* isolated from a handler (isolate 53) showed higher expression than the control sample, indicating that isolate's high toxigenic potential. The other genes analyzed in the study had of low expression in the evaluated growth conditions; the lowest level of expression (approximately 10^5 times lower than the expression level of the positive control sample) was detected in 1 gene of *S. aureus* isolated from cheese (isolate 41).

Low expression of toxigenic genes in *S. aureus* or the absence of expression have been described in other studies (Derzelle et al., 2009; Xu et al., 2015). The expression of *Staphylococcus* spp. toxigenic genes is strongly associated with growth conditions in the medium, such as temperature, pH, oxygen rate, microbial growth phase, and glucose concentration (Regassa et al., 1991; Tsutsuura et al., 2013). Moreover, the expression of genes of other staphylococcal toxins such as SEB, SEC, SED, and TSST is conditioned by the action of 2-component signal transduction systems and transcription regulators that modulate the expression of virulence factors in *S. aureus*, such as Agr, saeRS, sigB, SarA and ROT systems (Cretenet et al., 2011; Andrey et al., 2015). The low expression of these regulators, influenced by

Figure 1. Levels of expression of *sea*, *tsst-1*, and *sec* genes by *Staphylococcus aureus* strains. *Staphylococcus aureus* 36/2014 strain used as a positive control for *sea* was provided by Fundação Ezequiel Dias (Belo Horizonte, Minas Gerais, Brazil). Error bars represent the SE from the relative quantification mean for each *Staphylococcus aureus* strain.

several environmental factors and genetic interactions, is associated with a substantial loss of transcription levels of *seb*, *sec*, *sed*, and *tsst-1* and, therefore, the low production of these toxins (Queck et al., 2008; Even et al., 2009; Thoendel et al., 2011; Rudkin et al., 2012).

Importantly, the absence or low expression of enterotoxin genes in the studied staphylococcal isolates does not exclude the possibility that these bacteria could produce a large amount of toxin in foods. In planktonic systems such as the broth culture media used in our work, microorganisms may have a different gene expression profile than they do in multicellular environments such as foods, in which bacteria appear to be associated in different ways, and cellular interaction through molecular signaling is prevalent (Even et al., 2009; Cretenet et al., 2011; Valihrach et al., 2014). In this regard, Cretenet et al. (2011), using a transcriptomic approach to study the expression of virulence of *S. aureus* in a cheese matrix under the influence of *Lactococcus lactis*, showed that SEA expression was slightly increased in the cheese matrix when compared with the chemically defined medium, due to the presence of an acid environment and greater oxidative stress, capable of activating the prophage encoder of *sea*.

In Vitro Production of Staphylococcal Toxins

We also investigated the production of toxins in the staphylococcal isolates using the OSP technique. In this assay, only 2 staphylococcal isolates (1 from a handler and the other from an endogenous starter culture) were TSST producers. The low frequency and even absence in vitro of toxin-producing staphylococcal isolates have been reported in several studies, including staphylococcal isolates from outbreaks and clinical infections (de Lourdes R. S. da Cunha et al., 2007; Pedroso et al., 2016). The authors pointed to the experimental growth conditions and the low efficiency of the quantification techniques in detecting strains of low production as justification for the results. This assumption also appeared to apply to our study, considering that the 2 in vitro TSST-producing staphylococcal isolates were those that showed higher levels of *tsst-1* expression in qRT-PCR.

In Vitro Production of Hemolysin

We observed that 14 staphylococcal isolates (18.42%) were α -hemolysin producers, 29 (38.16%) were β -hemolysin producers, and 33 (43.42%) had no hemolysis pattern (γ -hemolysis). A higher proportion of γ -hemolysis and a lower proportion of α -hemolysis were described by Ariyanti et al. (2011), when they analyzed

the hemolytic activity of *S. aureus* isolated from different products of animal origin.

Hemolysins are toxins that aid in cell invasion and in evading the immune response of the host, and they are associated with the pathological changes observed during the course of staphylococcal infections (Otto, 2014). In our study, 43 staphylococcal isolates (56.58%) were hemolysin (α or β) producers, revealing their pathogenic potential. The high rate of staphylococcal isolates producing γ -hemolysis matched the finding that equine red blood cells were strongly affected by leucocidin, 1 of the proteins integrating the γ toxin 2-component system (Dinges et al., 2000).

Detection of agr Gene (Quorum-Sensing System)

We observed the presence of *agr* in 59 (77.63%) staphylococcal isolates. The Agr system is the main 2-component regulation system involved in modulating the expression of virulence factors in *Staphylococcus* spp. In general, it works by stimulating the production of extracellular proteins, such as α -toxin, β -hemolysin, TSST-1, and some SE. Also, it inhibits cell-wall-associated proteins, such as those responsible for biofilm formation, in response to increasing concentrations of the signaling molecule, the self-inducing peptide encoded by the *agr* gene (Queck et al., 2008).

The absence of *agr* in some staphylococcal isolates (22.37%)—especially those that expressed the *tsst-1* and *sea* genes in qRT-PCR and were TSST producers in the in vitro assay—suggest the performance of other quorum systems in modulating the expression of virulence factors in these microorganisms, such as SarA, sigma β , and ROT. In fact, the expression of *sea* has been described in the literature as independent of *agr* and mutations in some *tsst-1* negative regulators, such as *sigB*, *sarA*, and probably *rot*, in addition to environmental stimuli (growth rate, pH, and oxygen), and may increase the expression of TSST (Andrey et al., 2015). In the same way, studies have shown *agr*-independent ways to modulate biofilm formation. In a study by Beenken et al. (2010), a *sarA* mutation, which encodes the SarA protein, resulted in a lower capacity for biofilm formation in all clinical staphylococcal isolates analyzed, regardless of their *agr* functional status.

