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Tese

Diversidade genética, resistência a sanitizantes, formação de biofilme e expressão de genes de adesão em *Staphylococcus aureus* provenientes de leite

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Dedico

Aos meus pais, irmã e avó pelo apoio e amor incondicional

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Resumo

Kroning, Isabela Schneid. **Diversidade genética, resistência a sanitizantes, formação de biofilme e expressão de genes de adesão em *Staphylococcus aureus* provenientes de leite.** 2019. 112f. Tese (Doutorado) - Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. Universidade Federal de Pelotas, Pelotas.

Staphylococcus aureus é o terceiro micro-organismo mais envolvido em doenças transmitidas por alimentos no Brasil. A sua capacidade de formar biofilme em diferentes superfícies da indústria de alimentos é um fator de risco para o consumidor, devido a possibilidade de contaminação dos produtos alimentícios. Os objetivos deste estudo foram verificar a diversidade genética, avaliar a capacidade de formação de biofilme e a expressão de genes de adesão em isolados de *S. aureus* provenientes de leite. Além disso, objetivou-se determinar o perfil de suscetibilidade a sanitizantes e detectar a presença de genes de resistência a sanitizantes. A técnica de *spa typing* foi utilizada para avaliar a diversidade genética. Para avaliação da capacidade de formação de biofilme foram utilizadas microplacas de poliestireno para selecionar diferentes perfis de formação de biofilme. Após foram selecionados nove isolados de diferentes perfis de formação de biofilme (três fracos formadores de biofilme, três moderados formadores de biofilme e três não formadores de biofilme) e estes foram avaliados quanto a sua capacidade de formar biofilme em aço inoxidável nas temperaturas de 7, 10 e 37 °C em caldo Triptona de Soja (TSB) e leite UHT. Foi utilizada a técnica de PCR quantitativa em Tempo Real (RT-qPCR) para avaliar a expressão dos genes de adesão (*cna* e *ebpS).* Para verificar a suscetibilidade aos sanitizantes cloreto de benzalcônio e clorexidina utilizou-se o método de microdiluição em caldo e PCR para detectar genes de resistência a sanitizantes. Dos 31 isolados de *S. aureus* avaliados, 18 (58%) produziram exopolissacarídeo. Pela avaliação em poliestireno, verificou-se que 14 isolados (45%) apresentaram capacidade de produzir biofilmes nessa superfície. Dos nove isolados selecionados com diferentes perfis de formação de biofilme verificou-se que todos produziram biofilme em aço inox em todas temperaturas e meios de cultivo testados, independente de seu perfil de formação de biofilme em poliestireno. Com relação à presença dos genes de adesão, os genes *fnbA, fnbB, clfB, ebpS* e *cna,* foram encontrados em 48%, 3%, 52%, 80% e 62% dos isolados, respectivamente. A RT-qPCR revelou variação nos níveis de expressão dos genes *ebpS* e *cna*, entretanto, apenas no isolado C2, na temperatura de 10 °C, houve expressão significativamente maior dos dois genes testados em relação as demais temperaturas de multiplicação. Os isolados apresentaram perfil de resistência ao cloreto de benzalcônio (90,3%) e a clorexidina (100%); além disso, os genes *mepA, norA* e *norB* foram os mais prevalentes, com frequências de 77,4%, 80,6% e 100%, respectivamente. O *spa* type t127 foi o mais prevalente entre os isolados, sendo que esse *spa* type apresenta uma relação genética com isolados de origem humana, podendo ser a fonte de transmissão de *S. aureus* para o leite. Os resultados obtidos no presente estudo ressaltam a importância do monitoramento de *S. aureus* no ambiente da indústria de alimentos, uma vez que este micro-organismo é capaz de formar biofilme em diferentes superfícies, temperaturas e meios de cultivo e apresenta resistência fenotípica e genotípica a sanitizantes.

Palavras-chave: Biofilme; cloreto de benzalcônio; clorexidina; RT-qPCR; *spa typing*

Abstract

Kroning, Isabela Schneid. **Genetic diversity, resistance to sanitizers, biofilm formation and expression of adhesion genes in *Staphylococcus aureus* from milk.** 2019. 112f. Thesis (Doctor degree) - Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. Universidade Federal de Pelotas, Pelotas.

Staphylococcus aureus is the third microorganism most involved in foodborne diseases in Brazil. The ability to form biofilm on different surfaces of the food industry is a risk factor for the consumer, due to the possibility of contamination of food products. The aims of this study were to verify the genetic diversity, as well as to evaluate the capacity of biofilm formation and the expression of adhesion genes in *S. aureus* isolates from milk. In addition, it was aimed to determine the susceptibility profile to benzalkonium chloride and chlorhexidine sanitizers and to detect the presence of the main resistance genes to sanitizers in these isolates. The *spa* typing was used to evaluate genetic diversity. For evaluate the ability of biofilm formation, firstly polystyrene microplates were used to select different biofilm formation profiles. After this, nine isolates from different biofilm formation profiles (three poor biofilm producer, three moderate biofilm producer and three non-biofilm producers) were selected, and these were evaluated for their ability to form biofilm on stainless steel at temperatures of 7, 10 and 37 °C in Tryptone Soy Broth (TSB) and UHT milk. Real-time quantitative PCR (RT-qPCR) was used to evaluate the expression of adhesion genes (*cna* and *ebpS*). To verify the susceptibility to sanitizers benzalkonium chloride and chlorhexidine the broth microdilution method was used and the PCR was used to detect the main resistance genes to sanitizers. Of the 31 isolates of *S. aureus* evaluated, 18 (58%) produced exopolysaccharide. From the polystyrene evaluation, 14 isolates (45%) were able to produce biofilms on this surface. Of these, nine isolates with different biofilm formation profiles (weak biofilm producer, moderate biofilm producer and no biofilm producer) were selected to verify their ability to produce biofilm on stainless steel surface, and all isolates produced biofilms at temperatures of 7, 10 and 37 °C, in TSB and in UHT milk, independent of polystyrene formation profile. Regarding to the presence of the genes of adhesion *fnbA, fnbB, clfB, ebpS* and *cna,* were found in 48%, 3%, 52%, 80% and 62% of the isolates, respectively. The real-time quantitative PCR (RT-qPCR) technique showed variation in expression levels of the *ebpS* and *cna* genes; however, only in the C2 isolate at a temperature of 10 °C, there was a significantly higher expression of the two genes tested in relation to other multiplication temperatures. The isolates showed a resistance profile to benzalkonium chloride (90.3%) and chlorhexidine (100%); in addition, the *mepA, norA* and *norB* genes were the most prevalent, with frequencies of 77.4%, 80.6% and 100%, respectively. The *spa* type t127 was the most prevalent among the isolates, and this *spa* type has a very high genetic relation with isolates from human sources, being able to be the source of transmission of *S. aureus* to milk. The results obtained in the present study highlight the importance of monitoring *S. aureus* in the food industry environment, since this microorganism is able to form biofilm in different surfaces, temperatures and culture media and harbor phenotypic resistance and genotypic to sanitizers.

Key-words: biofilm; benzalkonium chloride; chlorhexidine; RT-qPCR; *spa* typing

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1 Introdução

O gênero *Staphylococcus* é composto por 53 espécies e 27 subespécies, pertencendo à família Staphylococcaceae (EUZÉBY, 2018). Dentre as espécies pertencentes a este gênero, destaca-se *Staphylococcus aureus,* que apresenta morfologia de cocos, Gram-positivos, anaeróbios facultativos, imóveis, produtor de enterotoxinas e tendem a formar agrupamentos em formato semelhante a cachos de uva. São micro-organismos mesófilos que se desenvolvem entre 7ºC e 47,8ºC e podem produzir mais de 30 fatores de virulência (KONEMAN et al., 2012; TRABULSI et al., 2008).

Staphylococcus aureus é o terceiro micro-organismo mais envolvido em doenças transmitidas por alimentos (DTA) no Brasil (BRASIL, 2017). Nos Estados Unidos da América, estima-se que ocorram 241 mil casos de DTA por ano em decorrência da intoxicação causada por esse micro-organismo (SCALLAN et al., 2011). Em 2015, na Europa, foram relatados 434 surtos de origem alimentar causados por toxinas estafilocócicas, os quais representam 9,9% de todos os surtos ocorridos no período (EFSA, 2016).

Devido à diversidade genética de *S. aureus,* a combinação de uma ou mais técnicas de tipificação se faz necessária para melhor caracterizar diferentes isolados. O método de *spa typing* tem como alvo a região X do gene *spa*, da proteína A de *S. aureus* que é considerada um potente fator de virulência que contém uma repetição em tandem (cerca de 24 pb de comprimento). Este *locus* é altamente polimórfico, devido a uma região interna de repetições curtas e variáveis não só em número, mas também devido a substituições de nucleotídeos dentro das unidades de repetição individuais (FRÉNAY et al., 1996). A diversidade de *spa* varia de acordo com o tipo de repetições e seus números de cópias, onde a diversidade dessa região parece surgir da deleção e duplicação de unidades repetitivas e, também, por pontos de mutação.

A tipagem por *spa* tornou-se muito relevante, uma vez que se baseia no sequenciamento de um único *locus*, sendo uma técnica menos dispendiosa e menos demorada do que outras técnicas já consolidadas, como por exemplo *Multilocus*

sequence typing (MLST) e *Pulsed-field gel electrophoresis* (PFGE) (MALACHOWA et al., 2005). Vale ressaltar que a combinação de técnicas de genotipagem pode aumentar o poder discriminatório para caracterizar diferentes isolados (ALBA et al., 2015; WANG et al., 2018).

Além da diversidade genética, outra capacidade de *S. aureus* muito explorada é a de formar biofilme em diferentes superfícies da indústria de alimentos é um fator de risco para o consumidor, devido a possibilidade de contaminação dos produtos alimentícios com estes micro-organismos patogênicos. Os biofilmes são populações de micro-organismos que se multiplicam sobre uma superfície e estão envoltos por uma matriz composta por proteínas, glicoproteínas, glicolipídeos, polissacarídeos, e DNA extracelular (WELLS et al., 2011). Atualmente, encontram-se poucos estudos na literatura avaliando isolados de *S. aureus* provenientes de alimentos em condições que simulam a formação de biofilme em superfícies de contato, meios de cultivo e temperaturas encontradas no ambiente da indústria de alimentos, de forma a verificar o comportamento destes micro-organismos e buscar maneiras de combater o biofilme formado.

Durante a formação de biofilme, uma etapa importante é a adesão a superfície. Em *S. aureus*, a adesão inicial a diferentes superfícies, incluindo superfícies abióticas e tecidos vivos, ocorre através das adesinas conhecidas como Componentes da Superfície Microbiana Reconhecedores de Moléculas Adesivas da Matriz (*Microbial Surface Components Recognizing Adhesive Matrix Molecules* - MSCRAMMs). Essas MSCRAMMs podem se aderir a um ou mais sítios da matriz extracelular, como a elastina (gene *ebpS*), laminina (gene *eno*), colágeno (gene *cna*), fibronectina A e B (genes *fnbA* e *fnbB*), fibrinogênio (gene *fib*) e os fatores de aglomeração (genes *clfA* e *clfB*) (SEO et al., 2008; ROHDE et al., 2010).

De acordo com Stanley e Lazazzera (2004) os genes relacionados à formação de biofilme são regulados em resposta a fatores ambientais, tais como baixo teor de glicose, anaerobiose, falta de ferro, alta osmolaridade e temperatura, sendo a expressão variável dependendo dos isolados avaliados (JOHLER, S.; STEPHAN, 2010). Sendo assim, quantificar a expressão gênica é fundamental em muitas áreas de pesquisa e, neste contexto, a técnica de PCR quantitativa em tempo real (RT-qPCR) se tornou referência para a quantificação de ácidos nucleicos (BUSTIN et al., 2009) e da expressão gênica (SCHMITTGEN; LIVAK, 2008), sendo uma importante ferramenta durante a formação de biofilme.

Além da formação de biofilme, outro fator preocupante na disseminação de *S. aureus* na indústria de alimentos é a resistência aos sanitizantes utilizados na limpeza das superfícies, uma vez que o seu uso inadequado pode proporcionar uma exposição subletal, contribuindo para o desenvolvimento de resistência dos micro-organismos, bem como para a troca de genes de resistência entre bactérias (GOESSWEINER-MOHR et al., 2014; BUZÓN-DURÁN et al., 2017).

Dentre os sanitizantes utilizados na indústria de alimentos destacam-se os compostos quaternários de amônio e as biguanidas. O primeiro apresenta mecanismo de ação físico, causando danos na membrana citoplasmática devido as alterações nas propriedades físicas e bioquímicas; já as biguanidas são compostos catiônicos que atuam na membrana celular, causando interação eletrostática com os fosfolipídios da membrana citoplasmática, modificando a sua permeabilidade e, consequentemente, dissolvendo-a (WESSELS; INGMER, 2013; FERNÁNDEZ-FUENTES et al., 2014).

Os genes de resistência aos compostos quaternários de amônio (cloreto de benzalcônio) e as biguanidas (clorexidina) relatados na literatura, compreendem bombas de efluxo, que estão tipicamente presentes em plasmídeos, como por exemplo os genes *qacA, smr, qacG, qacH, qacJ, mepA, norA* e *norB.* Sendo assim, pode ocorrer transferência horizontal desses genes, disseminando a resistência aos sanitizantes (ARGUDÍN et al., 2016).

Entretanto, a presença de genes de resistência a sanitizantes utilizados na indústria de alimentos é pouco pesquisada em micro-organismos isolados de alimentos e o conhecimento sobre os determinantes genéticos dessa resistência é de extrema importância para a implementação de estratégias nas práticas de higienização e sanitização no ambiente de processamento de alimentos.

Esta tese foi estruturada em 3 capítulos, sendo que o primeiro capítulo corresponde a um manuscrito de revisão sobre a formação de biofilme, resistência fenotípica e genotípica a sanitizantes e alternativas para remoção de biofilme. O segundo capítulo corresponde a um manuscrito sobre a diversidade genética de *S. aureus* e a resistência fenotípica e genotípica a sanitizantes e o terceiro capítulo trata da prevalência e expressão de genes de adesão em *S. aureus* durante a formação de biofilme na superficie de aço inox em diferentes meios de cultivo e temperaturas.

Sendo assim, os resultados obtidos no presente estudo, contribuirão para o entendimento dos mecanismos de formação de biofilme, da resistência fenotípica e genotípica a sanitizantes, bem como da diversidade genética de *S. aureus*, assim como poderão fornecer uma base para mais estudos acerca deste importante micro-organismo patogênico.

