UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ENGENHARIA DE ALIMENTOS

ANDERSON CLAYTON DA SILVA ABREU

METAGENOMICS OF THE 16S GENE AND RESISTANCE ASSESSMENT OF *Staphylococcus* SPP. ISOLATED FROM ORGANIC AND CONVENTIONAL PRODUCTION OF MINAS FRESH CHEESE IN THE STATE OF SÃO PAULO - BRAZIL

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CAMPINAS

2021

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Thesis presented to the Faculty of Food Engineering of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor of Food Science

Tese apresentada à Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas, como parte dos requisitos exigidos para a obtenção do título de Doutor em Ciência de Alimentos

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ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO ANDERSON CLAYTON DA SILVA ABREU, E ORIENTADO PELA PROFA. DRA. NATHÁLIA CRISTINA CIRONE SILVA.

CAMPINAS

2021

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Engenharia de Alimentos
Claudia Aparecida Romano - CRB 8/5816

Abreu, Anderson Clayton da Silva, 1991-
Ab86m Metagenômica do gene 16S e estudo da resistência de *Staphylococcus* spp. isolados de laticínios de produção orgânica e convencional de queijo Minas Frescal do estado de São Paulo - Brasil / Anderson Clayton da Silva Abreu. – Campinas, SP : [s.n.], 2021.

Orientador: Nathalia Cristina Cirone Silva.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos.

1. Perfil de comunidade microbiana. 2. Sequenciamento de nova geração. 3. Antibióticos. 4. Biofilmes. 5. Desinfecção e desinfetantes. I. Silva, Nathalia Cristina Cirone. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Metagenomic of the 16S gene and resistance assessment of *Staphylococcus* spp. isolated from organic and conventional production of Minas Fresch cheese in the state of São Paulo - Brazil
Palavras-chave em inglês:
Microbial community profiling
Next-generation sequence
Antibiotics
Biofilms
Disinfection and disinfectants
Área de concentração: Ciência de Alimentos
Titulação: Doutor em Ciência de Alimentos
Banca examinadora:
Nathalia Cristina Cirone Silva
Anderson de Souza Sant'Ana
Dirce Yorika Kabuki
Helio Langoni
Tiago Tomazi
Data de defesa: 22-06-2021
Programa de Pós-Graduação: Ciência de Alimentos

Identificação e informações acadêmicas do(a) aluno(a)
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- Currículo Lattes do autor: http://lattes.cnpq.br/7201986719225438

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AGRADECIMENTO

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001. Programa de Excelência Acadêmica (PROEX): nº 23038.000795/2018-61. Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP): processo n° 2016/02118-7 e 2018/00986-7.

Esta pesquisa utilizou recursos do Laboratório Nacional de Biorrenováveis (LNBR), uma instalação nacional aberta do Ministério da Ciência, Tecnologia, Inovações e Comunicações (MCTIC) operada pelo Centro Nacional de Pesquisa em Energia e Materiais (CNPEM). Agradecemos a equipe LNBR pela assistência durante os experimentos.

Gostaria de agradecer a minha família por todo suporte, apoio na realização desse sonho, minha mãe Cleuza, minha irmã Edrielli, e meus queridos sobrinhos Hugo e Carmen. Em especial, agradecer aos meus falecidos avós José Manuel e Maria do Carmo que me deram todo um suporte inicial que me ajudou a ser quem sou hoje. E com um carinho muito especial agradecer a minha tia Maria José, que sem ela dificilmente cogitaria a oportunidade de ter vindo a Campinas para cursar UNICAMP.

Gostaria de agradecer a minha orientadora Nathália por ter me proporcionado a realização de um sonho que era trabalhar com pesquisa na área de biologia molecular e todo suporte durante a condução do estudo; aos meus queridos amigos de laboratório: Bruna, Victória, Karen, Jaqueline, Giovana, Gustavo, Rafaela, Léia, Maria Luiza, Jéssica e Luísa que transformaram a experiência de pesquisa em uma atividade agradável e divertida. A técnica do laboratório Norma que sempre me trouxe palavra de conforto e sabedoria. Gostaria de agradecer a oportunidade que a UNICAMP me deu por conhecer meus queridos amigos de Mossoró, Jovilma, Maria Gabriela, Ayala, Rosselvet e Felipe.

Agradeço também a minha amiga de longa data Jacqueline Florio e seu esposo Ronaldo, que incansavelmente sempre me ajudaram nas mais diversas situações.

Por fim agradeço imensamente a paciência, apoio, suporte do meu namorado Leonardo, que por mais que estivemos longe na maior parte do doutorado, nunca deixou de acreditar que eu seria capaz de concluir meus objetivos.

RESUMO

Atualmente a procura por produtos com menos aditivos e agrotóxicos tem estimulado o comercio de produtos orgânicos, entre eles o leite orgânico, que pode vir a ser utilizado para produção de queijo Minas Frescal, muito consumido no Brasil. Tanto o leite como o queijo são alimentos extremamente ricos em nutrientes, sendo assim ótimos meios de culturas para diferentes microrganismos, dentre os principais deles temos o gênero *Staphylococcus*, bactéria patogênica, capaz de desenvolver resistência a inúmeros antimicrobianos. Sendo então o leite orgânico produzido em sistema sem o uso de antibióticos, a presente pesquisa tem por objetivo caracterizar a diversidade bacteriana por meio de sequenciamento do gene 16S rRNA e avaliar a resistência antimicrobiana e persistência de *Staphylococcus* spp. isolados de laticínios orgânicos e convencionais produtores de queijo Minas Frescal localizados no estado de São Paulo. Foram realizadas duas coletas em 3 laticínios orgânicos e 3 convencionais, sendo 12 coletas no total. As amostras consistiam em queijo Minas, leite pasteurizado, leite cru e swabs de manipuladores, superfícies e utensílios da indústria. Foram selecionadas 96 amostras para o Sequenciamento de Nova Geração (NGS), e os dados foram tratados por bioinformática para identificar a prevalência de famílias e gêneros, enquanto os *Staphylococcus* spp., previamente isolados, foram avaliados quanto ao seu perfil de resistência antimicrobiana e formação de biofilme. Pelo NGS foi possível observar 51 famílias e 96 gêneros diferentes de bactérias, embora não tenhamos identificado grandes diferenças estatísticas na abundância relativa de microrganismos entre os ambientes convencional e orgânico. Não foi possível observar diferença estatística significativa entre orgânico e convencional quanto o perfil de resistência antimicrobiana genotípica e fenotípica de *Staphylococcus*, mas foi importante para caracterizar e realçar a possível presença de resistência antimicrobiana de *Staphylococcus* em lacticínios. No nosso estudo, não houve grandes diferenças significativas entre o perfil do microrganismo estudado e do microbioma, pois inúmeros são os fatores que podem influenciar as características microbiológicas de um alimento, como boas práticas de fabricação, carga microbiana inicial, contaminação cruzada, higiene na ordenha, problemas desde a produção na fazenda até o armazenamento final dos produtos. Esses fatores podem ocorrer independe do tipo de produção, sendo ela orgânica ou não.

Palavras-chaves: Perfil de comunidade microbiana, sequenciamento de nova geração, antibióticos, biofilmes, desinfecção e desinfetantes.

ABSTRACT

Currently, the demand for products with less additives and pesticides has stimulated the trade of organic products, including organic milk, which may be used to produce Minas Frescal cheese, widely consumed in Brazil. Both milk and cheese are extremely nutrient-rich foods, thus being great culture media for different microorganisms, among the main ones we have the genus *Staphylococcus*, a pathogenic bacterium, capable of developing resistance to numerous antimicrobials. Since organic milk is produced in a system without the use of antibiotics, the present research aims to characterize bacterial diversity by sequencing the 16S rRNA gene and to evaluate the antimicrobial resistance and persistence of *Staphylococcus* spp. isolated from organic and conventional dairy producers of Minas Frescal cheese from the State of São Paulo. Two sample collections were performed in 3 organic and 3 conventional dairy plants, with 12 collections in total. The samples consisted of Minas cheese, pasteurized milk, raw milk and swabs from manipulators, surfaces and utensils used in the industry. 96 samples were selected for New Generation Sequencing (NGS), and the data was assessed using treated by bioinformatics to identify the prevalence of families and genera, while previously isolated *Staphylococcus* spp. were evaluated for their antimicrobial resistance profile and formation of biofilm. Through the NGS it was possible to 51 families and 96 different genera of bacteria, although we did not identified major statistical differences in the number of microorganisms between the conventional and organic environments. It was not possible to observe a statistically significant difference between organic and conventional plants regarding the genotypic and phenotypic antimicrobial resistance profile of *Staphylococcus*, but it was important to characterize and highlight the possible presence of antimicrobial resistance of *Staphylococcus* in dairy products. In our study, there was no significant difference between the profile of the studied microorganism and the microbiome, since there are countless factors that can influence the microbiological characteristics of a food, such as good manufacturing practices, initial microbial load, cross contamination, milking hygiene, issues occurring in the transit from the farm to the final storage of products. These factors can occur regardless of the type of production, whether organic or not.

Keywords: Microbial community profiling, next-generation sequence, antibiotics, biofilms, disinfection and disinfectants.

SUMÁRIO

INTRODUÇÃO

Segundo a Instrução Normativa nº 76, de 26 de novembro de 2018, leite cru refrigerado é o leite produzido em propriedades rurais, armazenado sob refrigeração e enviado aos laticínios, sob serviço de inspeção oficial, ele deve se apresentar líquido branco opalescente homogêneo e conter odor característico. Suas características físico-químicas deverão atender ao teor mínimo de proteína total de 2,9 g / 100 g, lactose anidra de 4,3 g / 100 g, sólidos não gordurosos de 8,4 g / 100 g, sólidos totais de 11,4 g / 100 g, acidez titulável entre 0,14 e 0,18 expressa em gramas de ácido lático / 100 mL. Não deve apresentar substâncias estranhas, tais como agentes inibidores de crescimento microbiano, uso de aditivos, coadjuvantes, neutralizantes da acidez e reconstituintes da densidade ou do índice crioscópico, não deve apresentar resíduos de produtos de uso veterinário e contaminantes acima dos limites máximos previstos em normas complementares. Em relação ao seu aspecto microbiológico, no tanque de armazenamento individual ou de uso comunitário deve apresentar médias geométricas trimestrais de Contagem Padrão em Placas de no máximo 300.000 UFC / mL, Contagem de Células Somáticas de no máximo 500.000 CS / mL e no estabelecimento beneficiador antes do seu processamento Contagem Padrão em Placas de até 900.000 UFC / mL. O leite ao chegar a unidade de beneficiamento não poderá ultrapassar 9,0 ºC, e deverá ser resfriado a 4,0 ºC até o seu processamento (BRASIL, 2018).