Antimicrobial Resistance Profile of Staphylococcal Isolates

The staphylococcal isolates were negative for the *mecA* gene, as reported by Martins et al. (2015), although a staphylococcal isolate from cheese showed intermediate resistance to oxacillin in the disk diffusion

test. Oxacillin is the second antimicrobial considered for the study of *mecA* resistance in staphylococcal isolates. Cefoxitin resistance has been regarded as a surrogate marker for the detection of MRSA by disk diffusion and other methods (Skov et al., 2014). The observation of 1 isolate with intermediate resistance to oxacillin but negative for the *mecA* gene, may also be due to the fact that although *mecA* is the main gene associated with methicillin-resistant staphylococcal isolates, other genes may influence the expression of resistance, such as the essential factors for methicillin resistance and high resistance to methicillin (*hmt*; Peacock and Paterson 2015). In addition, some studies have associated the *mecC* gene, a *mecA* homolog, with the methicillin resistance phenotype in *S. aureus* (Concepción Porrero et al., 2014; Paterson et al., 2014). Thus, it is common to report isolates that are resistant to oxacillin but negative for the *mecA* gene (Mendonça et al., 2012; de Melo et al., 2014).

All staphylococcal isolates were sensitive to sulfamethoxazole-trimethoprim, cefoxitin, gentamicin, vancomycin, and chloramphenicol in the disk diffusion test. However, we did observe resistance to erythromycin in 4 staphylococcal isolates (5.26%), tetracycline in 21 isolates (27.63%) and penicillin in 51 isolates (67.11%). However, no staphylococcal isolate presented multi-drug resistance to the evaluated antimicrobials. The low occurrence of erythromycin-resistant staphylococcal isolates was also reported by Jamali et al. (2015) when they studied the antimicrobial resistance profile of *S. aureus* isolated from milk and dairy products in a province in Iran. It is important to mention that erythromycin is one of the “highest priority critically important antimicrobials” as described by the World Health Organization (World Health Organization, 2019). The high percentages of tetracycline and penicillin-resistant staphylococcal isolates in our study were expected because of the widespread and indiscriminate use of antimicrobials in the control and treatment of major infections in animals raised on dairy farms (Jamali and Radmehr, 2013).

Hierarchical Cluster Analysis

We grouped the 76 staphylococcal isolates into 6 clusters according to phenotypic characteristics (DNase and mannitol fermentation capacity), toxin production determined by the PCR (SEA, SEC, and TSST) and OSP method, hemolytic activity, biofilm formation (qualitative and quantitative), and antimicrobial resistance (oxacillin, erythromycin, penicillin and tetracycline; Table 2). The frequency distributions of clusters differed for hemolytic activity ($\chi^2 = 22.57$; $P = 0.012$); qualitative ($\chi^2 = 11.66$; $P = 0.040$) and quantitative

biofilm formation ($\chi^2 = 65.72$; $P < 0.001$); and erythromycin ($\chi^2 = 86.29$; $P < 0.001$), penicillin ($\chi^2 = 76.00$; $P < 0.001$), and tetracycline resistance ($\chi^2 = 72.00$; $P < 0.001$). The frequency distributions of clusters did not differ for DNase ($\chi^2 = 9.48$; $P = 0.09$); mannitol fermentation ($\chi^2 = 3.20$; $P = 0.67$); production of SEA ($\chi^2 = 1.07$; $P = 0.96$), SEC ($\chi^2 = 1.58$; $P = 0.90$), TSST ($\chi^2 = 5.64$; $P = 0.34$), or OSP ($\chi^2 = 5.38$; $P = 0.37$); *agr* detection ($\chi^2 = 3.97$; $P = 0.55$); and oxacillin resistance ($\chi^2 = 2.65$; $P = 0.75$).

We used correspondence analysis to visualize the relationship between these formed clusters, hemolytic activity, biofilm formation (qualitative and quantitative), and antimicrobial resistance (erythromycin, penicillin, and tetracycline) by means of a perceptual map (Figure 2). The first 2 dimensions explained 64.7% of the variability of the data.

The profile of each cluster can be described and discriminated by its main characteristics (Table 2 and Figure 2). Cluster 1 showed a considerable amount (40%) of non-slime-producing strains with enterotoxin TSST and strongly adherent biofilm production, and most isolates had α -hemolytic activity. This group was resistant only to penicillin. In cluster 1, we detected 1 isolate from an endogenous starter culture (20%), 1 from a handler (20%), and 3 from cheese (60%). Cluster 2 consisted of staphylococcal isolates sensitive to all antimicrobials. In cluster 2, we detected 5 isolates from raw milk (22.73%), 6 from handlers (27.27%), and 11 from cheese (50%). Cluster 3 was resistant to penicillin and contained the only group of isolates that were resistant to tetracycline. This cluster also showed a considerable amount (40%) of non-slime-producing isolates, similar to cluster 1. In cluster 3, we detected 5 isolates from raw milk (25%), 1 from an endogenous starter culture (5%), 3 from handlers (15%), and 11 from cheese (55%). Cluster 4 was the only cluster to show a considerable number of non-slime-producing isolates (52%) with non-adherent biofilm production (33%) and γ -hemolytic activity (48%). These isolates were resistant only to penicillin, similar to cluster 1. In cluster 4, we detected 3 isolates from raw milk (14.29%), 3 from endogenous starter cultures (14.29%), 4 from handlers (19.04%), and 11 from cheese (52.38%). Cluster 5 was the only cluster to show strongly adherent biofilm production, and most staphylococcal isolates had β -hemolytic activity. These isolates were sensitive to all antimicrobials, similar to cluster 2. In cluster 5, we detected 1 isolate from raw milk (25%), 1 from a handler (25%), and 2 from cheese (50%). Cluster 6 was resistant to penicillin and the only group of staphylococcal isolates that was resistant to erythromycin. In cluster 6, we detected 4 staphylococcal isolates from cheese (100%).