2 Objetivos

2.1 Objetivo geral

Verificar a diversidade genética, bem como avaliar a capacidade de formação de biofilme e verificar a presença e a expressão de genes de adesão em isolados de *S. aureus* provenientes de leite. Além disso, objetivou-se determinar o perfil de suscetibilidade aos sanitizantes cloreto de benzalcônio e clorexidina e detectar a presença dos principais genes de resistência a sanitizantes nesses isolados.

2.2 Objetivos específicos

- Verificar a relação clonal entre os isolados;
- Avaliar o perfil de suscetibilidade aos sanitizantes cloreto de benzalcônio e clorexidina e avaliar a presença dos principais genes envolvidos na resistência a esses compostos nestes isolados;
- Verificar a capacidade de formação de biofilme por isolados de *S. aureus* provenientes de leite, em superfícies de poliestireno e aço inoxidável;
- Detectar a presença dos principais genes responsáveis pela adesão nesses isolados;
- Quantificar os níveis de expressão gênica dos genes *cna* e *ebpS,* relacionados à adesão, em função dos diferentes perfis de formação de biofilme *in vitro*;

.

3 Capítulo I

3.1 Manuscrito 1 - Biofilm formation by *Staphylococcus aureus* at food industry environment: genetics aspects, phenotypic and genotypic characteristics of sanitizers resistance and alternatives to remove biofilm

Manuscrito de revisão a ser submetido ao periódico *Food Research International* (Fator de Impacto 3.520) e Qualis A1 na Área de Ciência de Alimentos

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Abstract

The biofilm formation by *S. aureus* is a recurring problem in the food industry. There are several genetic factors involved in the adhesion of *S. aureus* to surfaces, being the biofilm a way of protection for this bacterium. Besides that, due to the layers of the biofilm there is an increase in the microbial resistance to antimicrobials and sanitizers when compared to the planktonic cells. Due to the formation of biofilms and increased resistance to antimicrobials and sanitizers there are several fields to be explored in order to decrease the biofilm formation by this pathogen. This review provides an overview about the genetic aspects involved in biofilm formation by *S. aureus*, besides reviewing the characterization phenotypic and genotypic of resistance to the main sanitizers used in the food industry and potential alternatives that can be used for biofilm control. We have analysed and critically discussed around 110 articles covering the latest improvements in the field.

Keywords: antibiofilm, essential oils, nanoparticles, plants extract

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5. Conclusion
References

Abbreviations: AI 2, auto-inducers 2; CE, competitive exclusion; CFU, Colony forming units; EOs, Essential oils; EPS, extracellular polymeric substances; MATE, Multidrug and Toxic Compound Extrusion Family; MBIC, minimal biofilm inhibitory concentration; MIC, minimal inhibitory concentration; MFS, Major Facilitator Superfamily; MRSA, Methicillin resistant *Staphylococcus aureus;* MSCRAMM, Microbial surface components recognizing adhesive matrix molecules; PIA, polysaccharide intercellular adhesin; QACs, quaternary ammonium compounds;; RND, Resistance-Nodulation-Division Family; SMR, Small Multidrug Resistance Family;

1. Introduction

Staphylococus aureus is a Gram positive pathogenic bacterium frequently involved in foodborne intoxications, being considered a public health concern throughout the world (CDC, 2018; EFSA, 2016; Lira et al., 2016).

The biofilm formation by *S. aureus* in food contact surfaces is frequently reported (Lira et al., 2016; Vázquez-Sánchez, Cabo, Ibusquiza, & Rodríguez-Herrera, 2014) highlighting a serious concern for food industries, because the attachment can promote cell survival, causing cross-contamination, reduced product shelf life as well as foodborne diseases (Abdallah et al., 2015; de Souza et al., 2014). Biofilms have been defined as sessile bacterial communities irreversibly attached to a biological or abiotic surface, usually embedded in extracellular polymeric substances (EPS) (Donlan & Costerton, 2002).

The major constituents of staphylococcal biofilms can be composed of polysaccharides, cell-surface-secreted bacterial proteinaceous adhesins, extracellular DNA and teichoic acids (Heilmann, 2011). The genetic ability of *S. aureus* to form biofilms is strongly linked to the production of polysaccharide

intercellular adhesin (PIA) and adhesins called MSCRAMM (Microbial surface components recognizing adhesive matrix molecules) on the surface of the microorganism (Atshan et al., 2012; Tang, Chen, Li, Zeng, & Li, 2013).

The *in vitro* methods of evaluation of biofilm formation vary according to the surface of interest in the different studies, which can be from hospital materials such as catheters, or surfaces found in the food processing environments such as polystyrene, silicone, glass and stainless steel (Azelmad et al., 2016; de Souza et al., 2014; Di Ciccio et al., 2015).

EPS act as physical barriers and cells in biofilm exhibit increased resistance to environmental stresses such as desiccation, temperature, osmotic stress, antimicrobials, and sanitizers (Rode, Langsrud, Holck, & Møretrø, 2007; Vázquez-Sánchez, Habimana, & Holck, 2013). Biofilm-associated antimicrobial resistance is of great importance to the maintenance and spread of foodborne pathogens (Al-Shabib et al., 2017). Due to the proximity of the cells, grow as biofilm facilitates the emergence of antimicrobial resistance by mutation in *S. aureus.* The bacterial growth in biofilms increases horizontal transfer of plasmid-borne antimicrobial resistance determinants by conjugation (Savage, Chopra, & O'Neill, 2013).

Is well established that once formed biofilms are hard to eradicate. To be considered a potent antibiofilm agent, the compound should hydrolyze the biofilm matrix and disintegrate the preformed biofilms (Bazargani & Rohloff, 2016). In this context, to evaluate the efficacy of different antibiofilm agents is of great importance for biofilm formation control. Several studies have been carried out in order to develop strategies to remove biofilms, such as the use of vegetable oils and extracts, nanoparticles, enzymes and co-cultivation with beneficial bacteria (Campana et al.,

2017; Jalvo, Faraldos, Bahamonde, & Rosal, 2017; Kannappan, Gowrishankar, Srinivasan, Pandian, & Ravi, 2017; Son, Park, Beuchat, Kim, & Ryu, 2016).

In this review we provide information to elucidate the biofilm formation by *S. aureus* in the food industry, highlighting the genetic aspects involved, the phenotypic and genotypic characterization of resistance to the main sanitizers frequently used in the food industry and alternatives that can be used to avoid/remove the formed biofilm.

2. Genetic aspects involved in biofilm formation

The formation of the bacterial biofilm can be divided into four stages. Firstly, the initial binding of the bacterial cells occurs, then the aggregation and formation of multiple layers of cells followed by the maturation of the biofilm and finally the detachment of the cells from the biofilm to initiate a new cycle of biofilm formation elsewhere. The initial interactions occurring between the bacteria and the surface are non-specific and conducted by different forces: hydrophobic, electrostatic and Lifshitz-Van Der Waals forces, among others (Gutiérrez et al., 2012; Vázquez-Sánchez et al., 2013). There are several genetic aspects involved in the biofilm formation by *S. aureus* which will be described below.

2.1 Locus *ica*

Cell aggregation and biofilm formation are mediated by the products of a locus composed by *icaADBC* genes, which encode proteins essential for the production of PIA. The synthesis of PIA is encoded by the products of single chromosomal genes, which are organized into an operon structure (Atshan et al., 2012).

The *ica* locus consists of four genes (*icaA, icaD, icaB* and *icaC)*. The genes *icaA* and *icaD* exert a primary role in the exopolysaccharide synthesis. The gene

encodes for a transmembrane enzyme with *N*-acetylglucosaminyl transferase activity, necessary for the synthesis of the poly-*N*-acetylglucosamine polymer. The *icaA* alone exhibites a low N-acetylglucosaminyl transferase activity and represents the catalytic enzyme. Coexpression of *icaA* with *icaD* led to a significant increase in this activity (Gerke, Kraft, Süßmuth, Schweitzer, & Götz, 1998). On the other hand, the product of the *icaC* gene appears to translocate the poly-*N*-acetylglucosamine polymer to the bacterial cell surface, while the *icaB* product operates the deacetylation of the molecule (Vuong et al., 2004). Negative regulation is conferred by the *icaR* gene encoded at the *ica* locus but divergently transcribed from *icaADBC*. IcaR binds to the *icaADBC* promoter, upstream to the *icaA* start codon, and negatively regulates *ica* expression*; icaR* gene deletion results in increased expression of *icaABCD* genes (Cue et al., 2009; Jefferson, Pier, Goldmann, & Pier, 2004).

In a study with 60 different clones, characterized by *spa* typing, the *icaADBC* genes were found to be present in all the 60 clones tested indicating a high prevalence of these genes in *S. aureus* from clinical origin (Atshan et al., 2012). In food sources, Tang et al. (2013) detected the genes *icaAD* and *icaBC* in 87.50% both of isolates, while Kroning et al. (2016) detected *ica*A in 66.6% and *ica*D in 58.4% of isolates.

2.2 Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

In addition to the PIA, some surface proteins of *S. aureus* (binding factors, fibronectin proteins, protein A, collagen adhesin) also help in bacterial adhesion. The microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) constitute a largest family of surface proteins and are part of the aggregation and colonization of the extracellular matrix (Vazquez et al., 2011).

These MSCRAMMs can bind to host extracellular matrix factors including elastin (*ebpS*), laminin (*eno*), collagen (*cna*), fibronectin A and B (*fnbA* and *fnbB*), fibrinogen (*fib*), bone sialoprotein (*bbp*) and bacterial ligands clumping factors A and B (*clfA* and *clfB*). These proteins share a common signal sequence for secretion and for anchoring to the cell wall (Götz, 2002; Seo, Lee, Rayamahji, Kang, & Yoo, 2008).

Khoramrooz et al. (2016) found a significant association between the presence of *icaD*, *icaA*, *fnbA*, *clfA* and *cna* genes and biofilm formation in *S. aureus*. Although all evaluated genes were not present simultaneously in all biofilm-producing isolates, in all biofilm-producing isolates at least one of the related genes was detected. In another study, adhesion and biofilm formation genes were searched utilized PCR, among isolates of *S. aureus* from different sources, where the genes *clfB* (100%); *cna*, *eno*, *fib*, and *ebpS* (93.75%); *fnbA*, (87.50%); *fnbB* (68.75%) and *clfA* (25%); were detected (Tang et al., 2013).

2.3 Accessory gene regulator and quorum sensing in *S. aureus* biofilm formation

The expression of the operon *ica* and the biofilm production depends on several environmental conditions as well as the population density. Bacterial communities produce and secrete signaling molecules, called auto-inducers 2 (AI-2), that trigger a cascade of cellular responses. This mechanism of cell-cell communication, called quorum sensing, controls several processes in the cell, including biofilm formation. Ma et al. (2017) demonstrate that the inactivation of *luxS* gene, which encodes AI-2 synthase, resulted in increased biofilm formation and higher PIA production, demonstrating that the *LuxS*/AI-2 systems negatively regulates PIA-dependent biofilm formation via repression of *rbf* expression in *S.*

aureus. The *rbf* gene is involved in the positive regulation of a protein or proteins that are important for biofilm formation. Moreover, AI-2 activated the transcription of *icaR*, a repressor of the *ica* operon, and subsequently decreased the levels of *icaA* transcription (Yu, Zhao, Xue, & Sun, 2012). Thus, in the case of *S. aureus*, the quorum sensing negatively regulates the formation of biofilm.

In *Staphylococcus* spp. the well-characterized quorum sensing system is called Agr. The Agr system consists of four genes (*agrA, agrC, agrD* and *agrB*) that are co-transcribed. The system is organized into a two-component transmembrane transduction complex (AgrA and AgrC), a pro-signaling peptide (AgrD), and a membrane component (AgrB), responsible for the externalization of the modified signaling peptide. The effector molecule of the Agr system is a regulatory RNA, the RNAIII, whose synthesis is dependent on the activation of the Agr system and driven by the Agr P3 promoter system. Among the virulence factors of *S. aureus* that are controlled by the Agr system is the production of PIA. When secreted, PIA is detected by AgrC, which activates the AgrA regulator. In turn, AgrA regulates the gene transcription positively, including those genes encoding the extracellular proteases involved in biofilm dispersion (Boles & Horswill, 2008).

Therefore, *agr* system control the production of matrix-degrading enzymes. Activation of the *agr* system is generally accepted as being inhibitory towards biofilm maturation. *Staphylococcus aureus* will not form a biofilm under conditions of high *agr* activity, and reactivation of *agr* in a mature biofilm results in disassembly (Boles & Horswill, 2008; Yarwood, Bartels, Volper, & Greenberg, 2004). Tan et al. (2015) demonstrated that strains of *S. aureus* with the locus *agr* inactivated form more robust biofilms compared to wild type strains.

2.4 Other genes involved in biofilm formation

A number of staphylococcal strains exhibit PIA-independent biofilm formation. In several strains, to secrete proteins and extracellular DNA appears to substitute PIA. Differently, surface proteins such as serine-aspartate repeat protein (SdrC) (Barbu, Mackenzie, Foster, & Höök, 2014), surface protein G (SasG) (Geoghegan et al., 2010), surface protein C (SasC) (Schroeder et al., 2009), Protein A (spa) (Merino et al., 2009) and biofilm-associated protein (Bap) (Cucarella et al., 2001) have been documented to contribute to biofilm formation in *S. aureus*.

In another study, adhesion and biofilm formation genes were searched among isolates of *S. aureus* from different sources, where the genes *clfB* and *sasG* (100%); *cna*, *eno*, *fib*, and *ebpS* (93.75%); *fnbA*, (87.50%); *fnbB* (68.75%); *sasC* (31.25%); *clfA* (25%); and *pls* (12.50%) were detected by PCR (Tang et al., 2013).

3. Resistance to sanitizers in biofilm environment

It is known that bacterial cells in biofilms are protected of various environmental stresses, leading to resistance to antimicrobials and sanitizers (Buzón-Durán, Alonso-Calleja, Riesco-Peláez, & Capita, 2017). The entire biofilm structure and its spatial organization are involved in the biofilm resistance to sanitizers, although other factors related to the physiological state of the cells may be responsible for the biofilm sanitizers resistance (Abdallah et al., 2015; Morente et al., 2013).