Já o leite pasteurizado é o leite fluido submetido a um dos processos de pasteurização previstos na legislação vigente, envasado automaticamente em circuito fechado e destinado a consumo humano, sendo proibida a pasteurização de leite previamente envasado. O leite pasteurizado é classificado conforme o conteúdo de gordura, sendo ele leite integral (teor de gordura mínimo 3,0 g / 100 g), leite semidesnatado (teor de gordura 0,6 a 2,9 g), ou leite desnatado (teor de gordura máximo de 0,5 g / 100 g), sendo o produto final deverá ser mantido sob refrigeração de 4,0 °C (BRASIL, 2018).

No Brasil (FEIL et al., 2020) e no mundo a busca por alimentos orgânicos tem crescido (BRITWUM; BERNARD; ALBRECHT, 2020; GONZÁLEZ et al., 2019; KATT; MEIXNER, 2020; KUSHWAH et al., 2019), entre eles o leite orgânico (BRITO;

SILVA, 2020; SCHWENDEL et al., 2015). Segundo a Instrução Normativa Nº 46, de 06 de outubro de 2011, para obtenção e manutenção da saúde dos animais do sistema orgânico, deve-se utilizar o princípio de prevenção, como alimentação adequada (da própria unidade de produção ou de outra sob manejo orgânico), manter as vacas preferencialmente em regime livre ter acesso a pastagem, com objetivo de promover as defesas imunológicas. No caso de doenças ou ferimentos em que o uso das substâncias naturais não estejam fazendo efeito, e o animal esteja em sofrimento ou risco de morte, poderão ser empregados produtos quimiossintéticos artificiais (BRASIL, 2011). Ao contrário da produção convencional onde é permitido o uso de quimiossintéticos artificiais e rações não orgânicas.

Tanto o leite orgânico como o convencional podem vir a ser submetidos a produção do queijo Minas Frescal no Brasil (CÂNDIDO et al., 2020). Segundo a Instrução Normativa nº 4 de 01 de março de 2004, entende-se por Queijo Minas Frescal, o queijo fresco obtido por coagulação enzimática do leite com coalho e/ou outras enzimas coagulantes apropriadas, complementada ou não com ação de bactérias lácticas específicas, é um queijo semi-gordo, de muito alta umidade, a ser consumido fresco, sendo o armazenamento deste produto não deverá ultrapassar 8,0ºC (BRASIL, 2004).

Sendo assim tanto o leite como o queijo Minas Frescal são produtos com características físico-químicas que abrigam variedades de nutrientes que tornam os laticínios favoráveis a presença de diversos microrganismos (PREZZI et al., 2020; VERRAES et al., 2015). Para caracterizar a diversidade bacteriana de alimentos, uma nova abordagem tem surgido na área da biologia molecular, que seria a caracterização da diversidade bacteriana utilizando-se o gene 16S rRNA por meio do Sequenciamento de Nova Geração (NGS) (BENGTSSON-PALME, 2017; JAGADEESAN et al., 2019; PARENTE; RICCIARDI; ZOTTA, 2020; YEUNG, 2012).

O NGS é baseado no conceito paralelo massivo que produz um grande número de 'leituras' relativamente pequenas. Cada leitura relata a composição real de nucleotídeos de pequenos pedaços individuais de DNA usando detectores de alta resolução. A aplicação do NGS para avaliar a composição de um microbioma em alimentos e bebidas e é comumente realizada utilizando-se amplicons gerados por PCR, como o 16S para bactérias e o ITS para fungos (CARDINALI; CORTE; ROBERT, 2017).

A abordagem do NGS em microbiologia de alimentos é usado na maioria das vezes de duas formas: determinação de toda a sequência do genoma de um único isolado cultivado (por exemplo, uma colônia bacteriana, um vírus ou qualquer outro organismo), que é comumente referido como "sequenciamento do genoma completo" ou “metagenômica”, onde NGS é aplicado a uma amostra biológica gerando sequências de múltiplos (senão todos) microrganismos nessa amostra. A aplicação da metagenômica na área de microbiologia de alimentos oferece oportunidades interessantes para prever a presença ou emergência de patógenos e microrganismos deteriorantes, e é um potencial para caracterizar a microbiota desconhecida (JAGADEESAN et al., 2019).

Dentre todos os microrganismos que podem estar presentes nos alimentos, em especial as bactérias, que podem ser caracterizadas nos produtos lácteos, existe um gênero de bactéria que é bastante prevalente: *Staphylococcus* spp.

O gênero *Staphylococcus* spp. pertence à família Staphylococcaceae, atualmente existem 69 espécies de *Staphylococuccus* e 30 subespécies (PARTE et al., 2020). *Staphylococcus* são bactérias Gram-positivas em forma de cocos, de forma redonda com o tamanho de cerca de 1 µm (RUBAB et al., 2018), não formadora de esporos, anaeróbia facultativa, não móvel, são mesófilas (KRONING et al., 2016) e apresentam catalase positiva (ANVISA, 2008). É encontrada em todo o meio ambiente, capaz de sobreviver em condições quentes, secas e se desenvolver em ambientes salinos (RUBAB et al., 2018).

Esse gênero de bactéria pode ser dividido em dois grupos, sendo eles os estafilococos coagule positiva (ECP) ou estafilococo coagulase negativa (ECN) (ANVISA, 2008). Umas das principais espécies de ECP é o *S. aureus*, conhecido não apenas por infecções em humanos (ISHITOBI et al., 2018) e animais (NDAHETUYE et al., 2020a), mas também devido a sua capacidade de causar intoxicação alimentar (FETSCH et al., 2014; HENNEKINNE; DE BUYSER; DRAGACCI, 2012; SOLANO et al., 2013). Durante muito tempo os ECN foram mais explorados em pesquisas de infecções hospitalares (ROGERS; FEY; RUPP, 2009) ou mastite em bovinos (DE VISSCHER et al., 2015; SOARES et al., 2012), mas cada vez mais estão presentes em produtos alimentícios, principalmente em lacticínios (ABREU et al., 2021; CÂNDIDO et al., 2020; CICCONI-HOGAN et al., 2014; RUARO et al., 2013), muitas espécies de ECN são capazes de produzir enterotoxinas (CÂNDIDO et al., 2020; NUNES et al., 2016;

RODRIGUES et al., 2017), podendo vir a ser potencias patógenos em alimentos. Sendo assim a pesquisa de SCN é de extrema importância em produtos lácteos.

Staphylococcus oferecem inúmeros fatores de patogenicidade, como adesinas, hemolisinas, leucocidinas, produção de biofilmes, habilidade de invadir células epiteliais (COSTA et al., 2011), enterotoxinas (CÂNDIDO et al., 2020), resistência antibióticos (SILVA; RODRIGUES; SILVA, 2020) dentre outros, que colaboram para propagar-se e lesionar seus hospedeiros.

Os *Staphylococcus* são considerados um dos principais agentes infecciosos envolvidos na mastite bovina e também na saúde humana, esse gênero de bactéria tem um alto potencial patogênico, devido a genes que codificam fatores de virulência e resistência, eles podem afetar os seres humanos por meio de várias vias, entre elas os alimentos, sendo assim os animais produtores de alimentos como os bovinos, são importantes fontes de contaminações (PÉREZ et al., 2020).

O uso de antibióticos em animais produtores de alimentos, com fins terapêuticos ou não, tem contribuído para o desenvolvimento da resistência aos antimicrobianos. Essas bactérias podem acabar tendo acesso a cadeia alimentar por contaminação cruzada direta ou indireta, devido ao contato com solo, água, fezes ou falha nos programas de controle de qualidade (ALONSO et al., 2019).

Existem diversos mecanismos envolvidos na resistência a antibióticos, incluindo limitação da absorção do antimicrobiano, alteração dos alvos da droga, produção de enzimas que inativam os antibióticos e a ativação de bombas de efluxo que efetivamente removem os antibióticos (HASSANZADEH et al., 2020).

Com o surgimento de *Staphylcoccus* resistente a antibióticos, o *Staphylococcus aures r*esistente à meticilina (MRSA) tornou-se extremamente notável. O MRSA é uma das maiores causas de infecções no mundo (LIU et al., 2020b; SHALABY et al., 2020), algumas dessas infecções podem ser de alto risco, como septicemia, pneumonia necrotizante e síndrome do choque tóxico. A rápida detecção da infecção pode contribuir para eficácia do tratamento e redução de taxa de mortalidade (LIN et al., 2016; WANG et al., 2015). Atualmente, o MRSA é distribuído por toda parte do mundo e sua habilidade de adquirir mecanismos de resistência a antibióticos gera preocupação para a saúde da população. A princípio sua transmissão se dava em ambientes clínicos, mas nas últimas

décadas a sua presença foi relatada na comunidade, isso quer dizer relatos de MRSA fora do ambiente hospitalar, como por exemplo, em alimentos (ONICIUC et al., 2015).

Vêm sendo realizado levantamento quanto às questões que envolvem a transmissão de MRSA, uma vez que é crescente sua difusão entre animais e os alimentos em geral (ARIZA-MIGUEL et al., 2015; ONICIUC et al., 2015; RODRÍGUEZ-LÁZARO et al., 2015). Foi relatada a presença deste grupo de micro-organismos principalmente em produtos cárneos como carne crua de porco, carne bovina, cordeiro, frango, peru, coelho e também em produtos lácteos como leite e queijos (DOULGERAKI et al., 2017). Isso significa que a cadeia produtiva de alimentos é uma forma de transmissão de micro-organismos resistentes para os seres humanos (GUTIÉRREZ et al., 2012). Deste modo, o acompanhamento das características genéticas de MRSA é importante para compreender melhor sua evolução genética (ARIZA-MIGUEL et al., 2015).