Table 2. Cluster profiles based on phenotype (DNase and mannitol fermentation), toxin production determined by the PCR (SEA, SEC, and TSST) and OSP method, genotype (*agr*), hemolytic activity, biofilm formation (qualitative and quantitative), and antimicrobial resistance (oxacillin, erythromycin, penicillin, and tetracycline)

Cluster	N	Level ¹	Phenotype (%)		Toxin production ² (%)				Genotype (%)		Biofilm formation (%)				Antimicrobial resistance			
			DNase	Mannitol fermentation	SEA	SEC	TSST	OSP	agr	Hemolytic activity (%)	Qualitative	Quantitative	Oxacillin	Erythromycin	Penicillin	Tetracycline		
1	5	0	0	80	100	60	100	0	80	40	0	100	100	0	80			
	1	100	100	20	0	40	0	100	20	60	60	0	0	0	0			
	2	0	0	0	0	0	0	0	0	0	0	0	0	100	20			
	3	0	0	0	0	0	0	0	0	0	40	0	0	0	0			
2	22	0	5	82	95	73	100	18	27	14	9	100	82	100	100			
	1	95	91	18	5	27	0	82	36	86	91	0	18	0	0			
	2	0	0	0	0	0	0	0	36	0	0	0	0	0	0			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
3	20	0	10	80	100	80	100	30	15	35	25	100	100	0	0			
	1	100	90	20	0	20	0	70	40	65	75	0	0	0	0			
	2	0	0	0	0	0	0	0	45	0	0	0	0	100	100			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
4	21	0	19	81	95	90	90	29	0	52	33	95	100	0	100			
	1	100	81	19	5	10	10	71	38	48	67	5	0	0	0			
	2	0	0	0	0	0	0	0	62	0	0	0	0	100	0			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
5	4	0	25	100	100	100	100	0	0	0	0	100	75	100	100			
	1	75	100	0	0	0	0	100	75	100	0	0	25	0	0			
	2	0	0	0	0	0	0	0	25	0	0	0	0	0	0			
	3	0	0	0	0	0	0	0	0	0	100	0	0	0	0			
6	4	0	0	75	100	100	100	25	25	0	0	100	0	0	100			
	1	100	100	25	0	0	0	75	25	100	100	0	0	0	0			
	2	0	0	0	0	0	0	0	50	0	0	0	100	100	0			
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
χ ² test			9.477	3.202	1.066	1.578	5.642	5.380	22.567*	11.658*	65.721*	2.654	86.291*	76.000*	71.999*			
P-value			0.091	0.669	0.957	0.904	0.343	0.371	0.012*	0.040*	<0.001*	0.753	<0.001*	<0.001*	<0.001*			

¹For phenotype, enterotoxin production, genotype, and qualitative biofilm formation: level 0 = negative; level 1 = positive. For hemolytic activity: level 0 = α ; level 1 = β ; level 2 = γ . For quantitative biofilm formation: level 0 = not adherent; level 1 = weakly adherent; level 2 = moderately adherent; level 3 = strongly adherent. For antimicrobial resistance: level 0 = sensitive; level 1 = indifferent; level 2 = resistant.

²SEA = staphylococcal enterotoxin A; SEC = staphylococcal enterotoxin C; TSST = toxic shock syndrome toxin; OSP = optimum sensitive plate.

*The frequency distribution of the characteristic is different among the clusters by the chi-square test at a significance level of 5% ($P < 0.05$).

Figure 2. Perceptual map of the correspondence analysis between hemolytic activity, biofilm formation (qualitative and quantitative), and antimicrobial resistance (erythromycin, penicillin, and tetracycline), and the clusters (C). B- = no adherent biofilm; B+ = weakly adherent biofilm; B+++ = strongly adherent biofilm; CRA- = non-slime-producing strains; CRA+ = slime-producing strains; ErytS = sensitive to erythromycin; ErytI = indifferent to erythromycin; ErytR = resistant to erythromycin; H α = α -hemolytic activity; H β = β -hemolytic activity; H γ = γ -hemolytic activity; PenS = sensitive to penicillin; PenR = resistant to penicillin; TetraS = sensitive to tetracycline; TetraR = resistant to tetracycline.

CONCLUSIONS

Staphylococcal isolates from Minas artisanal cheese, raw milk, endogenous starter cultures, and handlers from Campo das Vertentes, Brazil, presented toxigenic potential, with a higher prevalence of *sea* and *tsst-1*, although these genes showed low expression. The presence of toxigenic staphylococcal isolates in samples of raw cow milk and on the hands of humans demonstrates the need to control the pathogen, considering the One Health concept aimed at avoiding staphylococcal poisoning related to cheese intake. Biofilm was the main virulence factor found, because more than half of the staphylococcal isolates were biofilm-forming. In contrast, none of the staphylococcal isolates carried the *mecA* gene or presented a pattern of multi-drug resistance to antimicrobials commonly used in veterinary and human medicine.