Furthermore, the inappropriate use of sanitizers (e.g., overdilution, reduced contact time, incorrect temperature, or pH) may provide sub-lethal exposure, contributing for the development of resistance as well as the genetic exchange of resistance genes among bacteria in the biofilm community (Buzón-Durán et al., 2017; Goessweiner-mohr, Arends, Keller, & Grohmann, 2014). Following, the sanitizers

quaternary ammonium compounds, peracetic acid, sodium hypochlorite and biguanides will be discussed regarding its modes of action, resistance mechanisms and performance on attached cells of *S. aureus*.

3.1 Phenotypic resistance to sanitizers

There are many studies evaluating the sanitizers action to inhibit or remove attached cells in the biofilm (Buzón-Durán et al., 2017; Iñiguez-Moreno, Gutiérrez-Lomelí, Guerrero-Medina, & Avila-Novoa, 2018; Johani et al., 2018; Kamaruzzaman et al., 2017) However, *S. aureus* cells in biofilm are more resistant to sanitizers than free-living cells, thus it is important understand how this resistance occurs in each sanitizer, to create new strategies to solve these problem in the food industry.

The quaternary ammonium compounds (QACs) (e.g. benzalkonium chloride, benzethonium chloride, cetalkonium chloride, cetylpyridinium chloride, cetrimonium, cetrimide) have physical mechanisms of action, causing damage in the bacterial membrane due to disorder in its physical and biochemical properties. These cationic antimicrobials also act in intercellular targets, and bind to DNA, contributing to its bactericidal effect (Wessels & Ingmer, 2013). The mechanisms that lead to QACs resistance include efflux pumps activity, altered fatty acid composition and other modifications in the bacterial membrane (Ferreira, Pereira, Pereira, Melo, & Simões, 2011).

On the other hand, the contact with sub-inhibitory concentrations of benzalkonium chloride reduces the biofilm-forming ability of *S. aureus*, suggesting that these compounds have anti-biofilm properties even at low concentrations (Buzón-Durán et al., 2017). Cabeça, Pizzolitto, & Pizzolitto, (2012) verified that the count of viable cells of *S. aureus* in biofilm was significantly reduced after treatments

with sanitizers, of which the quaternary ammonium compounds reduced the counts 5.9 log CFU.cm^{-2} to 2.8 log CFU.cm^{-2} in comparison with the positive control (biofilm cells not treated with disinfectants).

Peracetic acid is an oxidizing agent which contains several free radicals highly reactive, inhibiting or killing microorganisms by several mechanisms due to interaction with a number of cellular constituents (Morente et al., 2013; Wessels & Ingmer, 2013). Although organic matter can affect the efficacy of oxidizing agents in environmental isolates (Martin, Denyer, McDonnell, & Maillard, 2008), there are no reports of resistance to this compound in *S. aureus* and in other bacteria, owing to its wide spectrum that confers lower risks for mutations or adaptations of this complex agent (Morente et al., 2013).

In the study of Iñiguez-Moreno et al. (2018), the mono and dual-species biofilms of *S. aureus* and *Salmonella* were subjected to sanitization treatments and the most effective sanitizer was peracetic acid (3500 ppm) at 10 and 15 min of exposure at 25 and 50 ºC, where the recovery of bacterial cells both in stainless steel or polypropylene surface was not possible. This fact can be explained by its high oxidizing capacity and low molecular size that confers an advantage for its penetration inside the biofilm (Ibusquiza, Herrera, & Cabo, 2011).

The sodium hypochlorite is one of the most extensively used sanitizers in the food industry (Campbell-Platt, 2015). This compound is an oxidizing agent that acts on the cell wall as well as in other parts of the bacterial cell, showing a broad antimicrobial spectrum (Morente et al., 2013). The resistance mechanism to chlorine sanitizers is not well understood. However, it is known that environmental conditions (e.g. temperature, stress factors, organic matter) and utilization in sub-inhibitory

levels decrease the availability of free chlorine leading to resistance profile (Khan, Beattie, & Knapp, 2016; Martínez-Hernández et al., 2013).

The effectiveness of sodium hypochlorite (NaClO) against adhered cells of *S. aureus* was evaluated by Melo et al. (2014), and the results revealed that the number of cells adhered to polystyrene was significantly reduced in the presence of this compound (150 ppm) with an exposition time of 10 min at 37 ºC. Iniguez-Moreno et al. (2017) found that NaClO applied at 200 ppm for 15 min at 37 ºC was the best condition to remove adhered cells of *S. aureus* in polypropylene surface.

The group of biguanides (chlorhexidine, alexidine and polymeric forms) are cationic compounds that cause electrostatic interaction with the acid phospholipids in the bacterial cytoplasmic membrane, modification of membrane permeability and consequently, membrane dissolution (Wessels & Ingmer, 2013). Besides membrane damage and leakage of cellular constituents, this group inhibits membrane enzymes (Condell et al., 2012). The biguanide resistance mechanisms are not totally understood, although it is suggested that alterations in the phospholipids can contribute for the resistance mechanisms (Gnanadhas, Marathe, & Chakravortty, 2013).

In the study of Kamaruzzaman et al. (2017), the polyhexamethylene biguanide at 15 $mg.L^{-1}$ reduced between 28 to 37% of biofilms mass of *S. aureus* isolates from bovine mastitis. On the other hand, no significant reduction in *S. aureus* biofilm was observed for treatment with this compound (0.8 log reduction), while the chlorhexidine, another biguanide compound, was effective against *S. aureus* showing high removal of bacteria (6 log reduction) adhered in polycarbonate coupons (Johani et al., 2018).

3.2 Genes related to resistance against sanitizers

In *S. aureus* an important mechanism of sanitizers resistance relies on efflux systems, based on transmembrane proteins able to transport to the extracellular medium a single class or structurally unrelated compounds (Keith Poole, 2005). There are five major classes of efflux pump systems: (i) the Major Facilitator Superfamily (MFS), (ii) the ATP (adenosine triphosphate)-Binding Cassette Family (ABC), (iii) the Resistance-Nodulation-Division Family (RND), (iv) the Small Multidrug Resistance Family (SMR), and (v) the Multidrug and Toxic Compound Extrusion Family (MATE), that getting energy exploiting either an ion gradient (H+) or ATP cleavage (Morente et al., 2013).

Quaternary ammonium compounds (QACs) resistance gene group comprises inducible efflux pumps that are encoded by plasmids (Worthing, Marcus, Abraham, Trott, & Norris, 2018). These genes are found in several bacteria including *S. aureus* and can be divided into two families: the MFS, which includes *qacA* and *qacB*, and the SMR, which includes *smr, qacG*, *qacH*, and *qacJ* (Wassenaar, Ussery, Nielsen, & Ingmer, 2015). The presence of *qac* genes in *S. aureus* promote higher tolerance to QACs (benzalkonium chloride) and biguanides (chlorhexidine) (Liu et al., 2015).

The MATE harbor the efflux pump *mepA* gene previously described in the chromosome of *S. aureus* (Kaatz, Mcaleese, & Seo, 2005), which confers low-level resistance to QACs, biguanides and other compounds such as pentamidine, ethidium bromide and tigecycline (Fernández-Fuentes, Abriouel, Ortega Morente, Pérez Pulido, & Gálvez, 2014). The *sepA* gene is a chromosomally-encoded gene that has been identified as a staphylococcal efflux pump belonging to RND family that confers low-level resistance to benzalkonium chloride, chlorhexidine and the dye acriflavine (Hassanzadeh et al., 2017; Narui, Noguchi, Wakasugi, & Sasatsu, 2002; K. Poole,

2002). Other efflux pumps chromosomally located belonging to MFS are *norA, norB,* and *qacEΔ1* genes, that mediate resistance to several compounds, including QACs and biguanides (Marchi et al., 2015; Pal, Bengtsson-Palme, Rensing, Kristiansson, & Larsson, 2014).

According to Fagerlund, Langsrud, Heir, Mikkelsen, & Møretrø, (2016), despite the presence of several efflux pumps, apparently, biofilm condition is a much more powerful resistance mechanism. Furthermore, until now, there are no reports about resistance genes for oxidizing agents, such as peracetic acid and sodium hypochlorite sanitizers, and more studies are necessary to unravel these points.

3.3 Bacterial conjugation on biofilm environment

Studies have shown that on biofilm environment, there is an increase in the frequency of horizontal transfer events by the conjugation mechanism, thus promoting the propagation of antimicrobial resistance genes (Ryder, Chopra, & O'Neill, 2012; Savage et al., 2013). Considering that conjugative transfer is the most important means of spreading antimicrobial resistance among bacteria, these events are cause for concern and more attention should be paid to the role of biofilms in development and transfer of resistance (Goessweiner-mohr et al., 2014; Morente et al., 2013).

Águila-Arcos et al. (2017), found *S. epidermidis* isolates with higher biofilm-forming capacity harboring horizontal transfer genes of resistance and relaxase genes. Furthermore, antimicrobial resistance genes codifying resistance to gentamicin, erythromycin, tetracycline and vancomycin were detected in the isolates, indicating potential risk of the development and dissemination of multidrug-resistant bacteria. In another study, was verified that *S. aureus* biofilms increase the frequency

of plasmid transfer events by both conjugation and mobilization events (Savage et al., 2013).

According to Savage et al. (2013), the frequent increase of transfer events probably is due to close cell-to-cell contact occurring in the biofilm and the fact that the biofilm matrix may act stabilizing contacts between neighboring bacteria. Moreover, factors such as oxygen availability, cell density, and juxtaposition were shown to affect plasmid transfer in biofilms, although more studies are necessary to unravel these points (Stalder & Top, 2016).

It should be emphasized that the sanitizers resistance in biofilms is a major public health issue in the food industry, therefore, the effective control strategy is indispensable to avoid this concern.

4. Alternatives to synthetic sanitizers

Previous studies have shown that *S. aureus* strains of different origins and genetic composition can produce robust biofilms resisting the action of common biocides used at their recommended or even higher concentrations (Almatroudi et al., 2016; A. de Oliveira et al., 2016).

The resistance of the biofilm to the sanitizers compounds is worrying, and the development for alternatives that potentiate the action of these compounds or have their effect alone has been frequently reported and will be discussed below (Abdallah et al., 2015; Almatroudi et al., 2016; Vetas, Dimitropoulou, Mitropoulou, Kourkoutas, & Giaouris, 2017).

Considering that the role of biofilm in food spoilage and in the food industry is relevant, inhibition of biofilm formation by natural agents is expected to be safe and could also enhance the efficacy of antimicrobial strategies for controlling microbial food spoilage.

4.1 Biofilm removal by essential oils and plant extracts

The essential oils (EO) emerge as potential alternative as natural sanitizer with the possibility to use for biofilm control in the food industry. The broad-spectrum antimicrobial activity of EO has prompted, in the last years, an intensive research on their potential exploitation as antimicrobial agents, either as raw mixtures or individual constituents, acting in free cells as well as in attached cells, such as those formed by *S. aureus* (Espina, Berdejo, Alfonso, García-Gonzalo, & Pagán, 2017; Kim et al., 2015; Merghni, Marzouki, Hentati, Aouni, & Mastouri, 2016; Vázquez-Sánchez, Cabo, & Rodríguez-Herrera, 2014).

Vázquez-Sánchez, Galvão, Mazine, Gloria, & Oetterer, (2018) described that effective and environmentally-friendly alternatives to traditional sanitizers are necessary to reduce the emergence of antimicrobial-resistant bacterial strains in food environments. The authors verified that treatments based in single and combined applications of EO of *Lippia sidoides*, *Thymus vulgaris* and *Pimenta pseudochariophyllus* showed a higher efficacy than peracetic acid and sodium hypochlorite against *S. aureus* planktonic and attached cells on polystyrene and stainless steel under food-related conditions.

Al-Shabib et al. (2017) tested a flavonoid, called Rutin, as broad-spectrum biofilm control agent against mono and multi-species biofilm formed by *Escherichia coli* and Methicillin resistant *S. aureus* (MRSA). Sub-MICs (minimal inhibitory concentration) (1/16 x MIC to 1/2 x MIC) were used to assess the inhibition of biofilm formed by *E. coli* and *S. aureus* on polystyrene. Mono specie biofilm formation by *E. coli* and *S. aureus* was greatly reduced by Rutin at their respective 1/2 x MIC (600

µg.mL^{-1}). For multi-species biofilm formation, the reduction in biofilm production was concentration dependent.

Cui, Zhou, & Lin, (2016) encapsulating Salvia essential oil showed that 2 mg.mL^{-1} effectively eradicated the *S. aureus* biofilms. A decrease in biofilms metabolism was seen after exposure to EO of Salvia oil and was observed that the inhibition rate of biofilm *S. aureus* was higher with the increase in concentration of EO of Salvia oil.

Espina et al. (2017) utilized treatments with 1000 ppm of carvacrol or citral, (Sigma, Steinheim, Germany), at 45 °C for 60 min which were capable of reducing more than 5 log CFU.cm^{-2} of the sessile cells forming part of mature biofilms of *S. aureus*, *Listeria monocytogenes* or *E. coli*.

Adhered *S. aureus* (± 6.1 log CFU.cm^{-2}) were inhibited after 1 min and 10 min treatments using thymol and carvacrol, major constituents of oregano and thyme EO, at concentrations of MIC and 2.0 x MIC. Reductions of 1.47–1.76 log CFU.cm^{-2} and 1.87–2.04 log CFU.cm^{-2} were observed, by the same author, using 0.5 x MIC of thymol and carvacrol, respectively (Engel, Heckler, Tondo, Daroit, & da Silva Malheiros, 2017). Sub-inhibitory concentrations of *Syzygium aromaticum* and *Cinnamomum zeylanicum* EO, and their major constituents (eugenol and cinnamaldehyde, respectively), were also reported, decreasing biofilm formation of *S. aureus* on polystyrene and stainless steel surfaces (Budri et al., 2015).

Rodrigues et al. (2018) utilized the *Origanum vulgare* L. EO and just carvacrol, (the major constituent of *O. vulgare*) and both were effective to remove young and mature biofilms on stainless steel surfaces. A 10 min exposure to 10 µL.mL^{-1} of *O. vulgare* EO or 5 µL.mL^{-1} of carvacrol caused a decrease of ≥2 log CFU.cm^{-2} in the sessile cells counts of young and mature biofilms formed by *S. aureus.* Rodrigues et

al. (2017) utilized sub-MIC doses of *O. vulgare* EO and carvacrol, and also decreased the sessile cell counts of *S. aureus* on stainless steel surfaces over time (360 h).