A aquisição do mecanismo de resistência adquirido pelo *S. aureus* pode ser dividido em duas categorias: a mutação de um gene bacteriano no cromossomo ou aquisição de um gene de resistência a partir de outras bactérias por meio de troca genética (conjugação, transdução ou transformação) (BROWN-JAQUE et al., 2017).

Os *Staphylococcus aureus* meticilina sensível (MSSA) evoluiu para MRSA devido à aquisição do cassete cromossômico estafilocócico (SCC*mec*) que carrega o gene *mec*A, mecB e *mec*C, é um elemento genético móvel do gênero *Staphylococcus* (LIU et al., 2016).

O SCC*mec* é um elemento genético que transporta o gene *mec* (*mec*A, *mec*B e *mec*C) juntamente com os genes que controlam sua expressão, *mec*R1 (codificando o sinal transduzir proteína MecR1) e *mec*I (codificação da proteína repressora MecI), e atua como um portador para troca de informação genética entre cepas de *Staphylococcus* (LIU et al., 2016). O SCC*mec* transporta recombinases de sítio específicos nomeadas como recombinases de cassete cromossêmico (*ccr*), responsáveis pela mobilidade dos elementos. Em *S. aureus* já foram identificados três tipos de genes *crr*, *ccr*A, *ccr*B e *ccr*C (DOULGERAKI et al., 2017). Além dos genes complexos *mec* e *ccr*, existem regiões situadas entre elas, são conhecidas como regiões J (regiões de junção), são uma união altamente variáveis que podem ser chamadas de J1, J2 e J3, que contêm componentes não essenciais do elemento SCC*mec* , mas podem abrigar genes de resistência adicionais, tais como plasmídeos (KAYA et al., 2018; MARTÍNEZ-MELÉNDEZ et al., 2015).

Os SCC*mec* são classificados em tipos e subtipos. Os tipos SCC*mec* são definidos pelo tipo de complexo *mec* e complexo *ccr* presente na cepa. Os subtipos são baseados nas diferenças presentes nas regiões J dentro de um SCC*mec*. Os elementos SCC*mec* em *S. aureus* são classificados em diferentes tipos (I a XIV) com base na combinação de *mec* e *ccr* (SOUSA et al., 2020; URUSHIBARA et al., 2020).

Além da preocupação com a resistência a antibióticos, os *Staphylococcus* são capazes de desenvolver resistência a outros antimicrobianos, dentre eles, temos os sanitizantes, que são amplamente utilizados em diversos países na indústria de alimentos com intuito de atender padrões de qualidade e saúde. A resistência a sanitizantes tem surgido rapidamente, sendo um dos motivos por trás disso o abuso e uso indevido de desinfetantes. Quando se utiliza qualquer antimicrobiano, até mesmo os sanitizantes, causa uma pressão seletiva que dá origem a células resistentes, sendo que por meio da transferência vertical e horizontal de genes, essas bactérias resistentes são capazes de dar origem a outras bactérias resistentes (CARLIE; BOUCHER; BRAGG, 2020).

Dentre os principais sanitizantes utilizados no combate de *Staphylococcus* na indústria de alimentos, temos os Compostos de amônio quaternário (QAC) que são conhecidos pela capacidade de romper as membranas celulares dos microrganismos, sendo considerado como um eficaz antimicrobiano (ZHANG et al., 2018).

Os QACs são encontrados em produtos naturais antimicrobianos, como a berberina, nitrogênios terciários, poliaminas, norspermidina. A versão sintética de QACs são largamente produzidos e utilizados como desinfetantes (MINBIOLE et al., 2016).

Os primeiros QACs a serem comercializados foram a partir de 1930 (JIAO et al., 2017) com aprovação do cloreto de benzalcônio como agente antimicrobiano. Desde então vários QACs foram comercializados como antibacterianos utilizados em diferentes estabelecimentos, incluindo hospitais e instalações de saúde, várias indústrias e ambientes comerciais e residenciais (MINBIOLE et al., 2016). Os QACs são largamente utilizados na indústria de alimentos (SHAMSUDIN et al., 2012).

Atualmente são comercializados os seguintes produtos QACs: benzalcônio (BAC), cloreto de cetilpiridínio (CPC), cloreto de didecildimetilamônio (DDAC). A maioria deles são derivados monocíclicos com pouca variabilidade estrutural (MINBIOLE et al., 2016). Estruturalmente, os QACs são compostos contendo nitrogênio (N +), o átomo de N está ligado a quatro grupos diferentes por ligações covalentes. A

maioria dos sais de QAC é composta predominantemente por sais de cloreto ou brometo, enquanto os sais de iodeto tendem a exibir solubilidade diminuída (JIAO et al., 2017).

O seu mecanismo de defesa da bactéria, está relacionado com as bombas de efluxo (MINBIOLE et al., 2016) que são codificadas por plasmídeos (SHAMSUDIN et al., 2012). Na literatura já foram relatados os *qac*s A, B, C (*smr*), G, H e J (WORTHING et al., 2018).. Genes *qac*A e *qac*B confere uma susceptibilidade reduzida a uma ampla gama de cátions orgânicos antimicrobianos, incluindo QACs. O *smr* (também conhecido como *qac*C/D) codifica uma pequena proteína que confere menor susceptibilidade a QAC (SHAMSUDIN et al., 2012). Além da presença de bombas de efluxo codificadas por plasmídeo, a resistência pode ser associada ao aumento da expressão do gene cromossômico que codifica a bomba *nor*A (MARCHI et al., 2015).

A sequência de nucleótidos de *qac*A é quase idêntica à de *qac*B. As proteínas codificadas diferem em apenas um aminoácido no resíduo 323 (Asp em QacA e Ala em QacB). No entanto, proteína QacA confere maior resistência a cátions divalentes do que proteína QacB (HANAKI et al., 2011).

Os QACs são geralmente mais eficazes contra bactérias Gram-positivas do que nas Gram-negativas, pois elas têm múltiplas camadas bastante complexas (MINBIOLE et al., 2016). Mesmo o *Staphylococcus* sendo uma bactéria Gram-positiva pode adquirir resistência ao QAC (IÑIGUEZ-MORENO et al., 2018; SHAMSUDIN et al., 2012).

A resistência de *Staphylococcus* ao QAC está relacionada ao sistema de efluxo de QAC, que causa a resistência de QAC e biocidas catiônicos em células planctônicas. Por meio deste sistema, o *S. aureus* é capaz de repelir moléculas tóxicas, proporcionando a capacidade de sobrevivência na presença desses sanitizantes. Também a estrutura tridimensional dos biofilmes de *Staphylococcus* estão relacionados com a resistência ao QAC, devido as alterações fisiológicas nas células do biofilme (IÑIGUEZ-MORENO et al., 2018).

Além dos fatores de resistência já mencionados acima, os *Staphylococcus* tem a capacidade de produzirem biofilmes, que aumentam a capacidade deles se protegerem de antibióticos e sanitizantes e persistirem mais no ambiente (ARCIOLA et al., 2012). O *Staphylococcus* sobrevive em superfícies secas e pode persistir por muito tempo em um mesmo local, podendo ser transmitido entre pessoas, e por contato com superfícies contaminadas, permanecendo nas roupas e pele de indivíduos que entram em contato com tais superfícies (LIN et al., 2016). A capacidade dos *Staphylococcus* de formar biofilme permite a sobrevivência do micro-organismo em ambientes inapropriados, tais como nos

utensílios das indústrias de alimentos, o que permite a contaminação dos alimentos (GUTIÉRREZ et al., 2012).

A formação de biofilme é um importante mecanismo de persistência no ambiente, utilizado por muitos patógenos bacterianos. O biofilme pode ser definido como uma comunidade microbiana séssil embutida em um material viscoso amorfo (RAAFAT et al., 2019).

Basicamente o mecanismo de produção dos biofilmes são divididos em quatro etapas: 1) fixação das células; 2) associação e acúmulo de células em múltiplas camadas; 3) maturação do biofilme e 4) descolamento das células do biofilme em um estado planctônico para começar mais um ciclo de formação de biofilmes (ARCIOLA et al., 2012; GUTIÉRREZ et al., 2012; RAAFAT et al., 2019). A Figura 1 abaixo, representa as 4 etapas de formação de biofilme.

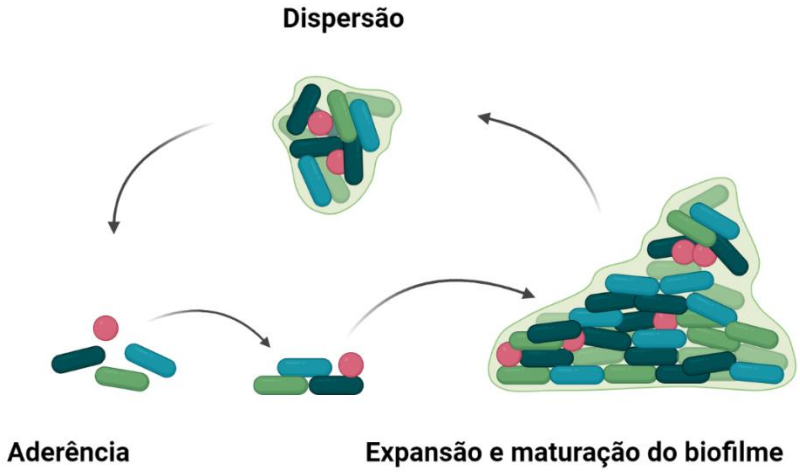

Figura 1 – Formação de biofilme (*Created with* BioRender.com).

A habilidade de formação de biofilmes de *Staphylococcus* está relacionada com a presença dos genes *bap*, *ica*A e *ica*D, característica associada à redução da suscetibilidade a antibióticos, pois reduz a disseminação do antibiótico nas camadas do biofilme (KRONING et al., 2016).

Um dos principais fatores de adesão das células se deve à produção do polissacarídeo adesina intercelular (PIA), que consiste em glucosaminilglicanos lineares β-1,6, cuja síntese é mediada pelo locus de adesão intercelular (*ica*ADBC) (ARCIOLA et

al., 2012), podendo também diminuir a eficiência dos desinfetantes, aumentando casos de contaminação de alimentos e causando prejuízos financeiros à indústria de alimentos (KRONING et al., 2016).