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REFERENCES

- Amaral, L. D., A. P. M. Romano, A. Nader Filho, and O. D. Ossi Júnior. 2005. Qualidade da água em propriedades leiteiras como fator de risco a qualidade do leite e a saúde da glândula mamária. *Arq. Inst. Biol. (Sao Paulo)* 71:417–421. [In Portuguese]
- Andrey, D. O., A. Jousselin, M. Villanueva, A. Renzoni, A. Monod, C. Barras, N. Rodriguez, and W. Kelley. 2015. Impact of the regulators SigB, Rot, SarA and sarS on the toxic shock Tst promoter and TSST-1 expression in *Staphylococcus aureus*. *PLoS One* 10:e0135579. <https://doi.org/10.1371/journal.pone.0135579>.
- Arciola, C. R., D. Campoccia, S. Gamberini, M. Cervellati, E. Donati, and L. Montanaro. 2002. Detection of slime production by means of an optimised Congo red agar plate test based on a colourimetric scale in *Staphylococcus epidermidis* clinical isolates genotyped for *ica* locus. *Biomaterials* 23:4233–4239.
- Ariyanti, D., S. I. O. Salasia, and S. Tato. 2011. Characterization of haemolysin of *Staphylococcus aureus* isolated from food of animal origin. *Indones. J. Biotechnol.* 16:32–37.
- Assis, G. B. N., F. L. Pereira, A. U. Zegarra, G. C. Tavares, C. A. Leal, and H. C. P. Figueiredo. 2017. Use of MALDI-TOF mass spectrometry for the fast identification of gram-positive fish pathogens. *Front. Microbiol.* 8:1492.
- Baran, A., A. Erdogan, T. Turgut, and M. C. Adiguzel. 2017. A review on the presence of *Staphylococcus aureus* in cheese. *Turkish J. Nature Sci.* 6:100–105.
- Basset, P., G. Prod'homme, L. Senn, G. Greub, and D. Blanc. 2013. Very low prevalence of methicillin-resistant *Staphylococcus aureus* carrying the *mecC* gene in western Switzerland. *J. Hosp. Infect.* 83:257–259. <https://doi.org/10.1016/j.jhin.2012.12.004>.
- Becker, K., C. Heilmann, and G. Peters. 2014. Coagulase-negative staphylococci. *Clin. Microbiol. Rev.* 27:870–926.