Campana et al. (2017) used three different essential oil-based microemulsions, formulated with *Cinnamomum cassia*, *Salvia officinalis,* or both, against *S. aureus* biofilms in different culture media and growth conditions, causing a reduction of > 3 log $CFU.cm^{-2}$ of *S. aureus* 24 h-old biofilms and desiccated biofilms, and up to 68% of biofilm removal after 90 min of exposure.

In addition to studies with EO, many authors have explored the potential of plant extracts to inhibit biofilm formation by *S. aureus.* Kannappan et al. (2017) explored the *in vitro* potential of *Vetiveria zizanioides* root extract (VREX) against the biofilm formation by *S. aureus,* verifying that 300 $mg.mL^{-1}$ of VREX inhibited the biofilm formation, therefore, this value was fixed as minimal biofilm inhibitory concentration (MBIC). Lee, Kim, Park, & Lee, (2017) investigated the antibiofilm activity of *Moringa oleifera* extracts which inhibited MRSA biofilm formation around 85%. In another study, the aqueous and the saline extracts of *M. oleifera* reduced approximately 2 log $CFU.cm^{-2}$ of *S. aureus* isolates on the stainless steel and polyvinyl chloride surfaces (A. M. De Oliveira, Da Silva Férnandes, De Abreu Filho, Gomes, & Bergamasco, 2018).

In a study utilizing gallic acid, the results showed that 0.25 $mg.mL^{-1}$ can inhibit biofilm formation, being that with increasing of gallic acid plant extract concentration, the inhibition of *S. aureus* biofilm formation was more significant (Liu, Pan, Li, Jie, & Zeng, 2017).

Gomes et al. (2018) tested various extracts, verifying that *Eucalyptus globulus* extract was the most effective against cells of *S. aureus*, followed by *Juglans regia*

and *Foeniculum vulgare* extracts. Fontaine et al. (2017) tested an extract of the elm leaf blackberry (*Rubus ulmifolius*) and ellagic acid rhamnoside, a bioactive component of botanical extract, and identified it as an inhibitor of *S. aureus* biofilm formation.

However, there are still strict limitations on the practical application of these compounds for the disinfection of industrial surfaces due to both their strong hydrophobic nature, which hampers their efficient rinsing out from surfaces after a disinfection program, causing corrosion of the surfaces and transmitting flavors to foods besides often presenting intense odor for application (Karampoula et al., 2016; Fong et al., 2014; Gutierrez et al., 2009). Thus, despite the promising results of EO to remove/reduce biofilm, many advances are still required for these compounds to be applied in practice in the food industry, especially with regard to their limitations of use.

4.2 Biofilm removal by competition, deletion or displacement

The use of lactic acid bacteria (LAB) can be considered as an alternative approach for reducing biofilm formation that has received attention and the proposed mechanisms for their antimicrobial activity include the competition for adhesion sites and nutrients and the production of growth-inhibiting compounds (Woo & Ahn, 2013).

Ouali et al. (2014) evaluated a *Lactobacillus pentosus* isolate that was able to strongly hamper the adhesion of *S. aureus* on abiotic surfaces as polystyrene and stainless steel. Pérez Ibarreche, Castellano, & Vignolo, (2014) reported that lactobacilli with biofilm forming capacity were able to control *L. monocytogenes* biofilms.

Moreover, Varma, Nisha, Dinesh, Kumar, & Biswas (2011) used the culture supernatant of *L. fermentum* and tested the adhesion of *S. aureus* in coincubation with *L. fermentum*, verifying that 2.5 and 5 $\mu g.mL^{-1}$ of *L. fermentum* was able to inhibit *S. aureus* growth.

Through the production of antimicrobial substances, Sadowska, Walencka, Wieckowska-Szakiel, & Rózalska (2010) showed the potent anti-staphylococcal activity of soluble factors produced by *L. acidophilus*. The supernatant obtained from *L. acidophilus,* containing bacteriocin-like inhibitory substances, was the most effective causing inhibition of growth and biofilm formation by *S. aureus.*

To inhibit the growth of hazardous microorganisms on food-contact surfaces and in food processing environments, biological methods which do not leave sanitizers residues, as well as do not affect sensorial properties of foods, nor cause corrosion of metals, can be utilized. These methods typically involved also the use of competitive exclusion (CE) microorganisms which have antagonistic activity against foodborne pathogens. The mechanisms of CE microorganisms to inactivate or prevent growth of pathogens are based on competition for attachment sites or nutrients, production of antimicrobial substances, or faster growth (Ukuku, Bari, Kassama, & Mukhopadhyay, S., Olanya, 2015).

In this way, strategies using the CE microorganisms to inactivate biofilm formation by foodborne pathogens in the food processing environments are of major importance. Son et al. (2016) tested the inhibition of *S. aureus* by antimicrobial biofilms formed by CE microorganisms on stainless steel and the populations of *S. aureus* biofilm exposed to biofilms of *Streptomyces spororaveus*, *Bacillus safensis* and *Pseudomonas azotoformans* decreased approximately 1 log CFU/coupon within 48 h.

4.3 Biofilm removal by nanoparticles and enzymes

Nanoparticles can be easily modified for surface charge and binding properties. They exhibit antimicrobial activity by affecting the cell permeability, by generation of reactive oxygen species or by competitive inhibition of key enzymes. Since their mode of action is completely different as compared to conventional antimicrobials, bacteria have lesser chance of developing resistance against nanoparticles (Forier et al., 2014; Gao et al., 2016).

Jalvo et al. (2017) conducted a study to verify the potential for biofilm removal of *S. aureus* with the use of titanium dioxide (TiO_2) nanoparticles. They found that most of the biofilm cells were damaged due to the photocatalytic effect of TiO_2. The efficacy of TiO_2 photocatalyst as an inhibitor of biofilm formation has been widely explored, but its effect as a biofilm component remover has received much less attention (Chorianopoulos, Tsoukleris, Panagou, Falaras, & Nychas, 2011; Jalvo et al., 2017).

Khiralla & El-Deeb (2015) described an alternative antimicrobial and antibiofilm agent, the selenium nanoparticles (SeNPs), which were biosynthesized by treatment of 1 mM SeO_2 with the free-cell supernatant of *Bacillus licheniformis* isolated from food wastes. The SeNPs in concentration of 20 $mg.mL^{-1}$ has sharp effect as an antibiofilm agent against *E. faecalis*, *S. aureus*, *E. coli* O157:H7, *S.* Typhimurium and *S.* Enteritidis, and all pathogens lost their ability to form biofilm.

Zero valent silver nanoparticles capped with capsaicinoids, containing *Capsicum annuum* extract*,* showed promising antibacterial activity on *S. aureus* with MIC 4/8 $\mu g.mL^{-1}$ and potent anti-biofilm potential, with reduction nearly 50% in *S. aureus* biofilm development (Lotha, Sundaramoorthy, Shamprasad, Nagarajan, & Sivasubramanian, 2018).

There is still a gap in the knowledge about the efficacy of enzymes against biofilms formed by specific bacterial strains and the optimal treatment necessary to effectively degrade the exopolysaccharide matrix (Watters et al., 2016). Few studies have investigated the ability of various enzymatic agents, for example, DNase I, dispersin B, α-amylase, lysostaphin and proteinase K to disperse or inhibit *S. aureus* biofilms (Craigen, Dashiff, & Kadouri, 2011; Di Poto, Sbarra, Provenza, Visai, & Speziale, 2009; Pettit, Weber, & Pettit, 2009; Shukla & Rao, 2017). Craigen, Dashiff, & Kadouri (2011) verified the ability of the α-amylase enzyme to remove preformed *S. aureus* biofilms. Preformed biofilms of *S. aureus* were incubated in the presence of 100 $mg.mL^{-1}$ of this enzyme and the biofilm reduction was extremely rapid, reducing the biofilm cells by 79% in 5 min and achieving a reduction of 89% after 30 min of incubation. The use of proteinase K to promote the biofilm dispersion of *S. aureus* isolates from mastitis showed great variation in its dispersion percentage. The four isolates tested showed varying percentages of dispersion, between 60% and 95.8%, after 24 h of treatment with proteinase K (Shukla & Rao, 2017).

Watters, Burton, Kirui, & Millenbaugh (2016) tested α-amylase, bromelain, lysostaphin and papain in biofilm preformed by *S. aureus.* The maximum levels of biofilm degradation achieved with lysostaphin ranged from 28% to 72%, whereas the maximal reductions ranged from 94% to 96%, 83% to 94%, and 85% to 94%, respectively, for α-amylase, bromelain, and papain. Moreover, other study reported nisin incorporated with 2,3-dihydroxybenzoic acid in nanofibers inhibited the biofilm formation by 88% after 24 h incubation in a methicillin-resistant strain of *S. aureus* (Ahire & Dicks, 2014).

5. Conclusion

In conclusion, there are several factors involved in biofilm formation by *S. aureus*, both with regard to environmental or genetic factors. Therefore, due to the danger related to biofilm formation in the food processing environment and the increased antimicrobial resistance among *S. aureus* strains, novel strategies are urgently required to control biofilm formation by this microorganism and dissemination of resistant strains. Knowledge of genetic factors involved in biofilm formation and the alternatives to inhibit the biofilm formation, associated to traditional procedures, offer a means of reducing this bacterium in the food industry environment. This is an area to be explored for the implementation of measures to control the *S. aureus* in food industry.

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4 Capítulo II

4.1 Manuscrito 2 - Genetic diversity and sanitizers resistance in *Staphylococcus aureus* from milk

Manuscrito a ser submetido ao periódico *Letters in Applied Microbiology* (Fator de Impacto 1.471) e Qualis A2 na Área de Ciência de Alimentos

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Significance and Impact of the Study: *Staphylococcus aureus* is an important agent of foodborne diseases. The use of quaternary ammonium compounds (QACs) and biguanides in food industries are extensively observed in Brazil, and the results of this study highlight the importance about the rational use of these compounds to prevent sanitizers resistance.

Abstract

Staphylococcus aureus is a pathogenic bacterium frequently associated with foodborne disease outbreaks. The aims of this study were to verify the genetic diversity of *S. aureus* isolated from milk, as well as to evaluate the susceptibility to sanitizers benzalkonium chloride (BC) and chlorhexidine (CHL) and to detect the presence of the main resistance genes to sanitizers. The genetic diversity was evaluated by *spa* typing and the isolates were tested for sanitizers resistance using the broth microdilution method. PCR was used for the detection of sanitizers resistance genes (*qacA, smr, qacG, qacH, qacJ, mepA, norA,* and *norB*). The *spa* type t127 was the most prevalent, and this *spa* type has a high genetic relation with human isolates, suggesting that humans are an important source to *S. aureus* to the milk. The isolates evaluated showed resistance profile to BC (90.3%) and all isolates (100%) were resistant to CHL. According to the PCR assays, the genes *mepA, norA and norB* were more prevalent, detected in 77.4%, 80.6% and 100% of isolates, respectively. In contrast, the genes *qacA* and *smr* were detected just in 9.6% and 3.2% of isolates, respectively. This study confirmed that *S. aureus* isolates from milk in the Southern Brazil show resistance profile to BC and CHL. The presence of efflux pumps was observed and these results are reported for the first time in Brazil, emphasizing the need to perform more studies in this field.

Keywords: Benzalkonium chloride; Chlorhexidine; Efflux pumps; Resistance genes; *spa* type

Introduction

Staphylococcus aureus is a Gram-positive bacterium related to human and animal diseases and commonly associated with foodborne disease outbreaks caused by staphylococcal enterotoxins (Scallan *et al.*, 2011; Umeda *et al.*, 2017).

Considering that *S. aureus* have become one of the most significant pathogens worldwide, molecular typing has proved to be an important tool to investigate the epidemiology among isolates. Various techniques have been developed to type *S. aureus* isolates, e.g. pulsed-field gel electrophoresis (PFGE) (Alkharsah *et al.*, 2018; Kroning *et al.*, 2018), protein A gene (*spa)* typing and *agr* typing (Bardiau *et al.*, 2016; Wang *et al.*, 2018). It is well established that the combination of genotyping methods could be useful and beneficial to better characterize different isolates.

Staphylococcal infections in humans and animals are commonly treated with antimicrobial agents. However, currently, antimicrobial resistance is considered a public health issue throughout the world (Normanno *et al.*, 2007). The resistance to sanitizers is also a worrying issue in view of its massive and uncontrolled use which has led to concerns about the development and emergence of resistant microorganisms. Moreover, the incorrect use of sanitizers (e.g., overdilution, reduced contact time, incorrect temperature, or pH), insufficient cleaning before disinfection, or the presence of residual concentrations of sanitizers may lead to nonlethal levels (Langsrud *et al.*, 2003; Seier-Petersen *et al.*, 2015).

The quaternary ammonium compounds (QACs) (benzalkonium chloride and dialkyl dimethyl ammonium chloride) have physical mechanism of action, causing damage in the membrane due to disorder in the physical and biochemical properties of the cell membrane (Wessels & Ingmer, 2013). The biguanides (chlorhexidine, alexidine and polymeric forms) are cationic compounds that act in the cell

membrane, causing electrostatic interaction with the acid phospholipids in the cytoplasmic membrane, modification of membrane permeability and consequently, membrane dissolution (Wessels & Ingmer, 2013; Fernández-Fuentes *et al.*, 2014).

Some studies described the phenotypic and genotypic resistance to compounds such as benzalkonium chloride (BC) and chlorhexidine (CHL) in *S. aureus* (Heir, Sundheim & Holck, 1999; Seier-Petersen *et al.*, 2015; Taheri *et al.*, 2016; Damavandi *et al.*, 2017). The QAC-resistance genes, such as *qacA, smr, qacG, qacH, qacJ, mepA, norA,* and *norB* in *S. aureus* are typically related to plasmids carrying antimicrobial-resistance genes and also confer resistance to biguanides (Argudín *et al.*, 2016).

No study about genotypic evaluation of sanitizers resistance of *S. aureus* isolates from food sources in Brazil have previously been performed. Therefore, the aims of this study were to verify the genetic diversity of *S. aureus* isolated from milk, as well as to evaluate the susceptibility to sanitizers benzalkonium chloride (BC) and chlorhexidine (CHL) and to detect the presence of the main resistance genes related with sanitizers resistance profile.