Dentro do locus *ica*ADBC, o gene *ica*A codifica a enzima N- acetilglucosaminil transferase, capaz de catalisar a síntese do polímero poli- N- acetilglucosamina. A expressão de apenas *ica*A gera baixa atividade enzimática. Mas quando o gene *ica*A é expresso juntamente com o gene *ica*D, a atividade da N- acetilglucosaminil transferase aumenta consideravelmente (ARCIOLA et al., 2012). Por mais que o locus *ica*ADBC seja extremamente importante para formação dos biofilmes, sua produção pode ser independente deste locus. A matriz extracelular de biofilmes também abriga proteínas adesivas que estão implicados na formação de biofilme, como a proteína associada à biofilme (denominado Bap). Este mecanismo de biofilme depende da capacidade dos *Staphylococcus* para expressar uma variedade de proteínas de adesão que permitem que as células se fixem e colonizem diversos tipos de superfícies. Essas proteínas servem para manter as células juntas dentro do biofilme, interagindo com outras proteínas de superfície das células próximas (ARCIOLA et al., 2012).

Tendo em vista as definições apresentadas para produtos lácteos orgânicos e convencionais e sobre aquisição de resistência de *Staphylococcus* spp. são levantadas duas principais hipóteses: se o impacto das diferenças do manejo orgânico para o convenciol seria o suficiente para diferir em relação de bactérias presentes em ambos os sistemas, e se a ausência do uso de antibióticos sintéticos no manejo orgânico seria capaz de apresentar isolados menos resistentes a antibióticos do que no sistema convencional.

Neste sentido, o objetivo deste estudo foi caracterizar e comparar a diversidade bacteriana de lacticínios orgânicos e convencionais de produção de queijo Minas Frescal, por meio de NGS e também de caracterizar resistência antimicrobiana do gênero *Staphylococcus* spp. correlacionando com a sua capacidade de persistência por meio da formação de biofilmes. Para tal, os seguintes objetivos específicos foram considerados:

- ✓ Realizar uma revisão de literatura a respeito da prevalência de MRSA em alimentos.
- ✓ Caracterizar a diversidade bacteriana de 3 laticínios orgânicos e 3 laticínios de produção convencional, utilizando amostras do produto acabado, matéria prima, manipuladores e ambiente da produção, por meio do NGS do gene 16S rRNA.

- ✓ Caracterizar a resistência antimicrobiana genotípica e fenotípica das cepas de *Staphylococcus* spp. por meio da realização de antibiogramas, PCR de genes de resistência a antibióticos e sanitizantes, e teste de eficácia de sanitizantes e determinações de Concentração Inibitória Mínima (CIM).
- ✓ Verificar a capacidade de formação de biofilmes de cepas de *Staphylococcus* spp. pré-selecionadas com genes de interesse, nas superfícies de aço inoxidável e avaliar a capacidade dos sanitizantes na redução dos biofilmes formados.

CAPÍTULO 1

Revisão de literatura: Methicillin-resistant *Staphylococcus aureus* in food and the prevalence in Brazil: a review

Artigo publicado no periódico "Brazilian Journal of Microbiology": Silva, A. C.; Rodrigues, M. X.; Silva, N. C. C. Methicillin-resistant *Staphylococcus aureus* in food and the prevalence in Brazil: a review. Brazilian Journal of Microbiology, 51, 347-356, 2020.

Brazilian Journal of Microbiology (2020) 51:347–356
https://doi.org/10.1007/s42770-019-00168-1

FOOD MICROBIOLOGY - REVIEW

Methicillin-resistant *Staphylococcus aureus* in food and the prevalence in Brazil: a review

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Received: 31 January 2019 / Accepted: 9 October 2019 / Published online: 30 October 2019

Abstract
Foodborne diseases (FBD) occur worldwide and affect a large part of the population, being a cause of international concern among health authorities. *Staphylococcus aureus* can be transmitted by contaminated food, and it is one of the pathogens that most cause foodborne outbreaks in Brazil. Currently, this organism's ability in developing resistance to antibiotics is notorious; methicillin-resistant *Staphylococcus aureus*—MRSA—is known for its resistance to methicillin, oxacillin, and others. MRSA is one of the leading causes of infections, becoming a major threat to human health worldwide due to the numerous toxins that can produce. At first, the transmission of MRSA occurred in clinical environments; but in recent decades, its presence has been reported in the community, outside the hospital environment, including food and food-producing animals around the world. In this review, information about MRSA was gathered to verify MRSA incidence in the world but especially in Brazil in food samples, food handlers, food-producing animals, and food processing environments. The studies show that MRSA is easily found and in certain cases with high frequency, thus representing a potential risk to public health.

Keywords Brazil · FBD · *mec*A · MRSA · Oxacillin

Introduction

Foodborne diseases (FBD) occur worldwide and affect a large part of the population, being a cause of international concern among health authorities [1]. FBDs have significant morbidity and mortality, it is estimated that thousands of people are hospitalized and some of them die from this cause. It is very difficult to estimate the actual number of FBD cases because not all of them are registered by the public health system due to misdiagnosis or underreporting [1].

Staphylococcus aureus can be transmitted by contaminated food [2]; and it is one of the pathogens that most cause foodborne outbreaks in Brazil [3]. This transmission is mainly due to the poor handling of food during processing [4, 5]. The consumption of food contaminated with toxins produced by *S. aureus* can lead to staphylococcal food poisoning, which may cause severe gastroenteritis, nausea, vomiting, diarrhea, and abdominal pain within 1 to 6 h after the consumption of contaminated food [6]. *S. aureus* can also lead to other diseases [2]; some of them severe, such as sepsis, endocarditis and necrotizing pneumonia [7]. This bacterium is found on human skin and it is commonly identified as a cause of hospital-acquired infections [8]. It is also the leading cause of bacterial infections in humans; around 20% of humans are persistent carriers of *S. aureus*, 30% are intermittent carriers, 50% of people do not carry this bacterium [7, 9, 10], and a third of people are asymptomatic carriers; the pathogen is commonly found in the nostrils, neck, axillae, groin, and rectum [11–13].

S. aureus spp. are non-spore forming Gram-positive bacteria in the form of cocci; they are non-mobile, mesophilic, biofilm-forming, and facultative anaerobes that produce enterotoxins [3]. They were first described by Sir Alexander Ogston in 1881, when the infection caused by this agent was fatal because of the lack of antibiotics [11]. Currently, this microorganism's ability in developing resistance to antibiotics is notorious. The resistance is usually acquired by horizontal

Responsible Editor: Mariza Landgraf.

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gene transfer, although mutation and selection are also important [14]. Infections caused by resistant strains are common in epidemic waves by one or more clones; methicillin-resistant *Staphylococcus aureus* (MRSA) is prominent in epidemic waves, being historically associated with hospitals and health units (healthcare-associated MRSA (HA-MRSA). Nowadays, it has emerged as a cause of community-associated infections (CA-MRSA), spreading rapidly among healthy individuals and its presence is a cause of concern due to resistance to various antibiotics, limiting treatment [14].

The incidence of CA-MRSA has been increasing [15–18]. Furthermore, CA-MRSA strains appear to be especially virulent [14]. It should be noted that CA-MRSA, HA-MRSA, and livestock-associated MRSA (LA-MRSA) have been found in foods intended for human consumption [15]. Researches have been showing the incidence of MRSA isolated from foods [6, 9, 19–21]. Studies from different geographical areas have revealed the presence of enterotoxins in MRSA isolates; in addition, the genetic relationship between enterotoxigenic isolates and isolates from human infections has been reported [15]. Therefore, this review aims to explore data that show the importance and incidence of MRSA isolated from foods around the world, and especially in Brazil where *S. aureus* is one of the main etiological causes of food poisoning outbreaks.

Antibiotic-resistant *Staphylococcus* spp.

Antibiotics correspond to a group of drugs that are commonly used in hospitals and in the community. However, pharmacological agents do not only affect the patients that use this, but also significantly intervene in the environment through the genetic modification of microorganisms [22].

The use of antibiotics has increased a lot over the years and, consequently, the exposure of these medicines to bacteria has also expanding [23]. *S. aureus* is a bacterial species known for its ability to become resistant to antibiotics [14]. For Chambers and DeLeo [14], exposure to antibiotics was, without a doubt, the most concentrated selective pressure exerted on the co-evolutive history of *S. aureus* with humanity.

The indiscriminate use of antibiotics stimulates the development of antibiotic resistance. The most useful antibiotics in the treatment of infections caused by *S. aureus* are β-lactams, including penicillin, methicillin, flucloxacillin, dicloxacillin, nafcillin, oxacillin, and cloxacillin [24].

Methicillin-resistant *S. aureus* (MRSA) are those who carry the *mec*A gene and are resistant to all penicillins, cephalosporins,s and carbapenem [24]. In MRSA cases, the antibiotic of choice has for a long time been vancomycin; however, other options have emerged such oxazolidinones, glycylcyclines, and lipopeptides [25]. Nevertheless, it is relevant to describe how antibiotic-resistant *S. aureus* arose, especially MRSA.

In 1928, Alexander Fleming discovered penicillin, thus making it possible to treat infections caused by *S. aureus*, starting the "Antibiotic Era" [14]. However, the use of penicillin to treat infections did not last long, as penicillin-resistant strains started to emerge [11]. In 1940, *S. aureus* became resistant to sulfonamide, and in 1944, it started becoming resistant to penicillin [11]. Thus, epidemic waves of antibiotic-resistant *S. aureus* began; in the 40s, the first wave was observed with resistance to penicillin, which still occurring today [14]. Methicillin and oxacillin were used in the 1960s to treat infections caused by *S. aureus*; however, some years later, resistant strains emerged, which were collectively known as MRSA [25]. In the following years, cases of resistance of *S. aureus* to different classes of antibiotics such as macrolides, fluoroquinolones, glycopeptides, aminoglycosides, and tetracyclines started being reported [26]. The second wave occurred almost immediately after the introduction of methicillin with the isolation of the first MRSA isolate, type I SCC*mec* (Staphylococcal Cassette Chromosome *mec*) [14]. The third wave happened in the mid-1970s with new MRSA strains that had new SCC*mec* types, type II and III, signaling a MRSA pandemic around the world; and the fourth and latest wave of antibiotic resistance arose in the late 1990s, with the emergence of MRSA strains in the community [14]. The discovery of CA-MRSA happened in the USA [27] when the strains were already resistant to several antibiotics, in addition to those of the beta-lactam type, and were not related to the hospital strains, which contained a new SCC*mec*, type IV, and a variety of virulence factors [14]. With the increase in MRSA, the use of vancomycin also increased in the treatment of infections caused by these bacteria; in this way, strains with intermediate resistance to vancomycin (Vancomycin-intermediate *Staphylococcus aureus* (VISA)) emerged, and in 2002, the first strains resistant to vancomycin (Vancomycin-Resistant *Staphylococcus aureus* (VRSA)) were identified [14].