- Becker, K., R. Roth, and G. Peters. 1998. Rapid and specific detection of toxigenic *Staphylococcus aureus*: Use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. *J. Clin. Microbiol.* 36:2548–2553.
- Beenken, K. E., L. N. Mrak, L. M. Griffin, A. K. Zielinska, L. N. Shaw, K. C. Rice, A. R. Horswill, K. W. Bayles, and M. S. Smeltzer. 2010. Epistatic relationships between *sarA* and *agr* in *Staphylococcus aureus* biofilm formation. *PLoS One* 5:e10790. <https://doi.org/10.1371/journal.pone.0010790>.
- Bergey, D. H., and J. Holt. 1994. *Bergey's Manual of Determinative Microbiology*. 9th ed. Williams and Wilkins, Baltimore, MD.
- Boucher, H., L. G. Miller, and R. R. Razouk. 2010. Serious infections caused by methicillin-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* 51(S2):S183–S197. <https://doi.org/10.1086/653519>.
- Breves, A., C. A. Miranda, C. Flores, I. Filippis, and M. M. Clementino. 2015. Methicillin- and vancomycin-resistant *Staphylococcus aureus* in health care workers and medical devices. *J. Bras. Patol. Med. Lab.* 51:143–152. <https://doi.org/10.5935/1676-2444.20150025>.
- Brown, A. F., J. M. Leech, T. R. Rogers, and R. M. McLoughlin. 2014. *Staphylococcus aureus* colonization: Modulation of host immune response and impact on human vaccine design. *Front. Immunol.* 4:507. <https://doi.org/10.3389/fimmu.2013.00507>.
- Carter, G. R. 1998. Fundamentos de Bacteriologia e Micologia Veterinária. Roca, São Paulo, Brazil. [In Portuguese]
- Castro, R. D., L. G. Oliveira, F. M. Sant'Anna, L. M. P. Luiz, S. H. C. Sandes, C. I. F. Silva, A. M. Silva, A. C. Nunes, C. F. A. M. Penna, and M. R. Souza. 2016. Lactic acid microbiota identification in water, raw milk, endogenous starter culture, and fresh Minas artisanal cheese from the Campo das Vertentes region of Brazil during the dry and rainy seasons. *J. Dairy Sci.* 99:6086–6096. <https://doi.org/10.3168/jds.2015-10579>.
- Cha, J. O., J. I. Yoo, J. S. Yoo, H. S. Chung, S. H. Park, H. S. Kim, Y. S. Lee, and G. T. Chung. 2013. Investigation of biofilm formation and its association with the molecular and clinical characteristics of methicillin-resistant *Staphylococcus aureus*. *Osong Public Health Res. Perspect.* 4:225–232. <https://doi.org/10.1016/j.phrp.2013.09.001>.
- Clarisse, T., S. Michèle, T. Olivier, E. Valérie, L. M. Vincent, H. Jacques-Antoine, G. Michel, and V. Florence. 2013. Detection and quantification of staphylococcal enterotoxin A in foods with specific and sensitive polyclonal antibodies. *Food Control* 32:255–261. <https://doi.org/10.1016/j.foodcont.2012.11.021>.
- CLSI (Clinical and Laboratory Standards Institute). 2015. Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Fifth Informational Supplement. CLSI document M100–S25. CLSI, Wayne, PA.
- Concepción Porrero, M., E. M. Harrison, J. F. Fernández-Garayzábal, G. K. Paterson, A. Díez-Guerrier, M. A. Holmes, and L. Domínguez. 2014. Detection of mecC-Methicillin-resistant *Staphylococcus aureus* isolates in river water: A potential role for water in the environmental dissemination. *Environ. Microbiol. Rep.* 6:705–708. <https://doi.org/10.1111/1758-2229.12191>.
- Costa, W. L. R., J. S. Ferreira, J. S. Carvalho, E. S. Cerqueira, L. C. Oliveira, and R. C. Almeida. 2015. Methicillin-resistant *Staphylococcus aureus* in raw meats and prepared foods in public hospitals in Salvador, Bahia, Brazil. *J. Food Sci.* 80:M147–M150. <https://doi.org/10.1111/1750-3841.12723>.
- Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Götz. 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun.* 67:5427–5433.
- Cretenet, M., S. Nouaille, J. Thouin, L. Rault, L. Stenz, P. François, J.-A. Hennekinne, M. Piot, M. B. Maillard, J. Fauquant, P. Loubière, Y. Le Loir, and S. Even. 2011. *Staphylococcus aureus* virulence and metabolism are dramatically affected by *Lactococcus lactis* in cheese matrix. *Environ. Microbiol. Rep.* 3:340–351. <https://doi.org/10.1111/j.1758-2229.2010.00230.x>.
- Cruzado-Bravo, M. L. M., N. C. C. S. Silva, M. X. Rodrigues, G. O. Silva, E. Porto, and G. L. Sturion. 2019. Phenotypic and genotypic characterization of *Staphylococcus* spp. isolated from mastitis milk and cheese processing: Study of adherence and biofilm formation. *Food Res. Int.* 122:450–460. <https://doi.org/10.1016/j.foodres.2019.04.017>.
- Cucarella, C., C. Solano, J. Valle, B. Amorena, I. Lasa, and J. R. Penades. 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J. Bacteriol.* 183:2888–2896. <https://doi.org/10.1128/JB.183.9.2888-2896.2001>.
- de Lourdes R.S. da Cunha, M., R. A. O. Calsolari, and J. P. A. Júnior. 2007. Detection of enterotoxin and toxic shock syndrome toxin 1 genes in *Staphylococcus*, with emphasis on coagulase-negative staphylococci. *Microbiol. Immunol.* 51:381–390. <https://doi.org/10.1111/j.1348-0421.2007.tb03925.x>.
- de Melo, D. A., I. S. Coelho, C. C. da Motta, A. C. C. M. Rojas, F. C. Dubenczuk, S. M. O. Coelho, and M. M. S. de Souza. 2014. Impairments of *mecA* gene detection in bovine *Staphylococcus* spp. *Braz. J. Microbiol.* 45:1075–1082. <https://doi.org/10.1590/S1517-83822014000300041>.
- Derzelle, S., F. Dilasser, M. Duquenne, and V. Deperrois. 2009. Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. *Food Microbiol.* 26:896–904. <https://doi.org/10.1016/j.fm.2009.06.007>.
- Di Ciccio, P., A. Vergara, A. R. Festino, D. Paludi, E. Zanardi, S. Ghidini, and A. Ianieri. 2015. Biofilm formation by *Staphylococcus aureus* on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. *Food Control* 50:930–936. <https://doi.org/10.1016/j.foodcont.2014.10.048>.
- Dias-Souza, M.-V., S. Andrade, A. P. Aguiar, and A. Monteiro. 2013. Evaluation of antimicrobial and anti-biofilm activities of *Anacardium occidentale* stem bark extract. *J. Nat. Prod.* 6:198–205.
- Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* 13:16–34. <https://doi.org/10.1128/CMR.13.1.16>.
- Even, S., C. Charlier, S. Nouaille, N. L. Ben Zakour, M. Cretenet, F. J. Cousin, M. Gautier, M. Coccagn-Bousquet, P. Loubière, and Y. Le Loir. 2009. *Staphylococcus aureus* virulence expression is impaired by *Lactococcus lactis* in mixed cultures. *Appl. Environ. Microbiol.* 75:4459–4472. <https://doi.org/10.1128/AEM.02388-08>.
- Fey, P. D. 2010. Modality of bacterial growth presents unique targets: How do we treat biofilm-mediated infections? *Curr. Opin. Microbiol.* 13:610–615. <https://doi.org/10.1016/j.mib.2010.09.007>.
- Figueiredo, A. M. S., F. A. Ferreira, C. O. Beltrame, and M. F. Côrtes. 2017. The role of biofilms in persistent infections and factors involved in *ica*-independent biofilm development and gene regulation in *Staphylococcus aureus*. *Crit. Rev. Microbiol.* 43:602–620. <https://doi.org/10.1080/1040841X.2017.1282941>.
- Foster, T. J., J. A. Geoghegan, V. K. Ganesh, and M. Hook. 2014. Adhesion, invasion and evasion: The many functions of the surface proteins of *Staphylococcus aureus*. *Nat. Rev. Microbiol.* 12:49–62. <https://doi.org/10.1038/nrmicro3161>.
- Gaventa, S., A. L. Reingold, A. W. Hightower, C. V. Broome, B. Schwartz, C. Hoppe, J. Harwell, L. K. Lefkowitz, S. Makintubee, D. R. Cundiff, and S. Sitze. 1989. Active surveillance for toxic shock syndrome in the United States, 1986. *Rev. Infect. Dis.* 11(Suppl. 1):S28–S34. https://doi.org/10.1093/clinids/11.Supplement_1.S28.
- Gerke, C., A. Kraft, R. Süßmuth, O. Schweitzer, and F. Götz. 1998. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. *J. Biol. Chem.* 273:18586–18593. <https://doi.org/10.1074/jbc.273.29.18586>.
- Grinholc, M., G. Wegrzyn, and J. Kurlenda. 2007. Evaluation of biofilm production and prevalence of the *icaD* gene in methicillin-resistant and methicillin susceptible *Staphylococcus aureus* strains isolated from patients with nosocomial infections and carriers. *FEMS Immunol. Med. Microbiol.* 50:375–379. <https://doi.org/10.1111/j.1574-695X.2007.00262.x>.
- Guidi, F., A. Duranti, S. Gallina, Y. Nia, A. Petruzzelli, A. Romano, V. Travaglini, A. Olivastri, V. Calvaresi, L. Decastelli, and G. Blasi. 2018. Characterization of a staphylococcal food poisoning outbreak in a workplace canteen during the post-earthquake recon-