Results and discussion

spa typing

Nine different *spa* types were identified among the 31 isolates. The *spa* type most prevalent was t127 (n=8), while t518 (n=2), t304 (n=2), and t002, t008, t189, t216, t948 and t5462 were represented only by one isolate. Thirteen isolates had no matching *spa* type in the database. Of these, eight presented the same repetition (New repetition 1 - NR1: isolates S2, S6, S8, S13, S14, S15, S16, S21). The other

new repetitions were designated as NR2, NR3, NR4, NR5 and NR6 (S12, 107, 152, 218 and 219, respectively). New spa types are generated by the rearrangement of the repeats in a recombination event or by generation of new repeats due to DNA polymerase error, deletion, or duplication events (Alkharsah *et al.* 2018) and reinforce the genetic diversity of isolates from this study.

Among the eight isolates belonging to *spa* type t127, five were grouped in the same PFGE pattern, belonged to *agr* type III and showed common antimicrobial resistance to sulfonamide (Kroning *et al*., 2018). Isolates showing *spa* type t518 presented genetic relatedness in the PFGE (80% similarity), showed the same *agr* type (*agr* IV), and carried the same resistance genes to sanitizers (*mepA, norA* and *norB).* Alkharsah *et al.* (2018) also described that there was clustering of some *spa* types within some PFGE groups, but they did not present the same phenotypic and genotypic characteristics, as the isolates from this study.

A study in Italy reported that most of the *spa* type t127 in *S. aureus* isolates associated with cattle showed very high genetic relatedness (between 90 and 100% PFGE similarity) with human origin isolates, suggesting that humans are an important source to transmitting *S. aureus* to the milk (Alba *et al.*, 2015). The *spa* type t127 was also prevalent in a study with goat milk (Cortimiglia *et al.*, 2015), and in other studies from dairy products and dairy plants (Carfora *et al.*, 2015; Papadopoulos *et al.*, 2018).

The *spa* typing technique has proved to be an important tool to investigate the epidemiology among isolates of *S. aureus* with high genetic diversity, and although the PFGE has being considered the gold standard, the combination of genotyping techniques could be beneficial to discriminate different isolates. Wang *et al.* (2018)

also highlighted the diverse genetic backgrounds of the *S. aureus* from raw milk by *spa* typing, *agr* typing and PFGE.

Minimal Inhibitory Concentration for BC and CHL

Twenty-eight *S. aureus* isolates evaluated in this study showed resistance profile to BC (90.3%) with MICs ranging from 4 to 8 $\mu g.mL^{-1}$, and all the 31 isolates (100%) were resistant to CHL, with MICs ranging from 4 to 16 $\mu g.L^{-1}$ (Table 1). Ignak, Nakipoglu & Gurler (2017) described MICs to BC ranging from 1 to 16 $\mu g.mL^{-1}$, and the MICs to CHL ranging from 0.75 to 12 $\mu g.mL^{-1}$ in *Staphylococcus* spp., similar results to those found in this study.

It was observed a multidrug-resistance profile in 54% of the isolates and these isolates showed cross-resistance to sanitizers tested. However, the isolates S3 and S4 were not multidrug-resistant and were susceptible to BC, demonstrating a different profile among isolates, suggesting that the isolates resistant to antimicrobials of clinic use also show resistance to sanitizers, in this study.

Cross-resistance between antimicrobials and sanitizers has also been reported in some studies. Wu *et al.* (2016) described cross-resistance among CHL, ciprofloxacin, gentamicin, amikacin, cefepime and meropenem. Moreover, Oliveira, Domingues & Ferreira (2017) reported cross-resistance between antimicrobials (ampicillin, erythromycin, vancomycin) and BC.

The inadequate use of sanitizers in food processing chain may facilitate the selection of strains that exhibit acquired sanitizers resistance and can carry genes encoding cross-resistance to antimicrobials of clinic use, thereby creating a new threat to public health (Gnanadhas, Marathe & Chakravortty, 2013).

Detection of BC and CHL resistance genes

The genes *mepA, norA* and *norB* were the most prevalent in this study, where 77.4% (24), 80.6% (25) and 100% (31) of isolates, respectively, carry these genes. In contrast, the genes *qacA* and *smr* were detected in 9.6% (3) and 3.2% (1) of isolates and the genes *qacG*, *qacH*, and *qacJ* were absent in the *S. aureus* evaluated. Curiously, the isolate S20 was the only one that harbored all genes related to QACs and biguanides sanitizers resistance (*norA, norB, mepA, qacA,* and *smr*) and the resistance gene *tetK,* encoding resistance to tetracyclines, as previously evaluated (Kroning et al., 2018). According to the statistical testing, the presence of *norA, norB, mepA, qacA,* and *smr* genes is associated with BC and CHL resistance ($p < 0.05$).

Quaternary ammonium compounds (QACs) resistance gene group comprises inducible efflux pumps that are encoded by plasmid-borne genes (Worthing *et al.*, 2018). These genes can be divided into two families: the Major Facilitator Superfamily (MFS), which includes *qacA* and *qacB* genes, and the Small Multidrug Resistance Family, which includes *smr, qacG*, *qacH*, and *qacJ* genes (Wassenaar *et al.*, 2015).

Low frequency, 9.6% of *qac* genes were observed in this study. Similar results were observed by Damavandi *et al.* (2017) and Hassanzadeh *et al.* (2017) that found low frequency of *qacA* gene (21.7% and 3.3%, respectively) in *S. aureus* from clinical sources. Heir *et al.* (1999) and Bjorland *et al.* (2005) detected low frequency of these genes in *S. aureus* from food origin.Taheri *et al.* (2016) also not detected *qacG, qacH*, and *qacJ* genes in *S. aureus* from clinical isolates.

The Multidrug and Toxic Compound Extrusion Family harbor the efflux pump *mepA* gene previously described in the chromosome of *S. aureus* (Kaatz, Mcaleese & Seo, 2005), which confers low-level resistance to QACs, biguanides as well other

compounds such as pentamidine, ethidium bromide and tigecycline (Fernández-Fuentes *et al.*, 2014).

Other efflux pumps chromosomally located belonging to MFS are the *norA, norB,* and *qacEΔ1* genes, that mediate resistance to several compounds, including QACs and biguanides (Pal *et al.*, 2014; Marchi *et al.*, 2015).

Liu *et al.* (2015) showed that genes *smr, norA* and *norB* were predominant in clinical *S. aureus* isolates. Hassanzadeh *et al.* (2017) described that the frequency of the genes *mepA, norA*, *norB*, and *smr* were 60%, 41.7%, 41.7%, and 30%, respectively, also in clinical *S. aureus* isolates. However, in isolates from food sources, the *norA, norB* and *mepA* genes are less researched and its frequency is low (Fernández-Fuentes *et al.*, 2014; Ammar *et al.*, 2016). The results of this study (*mepA* in 77.4%, *norA* in 80.6% and *norB* in 100% of isolates) are very interesting because antimicrobial resistance determinants often reside in mobile genetic elements which can be exchanged between different bacteria, propagating these genes and increasing the occurrence of resistant strains.

The *spa* type t127 was the most prevalent, and this *spa* type has a high genetic relation with isolates from human sources. This study confirmed that *S. aureus* isolates from milk in southern Brazil show resistance profile to BC and CHL. The use of QACs and biguanides in food industries is extensively observed in Brazil, and taking into account the results of this study, the rational use of these compounds is necessary to prevent sanitizers resistance in bacteria, as *S. aureus*. Almost 54% of isolates were multidrug-resistant and showed cross-resistance to sanitizers (BC and CHL), raising concerns regarding bacterial resistance. Furthermore, the presence of efflux pumps was observed (*norA, norB, mepA, qacA,* and *smr*), and these results, to our knowledge, were not reported in *S. aureus* isolates in Brazil.

Materials and Methods

Bacterial isolates

Thirty-one *S. aureus* isolates from milk obtained between 2008 and 2012 were evaluated. These isolates were previously characterized according to phenotypic tests (Gram, catalase, coagulase, hemolysis pattern, gelatinase activity), molecular characterization (thermonuclease enzyme for species identification, classical enterotoxins), *agr* typing, PFGE and antimicrobial resistance profiling against 15 antimicrobials, as well as presence of various resistance genes (Haubert *et al.*, 2017; Kroning *et al.*, 2018) (Table 1).

spa typing

The X region of the *spa* gene was amplified by using spa-1113f (5'- TAA AGA CGA TCC TTC GGT GAG C -3') and spa-1514r (5'-CAG CAG TAG TGC CGT TTG CTT -3') according to Ridom SpaServer recommendations. The amplified fragments were purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. Purified samples and oligonucleotides were subjected to sequencing at ACTGene Análises Moleculares (Brazil) and the DNA fragments sequencing was performed by the Sanger method using the AB 3500 Genetic Analyzer (Applied Biosystems™, USA). Thereafter, the identified sequences were assigned to the specific *spa* types according to the guidelines described at Ridom SpaServer database (http://www.spaserver.ridom.de).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for BC and CHL

The MIC for BC (Sigma-Aldrich, Irvine, UK) and CHL (Sigma-Aldrich, Irvine, UK) sanitizers were determined using the broth microdilution method in accordance with CLSI guidelines (CLSI, 2017). Firstly, *S. aureus* isolates were cultivated on Tryptone Soy agar (TSA, Oxoid, Hampshire, UK) at 37 ºC for 24 h. After incubation, the isolates were diluted on McFarland scale number 0.5 (10^8 CFU.mL^{-1}) in 0.85% (w/v) saline solution (Synth, Diadema, Brazil) and 10 µL were spotted on 90 µL Mueller-Hinton broth (MH, Oxoid, Hampshire, UK) in a microdilution plate, using variable concentrations of the compounds (ranging from 0.25 to 128 µg.mL^{-1}) with incubation at 37 ºC for 24 h. The MICs for BC and CHL were defined as the lowest concentration that prevented visible growth of the isolates. Isolates were considered resistant to BC and CHL with a MIC ≥ 3 and MIC ≥ 1 respectively (Taheri *et al.*, 2016).

The minimum bactericidal concentration (MBC) was evaluated from wells with no visible microbial growth. Aliquot of 100 µL were seeded on Petri dishes containing TSA and incubated at 37 °C for 24 h, followed by counts of viable cells. After this step, colonies were counted. The MBC was defined as the lowest concentration at which 99.9% of the initially inoculated cells were killed. The experiments were evaluated in three biological replicates.

Detection of BC and CHL resistance genes

Genomic DNA extraction followed the protocol described by Green & Sambrook (2012) with minor modifications. The PCR assays were performed in order to evaluate the resistance genes described in Table S1 in the supplementary

material. The reaction mixtures contained 12.5 μL of GoTaq® Green Master Mix 2x (Promega, Madison, USA), 1 μL of each oligonucleotide at a concentration of 10 or 20 ρmol, 2 μL of DNA (50 ng) and 8.5 μL of ultrapure water (Promega, Madison, USA) to a total volume of 25 μL. The mixtures were subjected to a MJ Research® PTC 100 thermocycler (Bio-Rad Laboratories, Hercules, USA). As control of the DNA samples, a PCR assay with *nuc* gene was performed previously. The cycling conditions followed the recommendations of the studies referenced (Table S1). After this, the PCR products were subjected to electrophoresis at 80 V for 70 min on a 1.5% (w/v) agarose gel (Invitrogen, Carlsbad, USA) in a 0.5 Tris/Acetate/EDTA buffer (TAE) using 1 kb DNA ladder molecular weight marker (Invitrogen, Carlsbad, USA). The amplified products were visualized in an UV transilluminator (Loccus, Cotia, Brazil).

Statistical analysis

Statistical analysis was performed by Chi-square test to determine the association of sanitizers resistance and presence of efflux pump genes, investigating differences among *S. aureus* isolates. A *p*-value of < 0.05 was considered statistically significant.

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Conflict of Interest

No conflict of interest declared

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Table 1 Characteristics of the 31 *Staphylococcus aureus* isolates from milk in the Southern Brazil

Isolate ID	Resistance phenotype[a*]	Resistance genotype*	MIC BC[b]	MIC CHL[c]	MBC BC[d]	MBC CHL[e]	Sanitizers resistance genes	PFGE pattern*	*agr* type*	*spa* type
S1	STR, TOB, CLI, SUL, TET	*ermB*	4	16	32	64	*mepA, norA, norB*	XI	III	t127
S2	AMP, TOB, TET	*tetK*, *tetL, ereB*	2	8	32	32	*mepA, norA, norB*	I	II	NR1[h]
S3	SUL	*tetB*	2	16	32	128	*mepA, norA, norB*	XI	III	t127
S4	ENR, SUL		2	4	32	32	*mepA, norA, norB*	IXb	IV	t518
S5	SUL		4	8	32	64	*norA, norB*	XI	III	t127
S6	OXA, CFO, CLI, SUL	*tetK, tetL*	4	8	16	32	*mepA, norB*	Xb	II	NR1[h]
S7	SUL	*tetB, ereB*	4	8	64	64	*mepA, norA, norB*	V	III	t127
S8	TOB, SUL, TET	*ereB*	4	16	32	32	*mepA, norA, norB*	Xa	I and II	NR1[h]
S9	PEN, AMP, OXA, TEI, ERY, CLI,		4	8	32	32	*mepA, norA, norB*	NT[f]	III	t948

	SUL									
S10	PEN, AMP, ERY, CLI, SUT, TET	*tetM*, Tn*916-1545*, *ermC, blaZ, dfrG*	4	8	16	32	*mepA, norB*	NT[f]	I	t5462
S11	TEI, SUL	*tetK, tetL*	4	16	16	64	*norA, norB*	XI	III	t127
S12	PEN, AMP, OXA, CFL, STR, TOB, TEI, ERY, CLI, ENR, SUL	*tetL*	8	16	64	128	*mepA, norB*	XII	ND[g]	NR2[h]
S13	TEI, SUL	*tetB, tetK, tetL, dfrA*	4	8	64	64	*mepA, norA, norB*	Xa	I	NR1[h]
S14	SUL	*tetK, tetL, ereB, dfrA*	4	8	32	64	*mepA, norA, norB*	NT[f]	I	NR1[h]
S15	PEN, AMP, OXA, CLI, SUL, TET		4	8	32	32	*mepA, norA, norB*	XVI	I	NR1[h]
S16	SUL	*tetK, tetL*	4	8	32	8	*mepA, norA, norB*	XVI	I and II	NR1[h]