Given the above, the concern about the presence, distribution, and incidence of resistant strains in any environment becomes evident; especially in health units where weakened individuals may be exposed to them, and in food production, where people from different age groups and with different health states may be contaminated during production and/or consumption.

Methicillin-resistant *Staphylococcus aureus*

The MRSA is one of the leading causes of infections [28]; 50% of the strains isolated in the USA and in European countries are Methicillin-resistant [25, 29]. Infections caused by MRSA generate higher expenses in the area of public health, and higher morbidity and mortality rate compared with non-resistant strains [25, 30]. Additionally, infectious diseases caused by MRSA are among the leading causes of death caused by infectious agents [11].

More people die each year from infections caused by MRSA than by HIV (human immunodeficiency virus) in the USA [11]. Thereby, methicillin resistance is a very serious health problem when implicated in human infections or in animals [31]. It is worth noting that the rapid detection of the infection may contribute to the effectiveness of treatment and reduction in the mortality rate [32, 33].

Currently, MRSA is spread around the world and its ability to acquire antibiotic resistance mechanisms raises concern; MRSA is often or can easily become resistant to multiple antibiotics, limiting treatment options [14]. At first, the transmission of MRSA occurred in clinical environments, but in recent decades, its presence has been reported in the community, outside the hospital environment [7]. However, investigating the origins of bacteria is complicated. Evidence indicates that resistant *S. aureus* can be spread in livestock operations and in hospitals, where antibiotics are widely used; thus, it could be disseminated within communities and the environment. It is worth mentioning that more researches are essential to determine how the transfers in fact occur [34].

As mentioned above, MRSA is classified as HA-MRSA, healthcare-associated methicillin-resistant *S. aureus*, CA-MRSA, community-associated methicillin-resistant *S. aureus* [35], or LA-MRSA, livestock-associated methicillin-resistant *S. aureus*. In an even more worrisome scenario, MRSA strains may become resistant to multiple antibiotics (multidrug-resistant MDR) [25]. The biggest problem is the ability of these bacteria to be transferred from animals to humans, causing infections [36]. Reports have been mentioned MRSA in animals, especially in pigs, but it can also affect calves [37], horses [38], and dogs [39]. LA-MRSA can be transmitted to humans who live in close contact with animals [40]. *S. aureus* can also be transmitted to humans through meat products, for example, due to their improper handling or cross-contamination during processing [34]. A recent study by Caggiano et al. [40] assessed healthy individuals who worked in the food industry, and the presence of *S. aureus* and MRSA among the individuals represented a risk to public health. LA-MRSA strains have been found in pork and chicken products in the USA, as well in raw turkey meat [34]. Caggiano et al. [40] conclude that the spread of *S. aureus* and MRSA in non-hospital environments, such as communities and in livestock, demands careful and continuous monitoring.

There are several methods to determine whether a *S. aureus* strain is methicillin-resistant; however, the one that is most often employed is the Kirby-Bauer method, which uses oxacillin and cefoxitin [25]. However, conventional culturing methods demand a lot of time; thus, methods based on polymerase chain reaction (PCR) and hybridization assays have been increasingly used as rapid methods for detection of MRSA [41]. The combination of methods has been widely employed for the detection of MRSA.

Resistance mechanism

The *mec*A gene, responsible for methicillin resistance in *S. aureus*, is the reason for these groups of microorganisms to be considered resistant to all beta-lactam antibiotic [42]. Methicillin resistance in *S. aureus* is mediated by the *mec*A gene, which encodes a new penicillin-binding protein (PBP), PBP-2a [43]. In MRSA strains, exposure to methicillin renders the four high-affinity binding proteins (PBPs) present inactive, whereas PBP-2a has low affinity to methicillin, allowing the growth of the cell, because it assumes the functions of the PBPs [43]. This resistance allows the biosynthesis of the cell wall, which is the target of β-lactam antibiotics, and occurs even in the presence of often inhibitory concentrations of antibiotics [44]. The regulation of the phenotype of resistance to methicillin and the production of PBP-2a are carried out by other genes, *mec*R1 and *mec*I; in addition, antibiotics with high PBP-2a affinity have shown effectiveness against MRSA in vivo [43].

These binding proteins decrease the ability of β-lactam antibiotics to act on bacteria [45]. In addition to the *mec*A gene, the *mec*C gene (previously called *mec*LGA251) has been recently described. This gene has been identified in strains isolated from, for example, milk collected from 465 herds in England in 2007 [46], and in cattle that would be submitted to slaughter between October 2011 and January 2012, mainly in Belgium and in France [47].

The resistance mechanism acquired by *S. aureus* can be divided in two categories: the mutation of a bacterial gene in the chromosome or the acquisition of a resistance gene from other bacteria through genetic exchange (conjugation, transduction, or transformation) [48].

The mobile genetic elements are related to PBP-2a, the penicillin-binding protein encoded by the *mec*A gene, which is found in SCC*mec*, the genetic element that encodes methicillin resistance; the resistance expression is controlled through transduction by a proteolytic signal, which corresponds to a sensor protein (*mec*R1) and a repressor (*mec*I) [44]. Molecular and biochemical mechanisms concerning methicillin resistance in *S. aureus* have been subject of studies, including regulatory events and those related to the structure of proteins [44].

The type of SCC*mec* can confer resistance to multiple antibiotics [13, 49, 50]. It transports site-specific recombinases, called cassette chromosome recombinases (*ccr*), which are responsible for the mobility of the elements. In *S. aureus*, three types of ccr genes have already been identified, *ccr*A, *ccr*B, and *ccr*C [51].

It is important to understand the origin and evolution of MRSA clones. The acquisition and diversity of different SCC*mec*s are crucial [52], and it is through that methicillin-susceptible *S. aureus* (MSSA) becomes MRSA. SCC*mec* carries either the *mec*A or *mec*C gene, regulatory genes, *ccr*AB or/and *ccr*C site-specific recombinase genes, and a variety of accessory genes encoding for a new specific

penicillin-binding protein (PBP2a) [53, 54]. The SCC*mec* element contains three J regions, besides the *mec* and *ccr* gene complexes, they were first report as the L-C, C-M, and I-R regions but were later changed to J regions. These regions constitute non-essential components of the cassette and may carry additional antimicrobial resistance determinants [55].

According to International Working Group on the Staphylococcal Cassette Chromosome elements (IWG-SCC), there are 11 official types of SCC*mec* recognized [56]. Some studies even mention the existence of 12 SCC*mec* [54] or even 13 SCC*mec* [52]. Traditionally, types I to III have been associated with MRSA strains from clinical isolates, while community (CA-) or livestock-associated (LA-) strains tend to harbor smaller and supposedly more mobile type IV SCC*mec* types and V [57, 58]. But it is currently possible to find several types of *Staphylococcus* SCC*mec* isolated from different types of food [21, 59, 60].

Methods used for identification and characterization of MRSA

There are several methods for identification of MRSA strains, phenotypically or genotypically. Strains can be screened on MRSA agar, after 18 to 24 h of incubation at 37 °C, colonies that appear pink are considered MRSA [59]. Other typical way are Kirby-Bauer standard disk-diffusion methods, which are commonly used to determine antimicrobial susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [25, 59, 61–63] or even the broth microdilution technique [64].

Some studies use resistance to oxacillin [59, 65] or cefoxitin [62, 64] or both [25] to determine if the strain is MRSA. It is also possible to determine MIC of *S. aureus* strains by the *E*-test according to the protocol suggested by the manufacturer [61].

Identification by polymerase chain reaction (PCR) [41, 59, 62–68] is also widely used. Specie confirmation can be performed using *nuc* gene; *mec*A gene is used to identify the methicillin-resistant strain [59, 62, 63, 65, 67, 68]. Other alternative for detection of MRSA is the use of kits based on PCR, a multiplex PCR and real-time PCR, to detect each SCC*mec* type and the chromosomal orfX-SCC*mec* junction [69]. In some cases when *S. aureus* is resistance to cefoxitin but is negative for the *mec*A gene, they can be tested for *mec*C gene, which also characterizes *S. aureus* as MRSA [67].

After confirmation of the *mec*A gene, it is important to submit the strains to additional molecular characterization. The techniques that are generally used are as follows: *staphylococcal* protein A (*spa*) typing [59, 61, 66–68], multilocus sequence typing (MLST) [59, 61, 64, 66, 67, 70], pulsed field gel electrophoresis (PFGE) [64–66], *Staphylococcal cassette chromosome mec* (SCC*mec*) typing [59, 66].

Methicillin-resistant *Staphylococcus aureus* isolated from food

MRSA has already been isolated from food, indicating that they are present as contaminants in the food production chain [6, 51, 71]. Recently, studies have been performed focused on transmission of MRSA, since its diffusion among food-producing animals and food has increased [2, 7, 72, 73].

The presence of this group of microorganisms was reported mainly in meats such as pork, beef, lamb, chicken, rabbit, and turkey, and also in dairy products, e.g. milk and cheese [51]. This means that the food production chain is a channel of transmission between resistant microorganisms and humans [4]. In this way, the monitoring of genetic characteristics of MRSA is important to better understand its genetic evolution [72].

The following table presents a data survey on studies conducted in different countries where MRSA was isolated from various kinds of food.