- struction of central Italy. *Toxins* (Basel) 10:523. <https://doi.org/10.3390/toxins10120523>.
- Hair, J. F., M. Wolfenbarger, A. H. Money, P. Samouel, and M. J. Page. 2003. *Essentials of Business Research Methods*. John Wiley and Sons, Milton, Australia.
- Hellemans, J., G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19. <https://doi.org/10.1186/gb-2007-8-2-r19>.
- Hennekinne, J. A., M. L. de Buyser, and S. Dragacci. 2012. *Staphylococcus aureus* and its food poisoning toxins: Characterization and outbreak investigation. *FEMS Microbiol. Rev.* 36:815–836. <https://doi.org/10.1111/j.1574-6976.2011.00311.x>.
- Hu, D. L., and A. Nakane. 2014. Mechanisms of staphylococcal enterotoxin-induced emesis. *Eur. J. Pharmacol.* 722:95–107. <https://doi.org/10.1016/j.ejphar.2013.08.050>.
- Huse, S. M., L. Dethlefsen, J. A. Huber, D. Mark Welch, D. A. Relman, and M. L. Sogin. 2008. Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLoS Genet.* 4:e1000255.
- Ibed, A. N., and S. S. Hamim. 2014. Molecular detection of methicillin resistant *Staphylococcus aureus* isolated from burns infection in Al-Nasiriyah city. *World J. Pharm. Sci.* 2:950–954.
- Instituto Brasileiro de Geografia e Estatística. 2018. Indicadores IBGE—Estatística da Produção Pecuária. Instituto Brasileiro de Geografia e Estatística, Rio de Janeiro, Brazil. [In Portuguese]
- Jain, A. K., M. N. Murty, and P. J. Flynn. 1999. Data clustering: A review. *ACM Comput. Surv.* 31:264–323. <https://doi.org/10.1145/331499.331504>.
- Jamali, H., M. Paydar, B. Radmehr, S. Ismail, and A. Dadrassnia. 2015. Prevalence and antimicrobial resistance of *Staphylococcus aureus* isolated from raw milk and dairy products. *Food Control* 54:383–388. <https://doi.org/10.1016/j.foodcont.2015.02.013>.
- Jamali, H., and B. Radmehr. 2013. Frequency, virulence genes and antimicrobial resistance of *Listeria* spp. isolated from bovine clinical mastitis. *Vet. J.* 198:541–542. <https://doi.org/10.1016/j.tvjl.2013.06.012>.
- Jarraud, S., C. Mougél, J. Thioulouse, G. Lina, H. Meunier, F. Forey, X. Nesme, J. Etienne, and E. Vandenesch. 2002. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect. Immun.* 70:631–641.
- Johler, S., G. Macori, A. Bellio, P. L. Acutis, S. Gallina, and L. Decastelli. 2018. Short communication: Characterization of *Staphylococcus aureus* isolated along the raw milk cheese production process in artisan dairies in Italy. *J. Dairy Sci.* 101:2915–2920. <https://doi.org/10.3168/jds.2017-13815>.
- Johler, S., D. Weder, C. Bridy, M. C. Huguenin, L. Robert, J. Hummerjohann, and R. Stephan. 2015. Outbreak of staphylococcal food poisoning among children and staff at a Swiss boarding school due to soft cheese made from raw milk. *J. Dairy Sci.* 98:2944–2948. <https://doi.org/10.3168/jds.2014-9123>.
- Joo, H. S., and M. Otto. 2012. Molecular basis of in vivo biofilm formation by bacterial pathogens. *Chem. Biol.* 19:1503–1513. <https://doi.org/10.1016/j.chembiol.2012.10.022>.
- Kateete, D. P., C. N. Kimani, F. A. Katabazi, A. Okeng, M. S. Okee, A. Nanteza, M. L. Joloba, and F. C. Najjuka. 2010. Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. *Ann. Clin. Microbiol. Antimicrob.* 9:23. <https://doi.org/10.1186/1476-0711-9-23>.
- Klotz, M., S. Oppé, K. Heeg, and S. Zimmermann. 2003. Detection of *Staphylococcus aureus* enterotoxins A to D by real-time fluorescence PCR assay. *J. Clin. Microbiol.* 41:4683–4687.
- Lamy, B., F. Laurent, O. Gallon, F. Doucet-Populaire, J. Etienne, and J. W. Decousser. 2012. Antibacterial resistance, genes encoding toxins and genetic background among *Staphylococcus aureus* isolated from community-acquired skin and soft tissue infections in France: A national prospective survey. *Eur. J. Clin. Microbiol. Infect. Dis.* 31:1279–1284. <https://doi.org/10.1007/s10096-011-1441-5>.
- Mack, D., W. Fischer, A. Krokotsch, K. Leopold, R. Hartmann, H. Egge, and R. Laufs. 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1, 6-linked glucosaminoglycan: Purification and structural analysis. *J. Bacteriol.* 178:175–183. <https://doi.org/10.1128/jb.178.1.175-183.1996>.