S17	PEN, AMP, SUL, TET	*blaZ*	8	8	32	32	*mepA, norA, norB, qacA*	VIII	I	t216
S18	PEN, AMP, OXA, TEI, ERY, CLI,		4	8	32	256	*mepA, norA, norB*	XIII	II	t002
S19	PEN, AMP, OXA, TEI, ERY, CLI, SUL		8	8	32	256	*mepA, norA, norB, qacA*	III	III	t127
S20	PEN, AMP, OXA, CFL, STR, TEI, ERY, CLI	*tetK*	8	8	32	64	*mepA, norA, norB, qacA, smr*	IV	ND[g]	t127
S21	PEN, AMP, OXA, CFO, CFL, TEI, ERY, CLI	*tetK, tetL, ereB*	8	8	32	32	*mepA, norA, norB*	XVI	ND[g]	NR1[h]
S22	SUL	*tetK, ereB*	4	8	32	32	*mepA, norA, norB*	XV	ND[g]	t189
C1	SUL, TET	*ereB*	4	8	16	64	*mepA, norA, norB*	NT[f]	ND[g]	t008
C2	TEI, SUL, TET	*tetK*, *tetL, ereB*	4	8	32	64	*mepA, norA, norB*	XI	III	t127

C3	SUL, TET	*tetK*, *tetL, ereB*	4	8	32	64	*mepA, norA, norB*	IXa	IV	t518
45	PEN, AMP, OXA, CFO, TOB, CLI	*blaZ*	4	8	16	128	*mepA, norA, norB*	VII	ND[g]	t304
93	PEN, AMP, TOB, CLI	*blaZ*	4	16	32	128	*norA, norB*	XIV	ND[g]	t304
107	PEN, AMP, OXA, CFO, CEF,TOB, TEI, ERY, CLI, TET	*tetL*, *tetM*, Tn*916-1545*	4	8	16	256	*NorB*	II	ND[g]	NR3[h]
152	PEN, AMP, OXA, CFO, CEF, STR, TEI, ERY, CLI	*strA*, *strB*	4	8	16	32	*norB*	VII	ND[g]	NR4[h]
218	PEN, AMP, OXA, CFO, CFL, TOB, TEI, ERY,	*tetM*, Tn*916-1545*, *ermB*	4	8	16	256	*norA, norB*	VIa	ND[g]	NR5[h]

219	CLI, TET PEN, AMP, OXA, STR, TOB, TEI, ERY, CLI, TET	*tetM*, Tn*916-1545*, *ermB*	4	8	16	256	*norB*	VIb	ND[g]	NR6[h]

[a]PEN: penicillin, AMP: ampicillin, OXA: oxacillin, CFO: cefoxitin, CFL: cephalothin, CEF: ceftiofur, STR: streptomycin, TOB: tobramycin, TEI: teicoplanin, ERY: erythromycin, CLI: clindamycin, ENR: enrofloxacin, SUL: sulfonamide, SUT: trimethoprim-sulfamethoxazole, TET: tetracycline.

[b]MIC BC: Minimum inhibitory concentration for benzalkonium chloride (µg.mL^{-1}).

[c]MIC CHL: Minimum inhibitory concentration for chlorhexidine (µg.mL^{-1}).

[d]MBC BC: Minimum bactericidal concentration for benzalkonium chloride.

[e]MBC CHL: Minimum bactericidal concentration for chlorhexidine.

[f]NT: Nontypeable.

[g]ND: Not detected.

[h]NR: New repeat lead to new *spa* type

*Haubert *et al*., 2017; Kroning *et al*., 2018

Supplementary material

Table S1 Oligonucleotides used in this study

Gene target	Sequence (5'–3')	Amplicon size (bp)	Program	Reference
qacA	fw: ACTACTGATATGATGACATCA rv: AGTTATATCAAGTGATTTGGG	1512	P1	Mereghetti, Quentin, Mee, & Audurier (2000)
qacG	fw: CAACAGAAATAATCGGAACT rv: TACATTTAAGAGCACTACA	670	P2	Taheri *et al*. (2016)
qacH	fw: ATAGTCAGTGAAGTAATAG rv: AGTGTGATGATCCGAATGT	550	P2	Taheri *et al*. (2016)
qacJ	fw: CTTATATTTAGTAATAGCG rv: GATCCAAAAACGTTAAGA	667	P2	Taheri *et al*. (2016)
norA	fw: TTCACCAAGCCATCAAAAAG rv: CTTGCCTTTCTCCAGCAATA	620	P3	Hassanzadeh *et al.* (2017)
norB	fw: AGCGCGTTGTCTATCTTTCC rv: GCAGGTGGTCTTGCTGATAA	213	P4	Hassanzadeh *et al.* (2017)
mepA	fw: ATGTTGCTGCTGCTCTGTTC rv: TCAACTGTCAAACGATCACG	718	P5	Hassanzadeh *et al.* (2017)
smr	fw: ATAGCCATAAGTACTGAAGTT rv: ACCGAAAATGTTTAACGAAAC	291	P1	Mereghetti, Quentin, Mee, & Audurier (2000)

fw: forward primer; rv: reverse primer. **P1:** 5 min at 95°C; 1 min at 95°C, 1 min at 53°C, 1 m at 72°C for 30 cycles; 7 min at 72°C; **P2:** 1 min at 95°C; 1 min at 95°C, 45 sec at 48°C, 1 min at 72°C for 30 cycles; 5 min at 72°C; **P3:** 4 min at 94°C; 30 sec at 94°C, 55 sec at 60°C, 55 sec at 72°C for 35 cycles; 5 min at 72°C; **P4:** 4 min at 94°C; 30 sec at 94°C, 55 sec at 62°C, 55 sec at 72°C for 35 cycles; 5 min at 72°C; **P5:** 4 min at 94°C; 30 sec at 94°C, 55 sec at 61°C, 55 sec at 72°C for 35 cycles; 5 min at 72°C**;**

5 Capítulo III

5.1 Manuscrito 3 – Biofilm formation on different surfaces, at different temperatures and culture media and expression of adhesion genes in *Staphylococcus aureus*

Manuscrito a ser submetido ao periódico *Food Research International* (Fator de Impacto 3.520) e Qualis A1 na Área de Ciência de Alimentos

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Abstract

Staphylococcus aureus are frequently associated with foodborne disease outbreaks and the biofilm formation by this bacterium in food industry is frequently reported**.** The aims of this study were to investigate the biofilm forming ability of *S. aureus* isolates from milk, through phenotypic analyses such as exopolysaccharide (EPS) production, biofilm forming on polystyrene and stainless steel surfaces and to evaluate detection of adhesion genes and to quantify expression of *cna* and *ebpS* genes. Of the 31 isolates, 18 isolates (58%) were positive for EPS production. The screening revealed 14 isolates (45%) with ability to produce biofilms on polystyrene and, of these, nine isolates with different biofilm profiles were selected to verify their ability to biofilm forming on stainless steel, and all these isolates produced biofilms on this surface. Regarding the adhesion genes *fnbA, fnbB*, *clfB*, *ebpS*, and *cna,* they were found in 48%, 3%, 52%, 80% and 62% of the isolates, respectively. The reverse transcriptase quantitative PCR (RT-qPCR) revealed variation in the expression levels of *ebpS* and *cna* genes, but only the expression of C2 isolate at 10 °C, was significantly higher in relation to the other growth temperatures, for both genes tested. It is highlighted that almost half of the *S. aureus* isolates showed ability to produce biofilms in polystyrene and, all nine selected *S. aureus* isolates present biofilm forming ability on stainless steel, evidencing the danger of *S. aureus* in the food industry and expression of *cna* and *ebpS* adhesion genes revealed that levels of expression from *ebpS* and *cna* gene are isolate dependent.

Keywords: adhesion, biofilm, RT-qPCR, stainless steel,

1 Introduction

Staphylococcus aureus is an important pathogen involved in foodborne outbreaks, often isolated from food and consequently in the food industries. The dairy food poisoning has increased over the years resulting in many public health problems worldwide (Cui, Zhou, & Lin, 2016; Scallan et al., 2011).

The biofilm formation by this bacterium in food contact surfaces is frequently reported (Lira et al., 2016; Vázquez-Sánchez, Cabo, & Rodríguez-Herrera, 2014), highlighting a serious concern for food industries. Biofilms are structured community of bacterial cells enclosed in a self-produced polymeric matrix and are capable of adhering to abiotic or biotic surfaces (Costerton, Stewart, & Greenberg, 1999; Donlan & Costerton, 2002).

The planktonic *S. aureus* cells in milk can be killed easily. On the other hand, the *S. aureus* biofilms can be until 1000 times more resistant to antimicrobials than the planktonic cells and when the milk is contaminated by *S. aureus*, this bacterium can form biofilms in surface of milk container (Bridier et al., 2015; Singh & Ray, 2014).

Adhesion is the fundamental step in the formation of biofilm communities which is facilitated by the expression of various microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Atshan et al., 2013). *Staphylococcus aureus* can express a variety of MSCRAMMs that interact with host extracellular ligands or surfaces, such as elastin binding protein (*ebpS*), laminin binding protein (*eno*), collagen binding protein (*cna*), fibronectin binding proteins A and B (*fnbA, fnbB*), fibrinogen binding protein (*fib*), clumping factors A and B (*clfA, clfB*), and bone sialoprotein binding protein (*bbp*) (Götz, 2002; Seo, Lee, Rayamahji, Kang, & Yoo, 2008).

Despite these evidences, the mechanism and/or process of biofilm formation in *S. aureus* is poorly understood and the studies on the expression profiles of genes involved in biofilm mechanism are still limited in number (Atshan et al., 2013; Kot, Sytykiewicz, & Sprawka, 2018; Liu, Pan, Li, Jie, & Zeng, 2017).

In this context, the aims of this study were to investigate the ability of *S. aureus* isolates from milk to form biofilm on polystyrene and on stainless steel surfaces, using different temperatures and culture media, and to perform genotypic analyses to detect the presence of adhesion genes and the expression of *cna* and *ebpS* genes.

2 Material and methods

2.1 Bacterial strains

A total of 31 *S. aureus* isolates from milk belonging to the culture collection of the Laboratório de Microbiologia de Alimentos (DCTA/FAEM/UFPel), previously identified according to the antimicrobial resistance profile, genetic relatedness, presence of classical enterotoxin genes and *agr* typing were evaluated (Kroning, Iglesias, Mendonca, Lopes, & Silva, 2018).

2.2 Exopolysaccharide (EPS) production by Congo Red agar analysis

The analysis of EPS production followed Lee, Bae, Han, & Lee, (2016), where the isolates were inoculated onto Congo Red agar (CRA) plates (37 $g.L^{-1}$ Brain Heart Infusion broth, 50 $g.L^{-1}$ sucrose, 10 $g.L^{-1}$ Agar Nº 1 (Oxoid, United Kingdom) plus 0.8 $g.L^{-1}$ Red Congo dye (Sigma, Brazil) and incubated at 37 °C for 24 h. Black colonies with a dry and crystalline aspect represented positive results. *Staphylococcus aureus*

ATCC 25923 and *S. epidermidis* ATCC 12228 were used as positive and negative controls, respectively.

2.3 Biofilm forming ability on polystyrene and stainless steel

Biofilm forming ability on polystyrene was assessed using 96-well microtiter plates, in accordance with the protocol proposed by Stepanović et al., (2007). The *S. aureus* bacterial growth was adjusted at a concentration of 0.5 on the McFarland scale (~1.5 x 10^8 CFU.mL^{-1}), and 20 µL were inoculated in 180 µL of Tryptic Soy broth (TSB, Acumedia, USA) followed by incubation at 37 °C for 24 h. The biofilm formed on the surface of each well was washed three times with 260 µL of phosphate buffered saline (PBS, Laborclin®, Brazil), to remove planktonic cells and fixed with 150 µL methanol (Synth®, Brazil) in each well, which was removed after 20 min of contact. Then, the biofilm was allowed to dry at room temperature before adding 150 µL of crystal violet (Laborclin®, Brazil). The optical density (OD) of each stained well was measured at 570 nm using an ELISA Plate Analyzer reader microplate (Robonick®, readwell PLATE, India). The negative control used was 0.85% saline water (w/v), and *S. aureus* ATCC 25923 was used as positive control.

Biofilm formation was evaluated using AISI 304 stainless steel coupons (0.366 µm roughness, 10 mm x 10 mm x 1 mm). Before use, the stainless steel coupons were sanitized according to the protocol proposed by Fontes Parizzi, De Andrade, De Sá Silva, Ferreira Soares, & Monteiro Da Silva (2004).

Nine isolates of *S. aureus* were selected from 31 isolates previously characterized for their biofilm formation ability in polystyrene microplates, three of which are non-biofilm producers, three are poor biofilm producers and three are

moderate biofilm producers, according to the criteria proposed by Stepanović et al. (2007). As a positive control, the standard strain *S. aureus* ATCC 25923 was used.

The bacterial growth was adjusted at a concentration of 0.5 on the McFarland scale, and 1 mL was inoculated in tubes containing 9 mL of TSB (Acumedia, USA) or UHT milk (Cooperativa Piá, Brazil) plus coupons (final concentration 10^7 CFU.mL^{-1}) followed by incubation at 7, 10 and 37 °C for 24 h. Subsequently, the stainless steel coupons were transferred to 5 mL of 0.1% (w/v) peptone water (PW, Oxoid, UK) and were immersed for 1 min to remove planktonic cells. The coupons were transferred to 10 mL of PW and vortexed for 2 min to remove the sessile cells (Andrade, Bridgeman, & Zottola, 1998; da Silva Fernandes, Fujimoto, Schneid, Kabuki, & Kuaye, 2014). Ten-fold dilutions were made in duplicate, and plated on Tryptic Soy agar (TSA, Acumedia, USA), followed by incubation for 24 h at 37 °C. The test was performed in triplicate. Biofilm formation data on polystyrene and stainless steel coupons were submitted to statistical analysis of variance (ANOVA) using Tukey's test ($p < 0.05$) with STATISTIX 8.0 software.

2.5 Detection of adhesion genes

Genomic DNA was extracted according to the protocol recommended by Matthews et al. (1997). For adhesion genes (*fnbA, fnbB, clfA, clfB, fib, ebpS, cna* and *eno*) detection the PCR reactions were performed using 25 µL of reaction mixture containing 12.5 µL of Master Mix 2x (Quatro G**,** Brazil), 1 µL of each primer (10 pmol), 8.5 mL of ultrapure water (Promega, USA) and 2 µL of template DNA (25 ng.µL^{-1}). The mixtures were placed in an MJ Research® PTC 100 using the following program: an initial step at 95 °C for 5 min and 40 cycles at 95 °C for 20 s, specific annealing temperature for each pair of primers (Table S1 in the supplementary

material) for 20 s and 72 °C for 20 s. The PCR products were subjected to electrophoresis at 80 V for 70 min on a 1.5% (w/v) agarose gel in 0.5x TAE buffer (Tris-acetate-EDTA) using a molecular weight marker of 1 Kb (Invitrogen, USA). The amplified products were visualized by UV trans-illumination (Loccus®, Biotecnologia, Brazil).