As shown in Table 1, a survey of MRSA research over the past 16 years has shown a great occurrence of this type of bacteria in meat, milk, and dairy products, becoming increasingly clear that MRSA is more present in products of animal origin or food-producing animals.

MRSA in Brazil

The data survey on the incidence of MRSA in food has been happening also in Brazil. Researches in different regions have been showing the incidence of MRSA in food and food-producing animals, with an emphasis on milk and dairy products and meat and meat products. Furthermore, the importance of researches conducted with samples of food processing environments and food handlers should also be highlighted [65, 89, 90]. Next, studies shall be presented to demonstrate the incidence of *S. aureus* and specifically MRSA in Brazilian territory. *S. aureus* is still a common contaminant in food in Brazil; it has been reported as one of the most prevalent causes of FBD in the country according to the Secretary of Health Surveillance [91], and it is commonly identified as the cause of diseases in food-producing animals.

In the Northeast of the country, Soares et al. [92] analyzed the presence of *S. aureus* in samples of food handlers in public schools of Camaçari, Bahia State. Swabs were collected out of the hands of 166 handlers, 53.3% of the samples were positive for the presence of *S. aureus*. The results indicated that the food handlers were using inadequate sanitary practices and rethink their training to ensure proper hygiene was essential [92]. Still in the Northeast, a study by Ferreira et al. [93] aimed to evaluate the presence of MRSA in food handlers of public hospitals in the city of Salvador, Bahia. The researchers collected swabs from the nostrils and hands of 140 food handlers in 10 public hospitals; 50% of the handlers had *S. aureus* on their hands or nostrils and 28.6% had MRSA. These authors

Table 1 Incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) in foods in different countries

Number of sample	Number of isolated	MRSA	Sample	Origin	Reference
N/M	846	5 (0.6%)	Cow milk	Michigan, USA	[74]
N/M	2132	38 (1.8%)	Cow milk	Wisconsin, USA	[75]
444	292	2 (0.7%)	Chicken	Japan	[76]
N/M	357	0 (0%)	Cow milk	North Carolina and Virginia, USA	[77]
1260	157	30 (19.1%)	Mutton, beef, camel, and poultry	Jordan	[78]
79	36	2 (5.5%)	Pork and raw beef	Holland	[79]
1634	160	6 (3.8%)	Cow milk and cheese	Italy	[80]
318	N/M	5 (1.6%)	Pork, chicken, rabbit, veal, and wild boar	Spain	[81]
2217	N/M	264 (11.9%)	Beef, veal, lamb and mutton, pork, chicken, turkey, poultry	Netherlands	[82]
79	36	2 (5.5%)	Retail meat	Holland	[9]
120	47	6 (12.8%)	Retail meat	USA	
N/M	402	31 (7.7%)	Retail meat	Canada	
583	N/M	1 (0.2%) 13 (26.5%)	532 human swabs without direct contact with pig breeding and 49 with direct contact	Netherlands	[19]
86	N/M	32 (37.2%)	Chicken, chicken meat products, turkey, turkey meat products	Germany	[83]
256	N/M	26 (10.15%)	Pork	USA	[84]
NM	583	28 (4.8%)	Wild boar meat	Berlin, Germany	[64]
100	7	2 (28.6%)	Fish-based ready-to-eat food	Greece	[85]
383	35	7 (20%)	Raw milk	Italy	[86]
323	85	7 (8.2%)	Nasal swabs of food industry workers	Italy	[40]
195	54	16 (29.6%)	Raw milk	China	[87]
93	54	19 (35,2%)	Clinical mastitis milk	Cairo, Egypt	[63]
3760	484	40 (8.3%)	Milk and milk derivatives	Italy	[59]
3290	913	41 (4.5%)	Raw meat	Iowa, USA	[88]
N/M	24	9 (37.5)	Milk	Switzerland and Italy	[68]
147	40	2 (5%)	Milk and swab	Brazil	[65]
372 303	N/M N/M	36 (9.7%) 5 (1.7%)	Bulk tank milk from conventional Bulk tank milk from organic	Germany	[67]

N/M, not mentioned

also concluded that there is great deficiency in the hygiene of food handlers, which could cause infection in patients [93].

André et al. [94] held in Goiânia a study conducted from February 2004 to March 2005, in which 24 milk samples, 24 samples of Minas Frescal cheese, and 92 samples of food handlers (46 of their hands and 46 of their nostrils) were collected, totaling 140 samples [94]. From these samples, 63 isolates of *S. aureus* were obtained, corresponding to 32.6% of the nasal swabs, 30.4% of the hand swabs, 67.7% of the milk swabs, and 70.8% of the cheese swabs. The researchers conducted the disk-diffusion test in agar to check the resistance of the strains. Disks impregnated with erythromycin (15 μg), ciprofloxacin (5 μg), tetracycline (30 μg), gentamicin (10 μg), vancomycin (30 μg), oxacillin (1 μg), and penicillin (10 μg) were used. The results showed that 23% of the isolates had resistance to some antibiotic and 5.5% were possibly MRSA [94]. Rodrigues et al. [21] analyzed *Staphylococcus* spp. isolates from three cheese processing plants including samples of raw milk, food handler, and cheese; a total of 100 isolates were characterized of which 88% were *S. aureus* and *mec*A gene was identified in six (6%) strains. In addition, Brazilian producers of milk derivatives were evaluated for the presence and diversity of *S. aureus*; interestingly, only 7.4% of the samples was positive for *S. aureus*, and no MRSA was found [89]. Corroborating these results, Silveira-Filho et al. [95] did not detect MRSA in samples of milk and milk derivatives collected in the Northeastern region of the country.

Costa et al. [20] carried out the isolation of *S. aureus* and identification of MRSA. Samples of different types of meats served in 10 hospitals of the city of Salvador, Bahia, were collected, and a total of 114 raw meat samples (30 chicken samples, 30 beef samples, 24 pork samples, and 30 fish

samples) were analyzed. Of the 114 raw meat samples, 28.1% were positive for MRSA. *S. aureus* was also isolated from 63 cooked meat samples (15 chicken samples, 15 beef samples, 15 pork samples, and 18 fish samples); of these, 9.5% contained MRSA. The high prevalence of MRSA in meat, mainly in food prepared for consumption, emphasizes the need for the best food handling practices in hospitals [20], and also for the best practices in the handling of animals.

A survey conducted with 552 milk samples from 15 dairy farms in the state of Paraíba identified 65 samples which tested positive for *S. aureus*, and of these, 20 had MRSA and none isolate was resistant to vancomycin [96]; in this case, 30.7% of the samples had MRSA. Recently, an outbreak of bovine mastitis caused by *S. aureus* in a Brazilian dairy farm was analyzed. Guimarães et al. [97] evaluated 115 milk samples from the herd affected by the disease and found that 53% of the samples had *Staphylococcus* spp.; of these, 98.4% were positive for *S. aureus*, and the presence of the *mec*A gene was identified in 48.3% of the *S. aureus* isolates. In total, 12.2% of the cases of mastitis were caused by MRSA; this high percentage raises concern for animal and human health [97]. On the other hand, Silva et al. [31] identified methicillin-sensitive *S. aureus* in milk samples from cows with mastitis. They obtained 56 MSSA isolates from 1484 milk samples of 518 cows in 11 different farms located in Brazil. In the same study, the researchers conducted molecular characterization, gene research, reinforcing the importance that these instruments have for characterizing *S. aureus* [31].

Rabello et al. [98] developed a research in the state of Rio de Janeiro in which they identified 227 *S. aureus* isolates in milk samples of cows with subclinical mastitis, with the exception of two that had clinical mastitis. The samples were collected from 18 herds distributed in 9 cities of Rio de Janeiro from July 2001 to July 2004. PCR analysis was performed to amplify 16S rRNA gene for bacteria species identification. The characterization of the strains was important to determine the cause of infection and develop control measures [98].

The study conducted by Monte [65] included 110 staphylococci isolated from 147 samples of 21 semi-extensive dairy farms in the Northeast of Brazil. Of these, 40 of them were *S. aureus*, most of them isolated from milk samples, two of which presented the *mec*A gene, indicative of MRSA. The other 70 isolates were coagulase negative, most of them from swabs (52.4%) and environmental samples (29.5%), 14 of these isolates were positive *mec*A.

Alves et al. [90] collected 64 samples from three dairy products from Minas Frescal artisanal cheese production located in the Midwest region of Goiás, Brazil. These samples include processing environments, raw materials, and final product. Those were confirmed by PCR amplification of the 16S rRNA gene, MLST, and antimicrobial susceptibility test, 33 isolates were confirmed as *S. aureus*, but only one was identified as MRSA (strain isolated from brine).

Given the above, it is possible to verify that the incidence of *S. aureus* and MRSA is common in food and in the food production chain, and that researches are still required for a better understanding of the distribution of MRSA and its diversity, with emphasis on analyses that allow verification of similarity of the strains and thus determinate or suggest their origin and, consequently, indicate preventive and control measures.

Conclusion

The use of antibiotics, and more specifically the indiscriminate use of antibiotics, in the treatment of infections caused by *Staphylococcus* generated a very serious public health problem: the resistance of *Staphylococcus* spp. to antibiotics. In this review, information about MRSA was gathered from studies published around the world. Herein, the incidence of MRSA in food samples, food handlers, food-producing animals, and food processing environments was presented. The situation is critical since VISA and VRSA are being reported in the treatment of infections caused by MRSA, revealing the difficulty to treat infections and the need for new antibiotics. The need for caution in the use of antibiotics in both human and animal health is emphasized.

In Brazil, researches support that *S. aureus* is widely present in the food production chain and in final products, thus representing a potential risk to public health. However, researches on MRSA in food in certain regions of the country are still scarce, which may be concealing a reality that is different from the one presented here. Finally, it is worth noting that studies on MRSA are still required because of their significance to public health.

Funding information This work was financially supported by grants from CAPES – Programa de Excelência Acadêmica (PROEX) (grant numbers 23038.000795/2018-61).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CAPÍTULO 2

Bacterial diversity in organic and conventional Minas Frescal cheese production using targeted 16S rRNA sequencing

Artigo publicado no periódico “International Dairy Journal”: Abreu, A. C. S.; Carazzolle, M. F.; Crippa, B. L.; Barboza, G. R.; Rall, V. L. M.; Rocha, L. O.; Silva, N. C. C. Bacterial diversity in organic and conventional Minas Frescal cheese production using targeted 16S rRNA sequencing. International Dairy Journal. 2021.