- Martins, J. M., É. Galinari, N. J. Pimentel-Filho, J. I. Ribeiro Jr., M. M. Furtado, and C. L. L. F. Ferreira. 2015. Determining the minimum ripening time of artisanal Minas cheese, a traditional Brazilian cheese. *Braz. J. Microbiol.* 46:219–230. <https://doi.org/10.1590/S1517-838246120131003>.
- Mégevaud, C., A. Gervais, U. Heininger, C. Berger, C. Aebi, B. Vaudaux, C. Kind, H.-P. Gnehm, M. Hitzler, G. Renzi, J. Schrenzel, and P. François. 2010. Molecular epidemiology of the nasal colonization by methicillin-susceptible *Staphylococcus aureus* in Swiss children. *Clin. Microbiol. Infect.* 16:1414–1420. <https://doi.org/10.1111/j.1469-0691.2009.03090.x>.
- Melo, T. A., T. F. dos Santos, M. E. de Almeida, L. A. G. Fontes Junior, E. F. Andrade, R. P. Rezende, L. M. Marques, and C. C. Romano. 2016. Inhibition of *Staphylococcus aureus* biofilm by *Lactobacillus* isolated from fine cocoa. *BMC Microbiol.* 16:250. <https://doi.org/10.1186/s12866-016-0871-8>.
- Mendonça, E. C. L., V. F. Marques, D. A. Melo, T. A. Alencar, I. S. Coelho, S. M. O. Coelho, and M. M. S. Souza. 2012. Caracterização fenotípica da resistência antimicrobiana em *Staphylococcus* spp. isolados de mastite bovina. *Pesqui. Vet. Bras.* 32:859–864. <https://doi.org/10.1590/S0100-736X2012000900008>.
- Morrison, J. M., and P. M. Dunman. 2011. The modulation of *Staphylococcus aureus* mRNA turnover. *Future Microbiol.* 6:1141–1150. <https://doi.org/10.2217/fmb.11.102>.
- Nielsen, A., M. Månsson, M. S. Bojer, L. Gram, T. O. Larsen, R. P. Novick, D. Frees, H. Frøkiær, and H. Ingmer. 2014. Solonamide B inhibits quorum sensing and reduces *Staphylococcus aureus* mediated killing of human neutrophils. *PLoS One* 9:e84992. <https://doi.org/10.1371/journal.pone.0084992>.
- Ono, H. K., Y. Sato'o, K. Narita, I. Naito, S. Hirose, J. Hisatsune, K. Asano, D. L. Hu, K. Omoe, M. Sugai, and A. Nakane. 2015. Identification and characterization of a novel staphylococcal emetic toxin. *Appl. Environ. Microbiol.* 81:7034–7040. <https://doi.org/10.1128/AEM.01873-15>.
- Ostyn, A., M. L. De Buyser, F. Guillier, J. Groult, B. Felix, S. Salah, G. Delmas, and J. A. Hennekinne. 2010. First evidence of a food poisoning outbreak due to staphylococcal enterotoxin type E, France, 2009. *Euro Surveill.* 15:1–4.
- Otto, M. 2014. *Staphylococcus aureus* toxins. *Curr. Opin. Microbiol.* 17:32–37. <https://doi.org/10.1016/j.mib.2013.11.004>.
- Paiva, R. G. 1991. Aprova da coagulase relacionada ao meio de cultivo de *Staphylococcus aureus* contendo diferentes concentrações de cloreto de sódio. *Rev. Patol. Trop.* 20:21–33.
- Parsonnet, J., M. A. Hansmann, M. L. Delaney, P. A. Modern, A. M. DuBois, W. Wieland-Alter, K. W. Wisemann, J. E. Wild, M. B. Jones, J. L. Seymour, and A. B. Onderdonk. 2005. Prevalence of toxic shock syndrome toxin 1-producing *Staphylococcus aureus* and the presence of antibodies to this superantigen in menstruating women. *J. Clin. Microbiol.* 43:4628–4634. <https://doi.org/10.1128/JCM.43.9.4628-4634.2005>.
- Paterson, G. K., E. M. Harrison, and M. A. Holmes. 2014. The emergence of mecC methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol.* 22:42–47. <https://doi.org/10.1016/j.tim.2013.11.003>.
- Peacock, S. J., and G. K. Paterson. 2015. Mechanisms of methicillin resistance in *Staphylococcus aureus*. *Annu. Rev. Biochem.* 84:577–601. <https://doi.org/10.1146/annurev-biochem-060614-034516>.
- Pedroso, S. H. S. P., S. H. C. Sandes, R. A. T. Filho, A. C. Nunes, J. C. Serufo, L. M. Farias, M. A. R. Carvalho, M. R. Q. Bomfim, and S. G. Santos. 2018. Coagulase-negative staphylococci isolated from human bloodstream infections showed multidrug resistance profile. *Microb. Drug Resist.* 24:635–647. <https://doi.org/10.1089/mdr.2017.0309>.
- Pedroso, S. H. S. P., S. H. C. Sandes, K. C. M. Luiz, R. S. Dias, R. A. T. Filho, J. C. Serufo, L. M. Farias, M. A. R. Carvalho, M. R. Q. Bomfim, and S. G. Santos. 2016. Biofilm and toxin profile:

- A phenotypic and genotypic characterization of coagulase-negative staphylococci isolated from human bloodstream infections. *Microb. Pathog.* 100:312–318. <https://doi.org/10.1016/j.micpath.2016.10.005>.
- Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* 8:151–156. <https://doi.org/10.1111/j.1472-765X.1989.tb00262.x>.
- Queck, S. Y., M. Jameson-Lee, A. E. Villaruz, T. H. Bach, B. A. Khan, D. E. Sturdevant, S. M. Ricklefs, M. Li, and M. Otto. 2008. RNAIII-independent target gene control by the agr quorum-sensing system: Insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol. Cell* 32:150–158. <https://doi.org/10.1016/j.molcel.2008.08.005>.
- Regassa, L. B., J. L. Couch, and M. J. Betley. 1991. Steady-state staphylococcal enterotoxin type C mRNA is affected by a product of the accessory gene regulator (agr) and by glucose. *Infect. Immun.* 59:955–962.
- Reinoso, E., S. Bettera, C. Frigerio, M. DiRenzo, A. Calzolari, and C. Bogni. 2004. RAPD-PCR analysis of *Staphylococcus aureus* strains isolated from bovine and human hosts. *Microbiol. Res.* 159:245–255.
- Robbins, R., S. Gould, and S. M. S. Bergdoll. 1974. Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. *Appl. Microbiol.* 28:946–950.
- Rudkin, J. K., A. M. Edwards, M. G. Bowden, E. L. Brown, C. Pozzi, E. M. Waters, W. C. Chan, P. Williams, J. P. O’Gara, and R. C. Massey. 2012. Methicillin resistance reduces the virulence of healthcare-associated methicillin-resistant *Staphylococcus aureus* by interfering with the agr quorum sensing system. *J. Infect. Dis.* 205:798–806. <https://doi.org/10.1093/infdis/jir845>.
- Sandes, S., L. Alvim, B. Silva, L. Acurcio, C. Santos, M. Campos, C. Santos, J. Nicoli, E. Neumann, and A. Nunes. 2017. Selection of new lactic acid bacteria strains bearing probiotic features from mucosal microbiota of healthy calves: Looking for immunobiotics through in vitro and in vivo approaches for immunoprophylaxis applications. *Microbiol. Res.* 200:1–13. <https://doi.org/10.1016/j.micres.2017.03.008>.
- Santos, V. M., H. B. Martins, I. S. Rezende, M. S. Barbosa, E. F. Andrade, S. G. Souza, G. B. Campos, P. S. Oliveira, D. S. Sousa, D. C. C. Da Silva, A. T. Amorim, J. Timenetsky, M. P. Cruz, R. Yatsuda, and L. M. Marques. 2014. Virulence factor profile of *Staphylococcus aureus* isolated from bovine milk from Brazil. *Food Nutr. Sci.* 5:1496–1505.
- Schroeder, K., M. Jularic, S. M. Horsburgh, N. Hirschhausen, C. Neumann, A. Bertling, A. Schulte, S. Foster, B. Kehrel, G. Peters, and C. Heilmann. 2009. Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. *PLoS One* 4:e7567. <https://doi.org/10.1371/journal.pone.0007567>.
- Silva, N., and V. C. A. Junqueira. N. F. A. Silveira, M. H. Taniwaki, R. A. Romeiro Gomes, and M. M. Okazaki. 2010. Manual de Métodos de Análise Microbiológica de Alimentos. Varela, São Paulo, Brazil.
- Simeão do Carmo, L., R. S. Dias, V. R. Linardi, M. José de Sena, D. Aparecida dos Santos, M. Eduardo de Faria, E. C. Pena, M. Jett, and L. G. Heneine. 2002. Food poisoning due to enterotoxigenic strains of *Staphylococcus* present in Minas cheese and raw milk in Brazil. *Food Microbiol.* 19:9–14. <https://doi.org/10.1006/fmic.2001.0444>.
- Simões, M., L. C. Simões, and M. J. Vieira. 2010. A review of current and emergent biofilm control strategies. *Lebensm. Wiss. Technol.* 43:573–583. <https://doi.org/10.1016/j.lwt.2009.12.008>.
- Skov, R., A. R. Larsen, A. Kearns, M. Holmes, C. Teale, G. Edwards, and R. Hill. 2014. Phenotypic detection of *mecC*-MRSA: Cefoxitin is more reliable than oxacillin. *J. Antimicrob. Chemother.* 69:133–135. <https://doi.org/10.1093/jac/dkt341>.
- Speziale, P., G. Pietroccola, T. J. Foster, and J. A. Geoghegan. 2014. Protein based biofilm matrices in staphylococci. *Front. Cell. Infect. Microbiol.* 4:171. <https://doi.org/10.3389/fcimb.2014.00171>.
- Sugimoto, S., F. Sato, R. Miyakawa, A. Chiba, S. Onodera, S. Hori, and Y. Mizunoe. 2018. Broad impact of extracellular DNA on biofilm formation by clinically isolated methicillin-resistant and -sensitive strains of *Staphylococcus aureus*. *Sci. Rep.* 8:2254. <https://doi.org/10.1038/s41598-018-20485-z>.
- Thoendel, M., J. S. Kavanaugh, C. E. Flack, and A. R. Horswill. 2011. Peptide signaling in the staphylococci. *Chem. Rev.* 111:117–151. <https://doi.org/10.1021/cr100370n>.
- Torres Filho, R. A., V. M. da Silva, L. M. Rodrigues, P. R. Fontes, A. L. S. Ramos, and E. M. Ramos. 2018. Classification of pork quality by hierarchical cluster analysis. *Br. Food J.* 120:1446–1456. <https://doi.org/10.1108/BFJ-10-2017-0589>.
- Tsutsuura, S., Y. Shimamura, and M. Murata. 2013. Temperature dependence of the production of staphylococcal enterotoxin A by *Staphylococcus aureus*. *Biosci. Biotechnol. Biochem.* 77:30–37. <https://doi.org/10.1271/bbb.120391>.
- Valihrach, L., B. Alibayov, K. Zdenkova, and K. Demnerova. 2014. Expression and production of staphylococcal enterotoxin C is substantially reduced in milk. *Food Microbiol.* 44:54–59. <https://doi.org/10.1016/j.fm.2014.05.020>.
- Verhoeven, P. O., J. Gagnaire, E. Botelho-Nevers, F. Grattard, A. Carricajo, F. Lucht, B. Pozzetto, and P. Berthelot. 2014. Detection and clinical relevance of *Staphylococcus aureus* nasal carriage: An update. *Expert Rev. Anti Infect. Ther.* 12:75–89. <https://doi.org/10.1586/14787210.2014.859985>.
- Vuong, C., S. Kocianova, J. M. Voyich, Y. Yao, E. R. Fischer, F. R. DeLeo, and M. Otto. 2004. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J. Biol. Chem.* 279:54881–54886. <https://doi.org/10.1074/jbc.M411374200>.
- Waters, E. M., S. E. Rowe, J. P. O’Gara, and B. P. Conlon. 2016. Convergence of *Staphylococcus aureus* persister and biofilm research: Can biofilms be defined as communities of adherent persister cells? *PLoS Pathog.* 12:e1006012.
- World Health Organization. 2017. Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics. WHO Press, Geneva, Switzerland.
- World Health Organization. 2019. Critically Important Antimicrobials for Human Medicine. 6th rev. WHO Press, Geneva, Switzerland.
- Xu, S. X., K. J. Kasper, J. J. Zeppa, and J. K. McCormick. 2015. Superantigens modulate bacterial density during *Staphylococcus aureus* nasal colonization. *Toxins (Basel)* 7:1821–1836. <https://doi.org/10.3390/toxins7051821>.

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