2.6 Total RNA extraction and cDNA synthesis

Three *S. aureus* (one non-biofilm producer (C2 isolate), one weak biofilm producer (S10 isolate), one moderate biofilm producer (S6 isolate) on polystyrene) were selected according to the PFGE pattern (Kroning, Iglesias, Mendonca, Lopes, & Silva, 2018), and the standard strain *S. aureus* ATCC 25923 as positive control and three temperatures (7, 10 and 37 ºC) were selected for the analyses.

The isolates were inoculated in TSA and incubated at 37 °C for 24 h. The bacterial growth was adjusted at a concentration of 0.5 on the McFarland scale, and 1 mL was inoculated in tubes containing 9 mL of TSB plus coupons (final concentration 10^7 CFU.mL^{-1}) followed by incubation at 7, 10 and 37 °C for 24 h. After incubation, the coupons passed through the washes cited above for removal of planktonic and sessile cells. Total RNA was extracted and purified with RiboPure-Bacteria kit (Invitrogen, USA) according to the protocol provided by the manufacturer. Following, the enzymatic DNA degradation was performed using DNase (Promega, USA). The concentration and purity of the RNA was measured using NanoVue™ Plus (GE Healthcare Life Sciences, USA). cDNA synthesis was performed employing total RNA (0.5 µg) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instruction. The resultant cDNA was stored at −20 °C.

2.7 Reverse transcriptase quantitative PCR (RT-qPCR)

The expression levels of mRNA of *cna* and *ebpS* adhesion genes were quantified by RT-qPCR by using specific oligonucleotide (Table S1). The reverse transcriptase quantitative PCR (RT-qPCR) experiments were conducted in three independents repetitions. The temperature of 37 °C was used as a reference control to standardize to experiment. The RT-qPCR reactions were carried out in a 25 µL reaction volume: 50 ng cDNA (1µL), 12.5 µL of Syber Green PCR Master Mix (Ludwig Biotec, Brazil), and 100 - 400 nM of each oligonucleotide (1 µL) and 9.5 µL of ultrapure water. The assay was performed in 96-well plates, and reactions were carried out in duplicate for each cDNA template. A RT-qPCR assay was performed using the LightCycler 96 PCR System (Roche Diagnostics, Switzerland), employing the cycling conditions cited above from PCR experiments. A melting curve was conducted following amplification in order to ensure the specificity of the amplified products. It was consisted of heating at temperatures between 55 ºC and 95 ºC at a ramp rate of 0.2 ºC s^{-1} with continuous fluorescence monitoring. To determine the RT-qPCR efficiencies and R2 values for each reaction, a dilution series was made from the cDNA template for each target gene. The Ct data were analyzed by $2^{-\Delta\Delta Ct}$ method with efficiencies (E) correction using software REST 2009 (Pfaffl, Horgan & Dempfle, 2002). The relative change in the gene transcription ratio (relative expression) for each target gene was calculated by normalizing gene expression to the *rpoB* reference gene. One Way ANOVA and Tukey post-test were used to determine significant differences ($p < 0.05$) between the mRNA levels for *cna* and *ebpS* genes. Software Prism 5 (GraphPad) was used for all statistical analysis.

3. Results

3.1 Exopolysaccharide (EPS) production

Eighteen isolates (58%) were positive for EPS production, presenting black colonies with a dry and crystalline aspect in CRA plates.

3.2 Biofilm forming ability on polystyrene

The screening of 31 isolates revealed that 14 isolates (45%) showed ability to produce biofilms in this surface (OD > 0.168), with a significant difference ($p < 0.05$) among the isolates (Fig. 1). Of these, 10 isolates (32%) were weak biofilm producer, four isolates (13%) were moderate biofilm producers and 17 isolates (55%) did not form biofilm.

3.3 Biofilm forming ability on stainless steel

All nine selected *S. aureus* isolates produced biofilms on stainless steel surface, in both culture media (TSB and UHT milk) and in all tested temperatures (7, 10 and 37 °C). It was observed no significant differences ($p > 0.05$) for biofilm formation ability between culture media were observed (Table 1).

At the temperature of 7 °C, the highest biofilm forming ability in TSB was shown by S3 and S21 isolates (5.78 and 5.72 log CFU.cm^{-2}) respectively. There were no significant differences ($p > 0.05$) between them and the other isolates, excepted to S1 isolate (4.67 log CFU.cm^{-2}) that presented significant differences from the others ($p < 0.05$). For biofilm forming ability in milk there were no significant differences ($p > 0.05$) between the isolates.

In the temperature of 10 °C, the highest biofilm forming ability in TSB was shown by the S7 and S6 isolate (6.88 and 6.30 log CFU.cm^{-2}, respectively), there

were no significant differences ($p > 0.05$) between them. In milk, the highest biofilm forming ability was shown by the 218 isolate (6.13 log CFU.cm^{-2}) with no significant differences ($p > 0.05$) to the other isolates, except to the S1 isolate (5.06 log CFU.cm^{-2}).

At 37 °C, the highest biofilm forming ability in TSB was observed by S3 isolate (7.91 log CFU.cm^{-2}) with no significant differences ($p > 0.05$) to the other isolates, excepted to the C2 isolate (6.81 log CFU.cm^{-2}) and 45 isolate (6.74 log CFU.cm^{-2}) that presented significant differences to the others ($p < 0.05$). In milk, the highest biofilm forming ability were found by the S6 and 218 isolates (7.88 log CFU.cm^{-2} for both), with no significant differences ($p > 0.05$) to the other isolates, except to the C2 isolate.

3.4 Detection of adhesion and biofilm formation genes

Regarding the adhesion genes, *ebpS* and *cna* were the most prevalent, being found in 80% (25/31) and 62% (19/31) of isolates, respectively. The *fnbA, fnbB* and *clfB* genes were found in 48% (15/31), 3% (1/31) and 52% (16/31), respectively. The *clfA, fib,* and *eno* genes were not detected in the isolates.

3.5 RT-qPCR

The relative expression of genes *cna* and *ebpS*, was evaluated during the biofilm formation in three *S. aureus* (S6, S10 and C2 isolates) selected according to the PFGE pattern (Kroning, Iglesias, Mendonca, Lopes, & Silva, 2018), and to be presented all adhesion genes tested. *Staphylococcus aureus* ATCC 25923 was used as positive control. These genes were selected because they were the most prevalent in the isolates evaluated in this study. The Fig. 2 shows the results from *cna* (A) and *ebpS* (B) relative gene expression at different temperatures.

The expression levels of *cna* gene in *S. aureus* ATCC 25923 (positive control) and in S10 isolate at different temperatures were not significant different between them. However, the C2 isolate growing at 10 °C showed significantly higher *cna* gene expression in relation to the other temperatures tested (p <0.05) and in relation to the control strain at the same temperature (p < 0.01). On the other hand, the S6 isolate did not show transcription of the *cna* gene at any temperature tested.

The expression levels of *ebpS* gene in *S. aureus* ATCC 25923 (positive control) as well as in the S10 and S6 isolates at different temperatures were not significant different between them. Again, when C2 isolate was growth up at 10 °C, the *ebpS* gene expression was significantly higher in relation to control strain growing at the same temperature (p <0.01). However, when C2 isolate was growth up at 7 °C, it did not express the *ebpS* gene.

4 Discussion

Staphylococcus aureus can present ability to adhere and form biofilms on surfaces in the environment of dairy farms and dairy industries, which facilitates their dissemination and persistence in these environments (Melchior et al., 2009).

In this study 31 isolates of *S. aureus* from milk were evaluated regarding their ability to produce EPS. From these 18 isolates (58%) were positive for EPS production and 13 (42%) were negative. Similar results were observed by Khoramrooz et al. (2016) which found that 68.8% of 80 *S. aureus* isolates were biofilm producer by CRA plate method, and by Melo et al. (2013) which found that 85% of *S. aureus* isolates were positive to EPS production.

For biofilm forming ability on polystyrene, almost half (45%) of the *S. aureus* isolates showed ability to produce biofilms (OD > 0.168), with a significant difference

(p <0.05) among the isolates (Fig. 1). Similar result was found by Lee et al. (2014) that also observed 45.2% of the *S. aureus* isolates from dairy farms showing biofilm forming ability on this surface. In this study two *S. aureus* isolates (S9 and 107) presented more ability to produce biofilms in the polystyrene microplate assay, with 0.4 and 0.35 OD values, respectively.

All nine selected *S. aureus* isolates produced biofilms on stainless steel surface, in both culture media (TSB and UHT milk) and in all tested temperatures (7, 10 and 37 °C). The UHT milk was used as a culture medium, in order to simulate the conditions found in the dairy industry, since stainless steel is the most used material in equipment and utensils, and the isolates of *S. aureus* are from milk.

It is important to note that there was a biofilm formation by the *S. aureus* isolates at 7 and 10°C. According to Brazilian legislation IN n° 76 (BRAZIL, 2018), 4 °C is the maximum temperature that milk can leave the farm and 7 °C is the maximum that the milk can be received in the dairy industry, therefore, there is risk of biofilm formation at these temperatures. Besides that, although milk temperatures in dairy industries are generally low (between 3 and 6 °C), higher temperatures (30 °C or more) may occur in some specific production lines, such as those producing cheese and yoghurts. Especially risky may be surfaces of plate heat exchanger pasteurizers (cooling sections) where bacterial biofilms can form and thus contaminate the product during the stream with more than 10^6 CFU.mL^{-1}. From this concentration *S. aureus* can produce thermoresistant enterotoxins, produced between 10 and 46°C, that preserve their biological activity after the pasteurization and ultra-pasteurization process, highlighting the relevance of the results obtained in this study (Flint, Bremer, & Brooks, 1997; Knight, Nicol, & McMeekin, 2004). Thus,

the choice of temperatures of 7 and 10 °C, were made because of Brazilian legislation and the minimum temperature that *S. aureus* can produce enterotoxins.

The counts of cells adhered on stainless steel in all temperatures tested varied between 4.65 – 7.88 log CFU.cm^{-2}. Ouali et al. (2014) showed that *S. aureus* isolates had a capacity to form biofilms, with cell counts to 10^8 log CFU.cm^{-2} on stainless steel AISI 304, within 24 h in 37 °C, and reported that the temperature appeared to play a positive role in the adhesion process.

Khoramrooz et al. (2016) related the presence of the genes *fnbA* (72.5%), *clfA* (50%), and *cna* (22%) in *S. aureus* isolated from bovine with subclinical mastitis. In this study, the *clfA, fib,* and *eno* genes were not detected. Tang et al. (2012) also not detected the *clfA* gene, while Pereyra et al. (2016) detected the gene *cna* in only 20% of strains (n = 4), already for the *fib* gene, He et al. (2014) detected this gene in 43.6% of isolates and Nemati, Hermans, Devriese, Maes, & Haesebrouck (2009) not detected *fib* gene, similar results to this study.

The Fig. 2 shows the results of *cna* (A) and *ebpS* (B) relative gene expression, at different temperatures. The option by TSB medium for RNA extraction, was due to higher levels of biofilm formation in stainless steel using this medium. Besides that, the *cna* and *ebpS* genes were the most prevalent found in our study. Thus, the expression of these two genes at different temperatures was evaluated for seeking a relationship between biofilm formation and expression of adhesion genes.

The *cna* gene is important to biofilm production and collagen binding capacity, the former being involved in the initial adhesion to surfaces, and the latter relevant to the diffusion of the infection (Elasri et al., 2002; Madani, Garakani, & Mofrad, 2017; Montanaro, Arciola, Baldassarri, & Borsetti, 1999). Peacock et al. (2002) highlighted that the presence of the *cna* gene is significantly more common in isolates with

potential invasiveness and this gene contribute to virulence in *S. aureus*. In this study, the *cna* gene were detected in 62% (19/31) of isolates by PCR.

About the expression, the C2 isolate growing at 10 °C showed significantly higher *cna* gene expression in relation to the other growth temperatures (p <0.05) and in relation to control strain at the same temperature (p <0.01). This isolate was selected on screening by their biofilm formation ability in polystyrene microplates, being no biofilm producer and negative from EPS production, but on stainless steel surface, this isolate produced biofilm (5.41 log $CFU.cm^{-2}$). Atshan et al. (2012) suggested that the expression of MSCRAMM and biofilm genes is isolate dependent and does not depend on your biofilm formation profile. The same authors related similar results to observed in this study, where the expression levels of some genes, such as *ebpS, cna* and other MSCRAMM, in weakly adherent isolates were found to be higher than in the strongly adherent isolates.

Some authors attribute that the variation between phenotypic and genotypic characterization in a isolate may be due to the heterogeneity in the genetic origins (Arciola, Baldassarri, & Montanaro, 2001; Atshan et al., 2012)

The elastin binding protein of *S. aureus* (EbpS) is a cell-surface-associated 25 kDa protein encoded by the *ebpS* gene, that promote adhesion and colonization by *S. aureus* on an abiotic surface or human tissue. (Downer, Roche, Park, Mecham, & Foster, 2002). However, some studies described that the *ebps* gene was essential for development of biofilm by *S. aureus* and by its survival in depleted nutritional niches (Atshan et al., 2013; Puah, Tan, Chew, & Chua, 2018).

For the *ebpS* gene expression, when C2 isolate growed up at 10 °C, its expression was significantly higher in relation to the control strain growing at the

same temperature (p <0.01). Nevertheless, growing at 7 °C, this isolate did not express the *ebpS* gene.

Kot et al. (2018) related that the expression levels of *ebpS* gene were higher under biofilm conditions during the first 6 h, both in the case of weakly and strongly adhering strains. On the other hand, Atshan et al. (2013) showed that the expression levels of *ebpS* gene in prolonged incubation for up to 48 h showed an up-regulation. Thus, in this study, the expression of *ebpS* gene may have occurred before or after 24 h, which was the time when it was quantified.

4 Conclusions

Almost half of *S. aureus* isolates from milk showed ability to produce biofilms in polystyrene. Among the *S. aureus* isolates selected based on its biofilm forming ability in polystyrene (three non-biofilm producers, three poor biofilm producers and three moderate biofilm producers) all produced biofilm on stainless steel, independent of its profile on polystyrene. The isolates carry adhesion genes, however, the expression levels of *cna* and *ebpS* genes in biofilm condition varied at different temperatures. Besides that, the expression levels of *cna* and *ebpS* genes are isolate-dependent and does not depend on your biofilm formation profile.