International Dairy Journal 122 (2021) 105139

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Bacterial diversity in organic and conventional Minas Frescal cheese production using targeted 16S rRNA sequencing

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ARTICLE INFO

Article history:
Received 6 December 2020
Received in revised form
6 June 2021
Accepted 8 June 2021
Available online 30 June 2021

ABSTRACT

Molecular identification and the study of bacteria in processed food products can be challenging or suffer limitations when using conventional methods. However, with Next Generation Sequencing (NGS), one can identify bacterial species with a significantly higher detection sensitivity using a 16S rRNA target gene. This study aimed to characterise the bacterial diversity of the organic and conventional production of Minas Frescal cheese, through the NGS analysis of the 16S rRNA gene. DNA was extracted from 96 samples, 48 from organic production and 48 from conventional production. The bacterial families and genera with the highest prevalence were identified. The most prevalent families were Enterobacteriaceae, Planococcaceae and Moraxellaceae. The NGS 16S rRNA tool proved to be very useful for bacterial characterisation, but it is still not possible to say whether a product contains more or less bacteria based on organic production, as there are several factors that contribute to contamination.

1. Introduction

Over the past decades, the search for health foods has increased worldwide (Britwum, Bernard, & Albrecht, 2020; González, Marquès, Nadal, & Domingo, 2019; Katt & Meixner, 2020; Kushwah, Dhir, Sagar, & Gupta, 2019); among health food, organic milk production generates important benefits for the environment, animal welfare and human health (Brito & Silva, 2020; Schwendel et al., 2015). According to the Brazilian Normative Instruction Nº. 46/2011 (Brasil, 2011): to obtain and maintain the health of animals in an organic system, the principle of prevention must be used; such as adequate food (from the production unit itself or from another source under organic management), preferably live in a free regime, practice regular exercise and have access to pasture to promote immune defences.

In the case of diseases or injuries in which the use of natural substances is not having an effect, and for which reason the animal is suffering or is at risk of death, synthetic antibiotic products may be used (Brasil, 2011); as opposed to conventional production where the use of synthetic antibiotics is allowed.

Among the consumed dairy products in Brazil, Minas Frescal cheese is the third most produced type of cheese (Lollo et al., 2015), with both organic and conventional milk used for its production (Cândido et al., 2020). According to Normative Instruction Nº. 4/2004: Minas Frescal cheese is produced by enzymatic coagulation of milk with rennet and/or other suitable coagulating enzymes, complemented or not with the action of specific lactic bacteria; it is an unripened, semi-fat cheese of very high humidity, to be consumed fresh, and which must be stored at temperatures lower than 8.0 °C (Brasil, 2004).

Primarily, Minas Frescal cheese production is comprised of the following stages: raw milk filtration; pasteurisation that can be slow (65 °C, 30 min) or fast (75 °C, 15 s); a coagulation preparation step, which consists of calcium chloride addition (this salt is required for increasing the content of soluble calcium in milk) and rennet to promote milk coagulation, thus converting liquid milk into cheese curd; treatment of the paste after coagulation, which consists of cutting the paste into cubes; stirring the cubes for 1 min and resting for 3 min, and then repeating this cycle for 30 min; hanging, which is necessary to give the cheese its characteristic

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https://doi.org/10.1016/j.idairyj.2021.105139
0958-6946/

shape; salting, both dry salting, which is adding salt to the surface of the paste, or salt application to the paste mixture; packaging into plastic bags; and storage under refrigeration (Silva, 2005).

Raw milk and Minas Frescal cheese both present physicochemical characteristics that contain various nutrients, such as carbohydrates, lipids, proteins, essential amino acids, enzymes, vitamins and minerals; making them an excellent culture media for several microorganisms (Delorme et al., 2020; Prezzi et al., 2020; Verraes et al., 2015).

The identification of microorganisms has always been a challenge for the food industry, especially in the dairy sector, since contamination can occur at different stages of production. Therefore, monitoring the bacterial diversity is important and can provide relevant information regarding product quality (Delorme et al., 2020).

Thus, compared with traditional molecular methods, Next Generation Sequencing (NGS) platforms offer significantly higher detection sensitivity when it comes to studying bacterial communities (Ercolini, 2013; Fuka et al., 2013; O'Sullivan et al., 2015). NGS of the bacterial 16S rRNA amplicons showed that the set of microorganisms can vary depending on the milk source, processing (using raw or pasteurised milk), and with the addition of several ingredients. It is also possible to identify genera that were not previously associated with cheese ecosystems (Prevotella and Helcococcus), and to identify specific microorganisms in certain types of cheese (*Arthrobacter* in goats' milk cheese) (O'Sullivan et al., 2015; Quigley et al., 2012). Based on NGS, the microbial communities of different types of cheese can be characterised. Therefore, it can provide information on the sensory properties of the product, as well as its overall quality. Additionally, one can identify the microbial communities throughout the production facilities (Bokulich & Mills, 2013; O'Sullivan et al., 2015).

Previously, the applicability and advantages of NGS have been outlined for the study of microbial ecology in several food products (De Filippis, Parente, E., & Ercolini, 2017). These included microorganism monitoring during curd fermentation, cheese ripening and investigation of the spatial distribution of microbes in different parts of the same cheese.

Although many studies have investigated the contribution of a single or a few microorganisms in dairy products, information on microbial communities is still lacking. Therefore, understanding the dynamics of the milk microbiota is of great importance to control the qualitative, sensory and biosafety characteristics of dairy products (Lessard, Viel, Boyle, St-Gelais, & Labrie, 2014; Marino et al., 2019; Tilocca et al., 2020).

Based on the lack of information on microbial communities associated with the production of Brazilian Minas Frescal cheese; this research aims to identify and characterise the bacterial diversity in organic and conventional Minas Frescal cheese production, raw material such as raw milk, pasteurised milk, swabs from food handlers and swabs from surfaces in the processing environment, through Next Generation Sequencing of the 16S rRNA gene.

2. Material and methods

2.1. Sample origins

The samples were obtained from three organic (A, B and C) and three conventional dairy producers (D, E and F), in different cities of the state of São Paulo. Sampling was conducted twice in each dairy company, with an interval of about one month. Samples were collected during each visit, from Minas Frescal cheese, pasteurised milk, raw milk, swabs of food handlers, environment and equipment (lira, floor, table, cold chamber, shape mold, drains, boards, tanks). The swabs were chosen randomly during the visual inspection of the dairy; focusing on points of interest for collection and the availability of the equipment and utensils at the time of collection.

It must be noted that due to the unavailability of the producer, it was not possible to carry out the second swab collection of Producer E lacquers, thus extra swab samples from the first E lacquer collection were sequenced, so that the number of swab samples remained uniform at 10 swabs per dairy).

All 6 dairies produce Minas Frescal cheese using whole pasteurised milk, the only exception being in the industries that process organic milk, where the animals are fed using the organic system and hardly use synthetic antibiotics; unlike the production of conventional milk. All Minas Frescal cheeses collected in the present study are produced only through enzymatic coagulation, there are no starter cultures used. Table 1 shows all the sequenced samples.

2.2. Total DNA extraction

DNA was extracted from samples of Minas Frescal cheese; 25 g were diluted in 225 mL of Butterfield's Phosphate Buffer solution, and a 1.8 mL aliquot was subjected to total DNA extraction. For milk samples and swabs (the swabs were inside test tubes with 9 mL of saline), 5 mL was centrifuged at 18,000×*g* for 10 min at room temperature. The supernatant was discarded, and the pellet was resuspended in 1.8 mL buffer solution and subjected to extraction using the PowerFood® Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA was quantified using NanoDrop (Thermo Fisher Scientific, Massachusetts, USA), and stored at −20 °C prior to PCR.

2.3. PCR amplification of 16S rRNA genes

For each DNA sample (n = 96), 5 ng were used as PCR template and a pair of 16S rRNA primers were used to reach the V4 region, producing an amplicon with 358 bp (Choo, Leong, & Rogers, 2015). PCR was conducted using 10 μL of 5 × Buffer (Thermo Fisher Scientific, Massachusetts, USA), 1 μL of 10 mM dNTP (Invitrogen, California, USA), 1 μL of 515F_V4 and 806R_V4 primer (Sigma–Aldrich, Missouri, USA), 1.5 μL of DMSO (Thermo Fisher Scientific), 1 μL Phusion ™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific), 15 μL of DNA and as much ultra-pure Milli-Q water as needed to complete the 50 μL reaction. Cycling conditions consisted of 98 °C for 30 s, 25 cycles of 98 °C for 25 s, 55 °C for 40 s and 72 °C for 1 min, followed by 72 °C for 10 min. A negative control consisting of reagents with ultrapure Milli-Q water was also included in the PCR assay. The amplicons were analysed using agarose gel electrophoresis, purified using Ampure XP beads (Beckman Coulter, Indianapolis, IN) and quantified using NanoDrop.

2.4. Library construction and sequencing

The 16S rRNA gene fragments were indexed using the Nextera XT Kit (Illumina, San Diego, CA, USA) according to the 16S Metagenomic Sequencing Library Preparation Guide (Illumina, 2013). Each PCR index reaction contained 10 μL of 5X Q5 Reaction Buffer (New England Biolabs Inc., Massachusetts, USA), 10 μL of 5X Q5 High GC Enhancer (New England Biolabs Inc.), 1 μL of dNTP 10 mM (Invitrogen, California, USA), 5 μL of Nextera XT Index Primer 1 (N7xx) and Nextera XT Index Primer 2 (S5xx), 0.5 μL of Q5® High-Fidelity DNA Polymerase (New England Biolabs Inc.), 8 μL of DNA (5 ng μL^{-1}), and as much ultra-pure Milli-Q water as needed to complete the 50 μL reaction. The Index PCR was cycled according to the 16S Metagenomic Sequencing Library Preparation Guide, and the libraries were cleaned using Ampure XP beads. The sequencing libraries were quantified using the Qubit (Invitrogen, CA, USA)

Table 1
Origin of collected samples from dairy farmers in São Paulo, Brazil.