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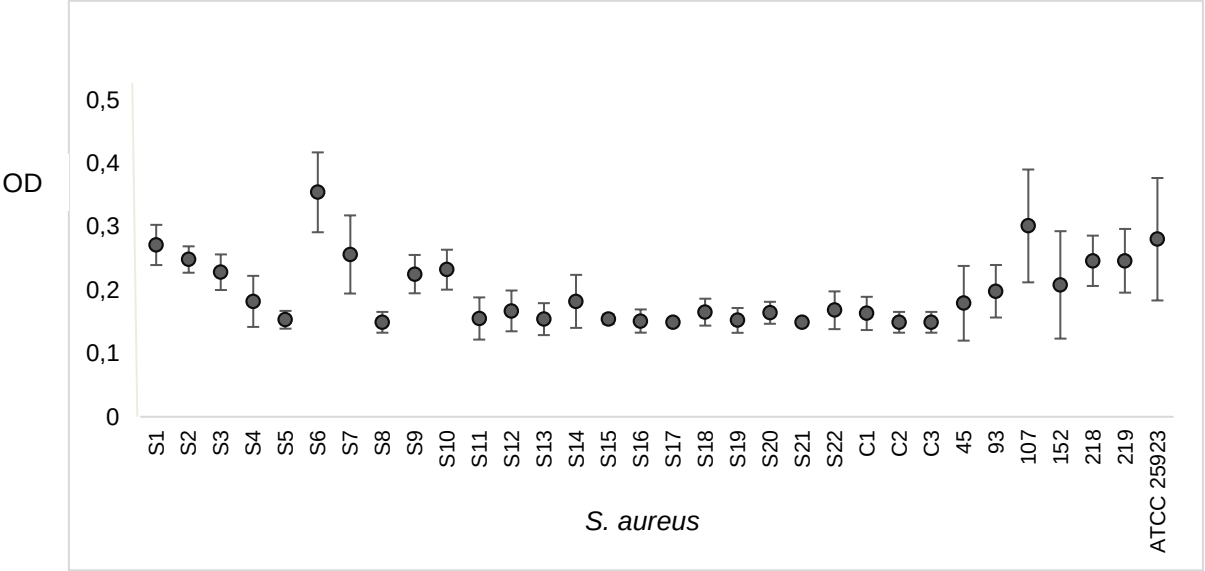
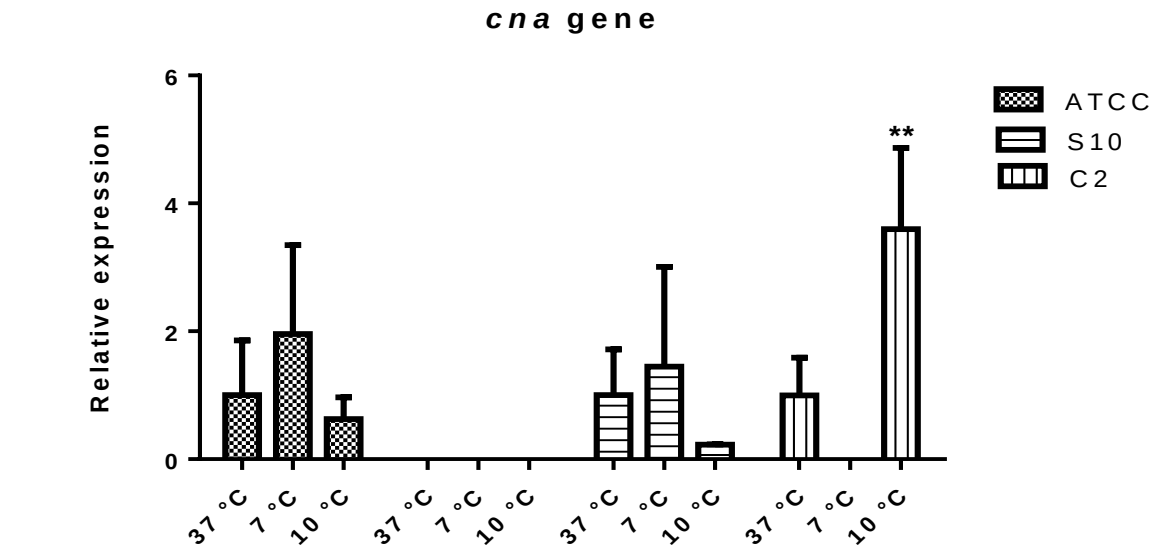
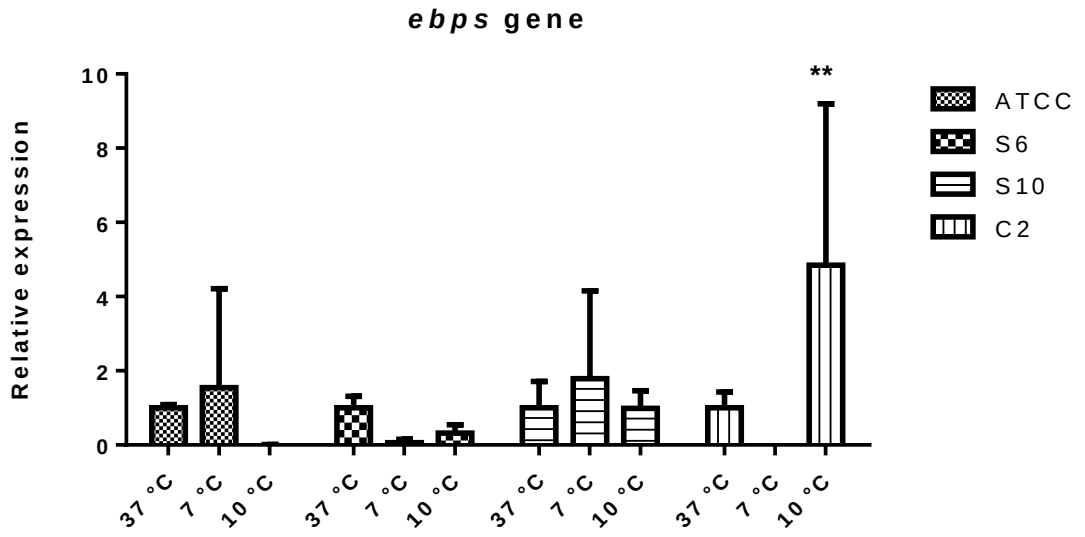


Figure 1. Biofilm formation on polystyrene microtiter plates results for all of the tested *Staphylococcus aureus* isolates and positive controls (*Staphylococcus aureus* ATCC 25923). The mean of three independent measurements is presented. Error bars indicate the standard deviation.



(A)



(B)

Figure 2. Relative biofilm gene expression of *cna* (A) and *ebps* (B) genes. The isolates S6, S10, C2 and *Staphylococcus aureus* ATCC 25923 (control) were submitted to growth at different temperatures (7, 10 and 37 °C). Relative gene expression was determined by RT- qPCR and calculated using the $2^{-\Delta\Delta Ct}$ method, employing *rpoB* mRNA expression as reference gene. Data were analyzed by One Way ANOVA and Tukey posttest. Asterisks (**) represent statistical difference to the control group ($p < 0.01$).

Table 1. Mean of the counts of *S. aureus* (log CFU.cm^{-2} ± standard deviation) adhered to stainless steel at 7, 10 and 37 °C in TSB and milk

Isolate	log CFU.cm^{-2}± SD[1]					
	7 °C		10 °C		37 °C	
	TSB	Milk	TSB	Milk	TSB	Milk
S21	5.72±0.05^{Ab}	4.65±0.03^{Ac}	5.66±0.35^{BCb}	5.42±0.31^{ABbc}	7.04±0.45^{ABCDa}	7.33±0.01^{ABa}
C2	5.27±0.02^{ABb}	5.36±0.08^{Ab}	5.41±0.01^{BCb}	5.38±0.37^{ABb}	6.81±0.03^{BCDEa}	7.41±0.01^{ABa}
45	4.70±0.04^{ABb}	5.14±0.28^{Ab}	4.99±0.02^{Cb}	5.44±0.01^{ABb}	6.74±0.18^{BCDEFa}	6.51±0.01^{BCDa}
S3	5.78±0.02^{Ab}	5.17±0.31^{Ab}	5.46±0.40^{BCb}	5.33±0.03^{ABb}	7.91±0.05^{Aa}	7.36±0.12^{ABa}
S10	5.33±0.01^{ABb}	5.12±0.22^{Ab}	4.80±0.39^{Cb}	5.63±0.05^{ABb}	6.92±0.20^{ABCDa}	7.27±0.38^{ABCa}
218	5.57±0.01^{ABbc}	4.77±0.22^{Ac}	4.73±0.06^{Cc}	6.13±0.02^{Ab}	7.55±0.12^{ABa}	7.88±0.02^{Aa}
S1	4.67±0.70^{Bb}	5.36±0.01^{Ab}	5.50±0.05^{BCb}	5.06±0.02^{Bb}	7.55±0.07^{ABa}	7.42±0.01^{ABa}
S6	5.66±0.01^{Abc}	5.15±0.12^{Ac}	6.30±0.09^{ABb}	5.48±0.10^{ABbc}	7.42±0.29^{ABCa}	7.88±0.02^{Aa}
S7	5.47±0.03^{ABb}	5.24±0.04^{Ab}	6.88±0.84^{Aa}	5.81±0.01^{ABb}	6.93±0.39^{ABCDa}	7.12±0.24^{ABCDa}
ATCC 25923	5.87±0.08^{Abc}	5.26±0.08^{Ac}	5.88±0.01^{Bbc}	5.37±0.02^{ABc}	6.74±0.18^{ABCDEFab}	7.25±0.05^{ABCa}

[1] Logaritmic of colony forming unit per cm^2 ± standard deviation

^{A,B,C} Means in the same line followed by the same capital letter are not significantly different by the Tukey's test ($p \geq 0.05$).

^{a,b,c} Means in the same column followed by the same lowercase letter are not significantly different by the Tukey's test ($p \geq 0.05$).

Supplementary material

Table S1 - Oligonucleotides used in this study for detection of adhesion and biofilm formation genes

Genes	Nucleotide sequence of primers (5'-3')	Annealing temperature	Amplicon size (bp)	Reference
icaA F	CTTGCTGGCGCAGTCAATAC	60	178	Pereyra et al., 2016
icaA R	CCAACATCCAACACATGGCA			
icaD F	CGCTATATCGTGTGTCTTTTGGA	60	164	Pereyra et al., 2016
icaD R	CGCTATATCGTGTGTCTTTTGGA			
icaB F	ATACCGGCGACTGGGTTTAT	60	140	Atshan et al., 2013
icaB R	TTGCAAATCGTGGGTATGTGT			
icaC F	CTTGGGTATTTGCACGCATT	60	209	Atshan et al., 2013
icaC R	GCAATATCATGCCGACACCT			
fnbA F	AAATTGGGAGCAGCATCAGT	60	138	This study
fnbA R	CGCTTACTTTATTATCAGCAGCT			
fnbB F	AAGTACAGATGGTCAAGTTATGGCGA	60	261	This study
fnbB R	CGACCATTAGCTGTTACTCCCCAAT			
clfA F	ACCCAGGTTCAGATTCTGGCAGCG	60	165	Atshan et al., 2013
clfA R	TCGCTGAGTCGGAATCGCTTGCT			
clfB F	TGGTGGAAGTGCTGATGGTGATTC	60	204	This study
clfB R	CCTGAGTCGCTGTCTGAGCCTGAG			
fib F	CGTCAACAGCAGATGCGAGCG	58	239	Atshan et al., 2013
fib R	TGCATCAGTTTTCGCTGCTGGTTT			
***ebpS* F	TCAAGCGAACAATCAAGCAC	55	149	This study
***ebpS* R	AACCGTAGTATTGAATTGCGATA			
***cna* F	TAAAGCGTTGCCTAGTGGAGACTATAT	62	189	This study
***cna* R	CCTTCCCAAACCTTTTGAGCA			
eno F	TGCCGTAGGTGACGAAGGTGGTT	58	195	Atshan et al., 2013
eno R	GCACCGTGTTCGCCTTCGAACT			
bap F	ATACTGATGGCGATGGTA	60	106	Xue et al., 2014
bap R	ACTGTGTCTTCTGTTGTAAT			
**rpoB* F	GCGAACATGCAACGTCAAG	60	121	This study
**rpoB* R	GACCTCTGTGCTTAGCTGTAATAGC			

*The *rpoB* gene was used as an endogenous normalizer for the expression quantification of adhesion genes in *S. aureus*

***cna* and ***ebpS* were used in qPCR.

6 Considerações finais

Através das análises realizadas, verificou-se que quase metade dos isolados de *S. aureus* foram capazes de produzir biofilme em poliestireno. Nove isolados foram selecionados pelo seu perfil de formação de biofilme em poliestireno, sendo três moderados formadores, três fracos formadores e três não formadores de biofilme nessa superfície. Todos os nove isolados apresentaram capacidade de formação de biofilme em aço inoxidável a 7, 10 e 37 °C em TSB e leite integral UHT, independente do perfil de formação de biofilme em poliestireno, o que representa um perigo para a indústria de alimentos, devido ao perigo associado à contaminação de alimentos pelos biofilmes formados.

Os isolados avaliados deste estudo também apresentam os genes de adesão *ebpS, cna fnbA, fnbB* e *clfB,* sendo os genes *ebpS* e *cna* os mais prevalentes. Pela análise de expressão gênica dos genes *ebpS* e *cna,* pode-se observar que os níveis de expressão variaram nas diferentes temperaturas, no entanto, foram isolado-dependentes, e não apresentaram relação com o perfil de formação de biofilme.

Os isolados apresentam perfil de resistência ao cloreto de benzalcônio e à clorexidina, bem como portam os genes de resistência *norA, norB, mepA, qacA* e *smr*. Além disso, observou-se resistência cruzada com antimicrobianos de uso clínico, o que demonstra a necessidade do uso racional e correto desses compostos na indústria de alimentos para evitar a disseminação da resistência a esses compostos.

Com relação a diversidade genética dos isolados, observou-se que o *spa* type t127 foi o mais prevalente entre os isolados, sendo que a literatura aponta que esse *spa* type apresenta relação genética com isolados de origem humana, podendo ser a fonte de transmissão de *S. aureus* para o leite.

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