Dairy (n = 6)	Collection (n = 12)	Sample (n = 96)
Organic		
A	1 and 2	Raw milk (1) Pasteurised milk (1) Cheese (1) Swabs (5): cold chamber, floor, food handler, lira, table
B	1 and 2	Raw milk (1) Pasteurised milk (1) Cheese (1) Swabs (5): cold chamber, floor, food handler, lira, table
C	1 and 2	Raw milk (1) Pasteurised milk (1) Cheese (1) Swabs (5): cold chamber, floor, food handler, lira, table
Conventional		
D	1 and 2	Raw milk (1) Pasteurised milk (1) Cheese (1) Swabs (5): floor, food handler, lira, shape mold, table
E	1	Raw milk (1) Pasteurised milk (1) Cheese (1)
	2	Raw milk (1) Pasteurised milk (1) Cheese (1) Swabs (10): board, cold chamber boxes, cold chamber floor, drain, food handler, floor processing, shape mold, table, tank
F	1 and 2	Raw milk (1) Pasteurised milk (1) Cheese (1) Swabs (5): cold chamber, floor, food handler, lira, table

method following the manufacturer's instruction, given a value in nanogram per microlitre, and then converted to nanomolar for quantification by qPCR KAPA Library Quantification kits (Kapa Biosystems, Cape Town, South Africa) according to the manufacturer. Finally, a library was prepared and denatured according to the MiSeq denaturation and dilution library guide (Illumina, 2013). The denatured library was subsequently sequenced using the MiSeq v3 reagent cartridge (Illumina) from the Brazilian Bioenewables National Laboratory (LNBR), located at the National Centre for Research in Energy and Materials (CNPEM).

2.5. 16S rDNA amplicon sequence analysis

The 250-bp paired-end FASTQ files were then inspected using FastQC (version 0.11.7) [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/]. Next, the primer adaptor sequences were trimmed out of the reads using Cutadapt (version 1.16) (Martin, 2011); keeping only the read pairs that contained the complete sequences of both the forward primer in the R1 read and the reverse primer in the R2 read.

Amplicon sequence variants (ASVs) and taxonomic inference of the 16S libraries were obtained using the QIIME2 pipeline (Bolyen et al., 2018). Firstly, the DADA2 algorithm (version 1.6.0) (Callahan et al., 2016), was configured to truncated R1 and R2 reads at 210 bp and 170 bp, respectively. Then, reads were filtered to remove the ones with more than 2 expected errors and ambiguous bases. Parameters of the error models were obtained by alternating sample inference with parameter estimation until convergence was achieved. The error models and dereplicated reads pooled from all samples were used as input for the dada function to obtain denoised sequences from R1 and R2 reads. Pairs of R1 and R2 reads with a minimum overlap length of 16 bp and no mismatches were then merged to obtain ASVs. Next, PCR chimeras identified with the consensus method were filtered out. Lastly, taxonomic assignment of the 16S ASVs was performed using the naive Bayesian classifier method. The 16S training dataset consisted of taxonomically assigned sequences from the SILVA database release 132 (https://www.arb-silva.de). Minimum bootstrap confidence was set to 50. Exact matching of 16S ASVs to database sequences was used to assign species to these fragments. The rarefaction curve and alpha diversity were estimated using QIIME2 (diversity core-metrics function) configured for a max-depth of the 4000 pairs of reads. The Shannon index was used to calculate alpha diversity.

2.6. Statistical analysis

Statistical analysis was performed with DESEQ2 (Love, Huber, & Anders, 2014) using the adjusted *p*-value ($padj \leq 0.05$), to compare the Amplicon sequence variants (ASVs) identified. In cases of $padj > 0.05$, *p*-value ≤ 0.05 was considered. Table 2 shows the planning of the statistical experiment used to compare the difference, not only between the organic and conventional, but also between the steps (difference between groups of samples). Thus, one can verify whether there is a statistical difference in bacterial diversity throughout the process, from raw milk to the final product (Minas Frescal cheese).

3. Results and discussion

3.1. Native microbiota assessment in organic and conventional Minas Frescal cheese production

This study evaluated the bacterial diversity of three organic and three conventional Minas Frescal cheese producers in the state of São Paulo, Brazil, including its processing line, food handler swabs, surface swabs, equipment, and samples of raw milk, pasteurised milk and Minas Frescal cheese.

Using NGS data, we were able to verify great bacterial diversity. We identified 51 different bacterial families; 40 in raw milk, 32 in pasteurised milk, 28 in Minas Frescal cheese and 42 from swabs

Table 2
Statistical experimental design for comparison between organic versus conventional products and for comparison between different stages of the production process.

Sample types	Purpose of comparisons
Organic dairy versus Conventional dairy	A comparison was made between the 45 samples of organic production and the 45 samples of conventional production; to verify which families and genera of bacteria could be different between the types of production.
Pasteurised milk versus Raw milk	A comparison was made between the 12 samples of pasteurised milk and the 12 samples of raw milk; to verify whether after the heat treatment the samples had different bacterial diversity.
Cheese versus Pasteurised milk	A comparison was made between the 12 cheese samples and the 12 pasteurised milk samples; to verify whether the final product had bacteria different from the pasteurised milk from which it may have been contaminated during the cheese making process.
Cheeses versus Swabs	A comparison was made between the 12 cheese samples and the 60 swabs (manipulations of food, equipment, industry surfaces); to verify whether there were different bacteria between them, the swabs being the representation of our industrial process, which may contaminate the cheeses.

(Supplementary material Fig. S1). We identified a total of 96 bacterial genera; 61 in raw milk, 41 in pasteurised milk, 39 in Minas Frescal cheese and 72 from swabs (Supplementary material Fig. S2). Figs. 1 and 2 show, respectively, the prevalence of families and genera above 1% of ASV (most prevalent), considering all samples. Figs. 3 and 4, show the prevalence of families and genera in organic and conventional products, respectively. Supplementary material Figs. S1 and S2 show all of the families and genera found in the 96 samples. The rarefaction curve (Supplementary material Fig. S3) shows a plateau indicating that the sequencing depth was sufficient to sample all microorganisms present in the conventional and organic environments. In addition, Fig. 5 shows the comparison of alpha diversity (Shannon index) between conventional and organic environments, revealing no statistically significant differences between them.

Although we identified substantial bacterial diversity in the samples, when comparing the samples of organic and conventional dairy products, there was no significant difference. Enterobacteriaceae was the predominant bacteria family in both organic (44.1%) and conventional (46.9%) Minas Frescal cheese processing lines. This family contains opportunistic pathogens (Purdy, Clark, & Straus, 2010) that can be identified in dairy products (Odenthal, Akineden, & Usleber, 2016), affecting their sensory characteristics (Westling et al., 2016), and producing gas in cheese (Tabla et al., 2016). In addition, many strains may present antibiotic resistance (Tepeli & Demirel Zorba, 2018). Within this family, depending on the class of coliform bacteria, species may be considered an indicator of sanitary hygienic quality (Metz, Sheehan, & Feng, 2020). Moreover, pathogenic bacteria such as some *Escherichia coli* strains, *Salmonella* and *Shigella species* may be also found in Minas Frescal cheese (Ribeiro Júnior et al., 2019b). Fig. 4 shows that *Escherichia-Shigella* were among the five most prevalent genera identified by this study in both organic (2.7%) and conventional (5.6%) products.

The second most prevalent family in organic and conventional dairy products was Planococcaceae, with a prevalence of 15.4% and 13.8%, respectively. Sant'Ana et al. (2019) analysed microbial shifts in artisanal cheese from Minas Gerais and found species of the Planococcaceae family in all samples of cheese rinds from all farms. The authors suggested that the microorganisms in this family may be specific to these cheeses and may play an important role in the ripening processes. In addition, the presence of Planococcaceae suggests that artisanal cheese from Minas is strongly associated with the environment and cheese handling environment. The processing environment forms different functional niches and selects the species that best perform in that environment, all of this occurs regardless of the inoculation (Bokulich & Mills, 2013; Goerges et al., 2008; Mounier et al., 2005; Sant'Anna et al., 2019).

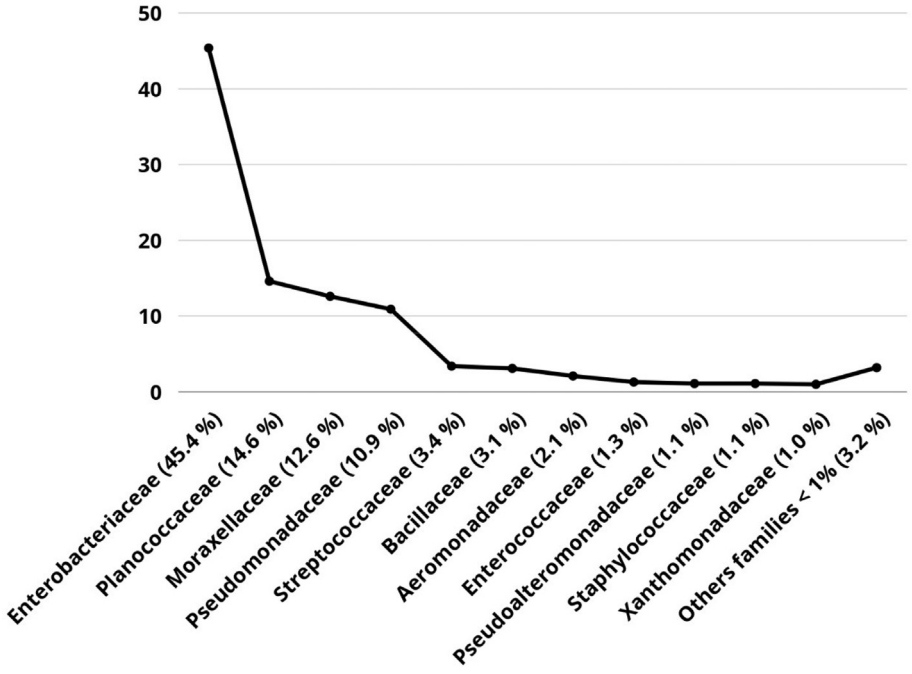

Fig. 1. Percentage of bacterial families above 1% of ASV, found in all of the samples.

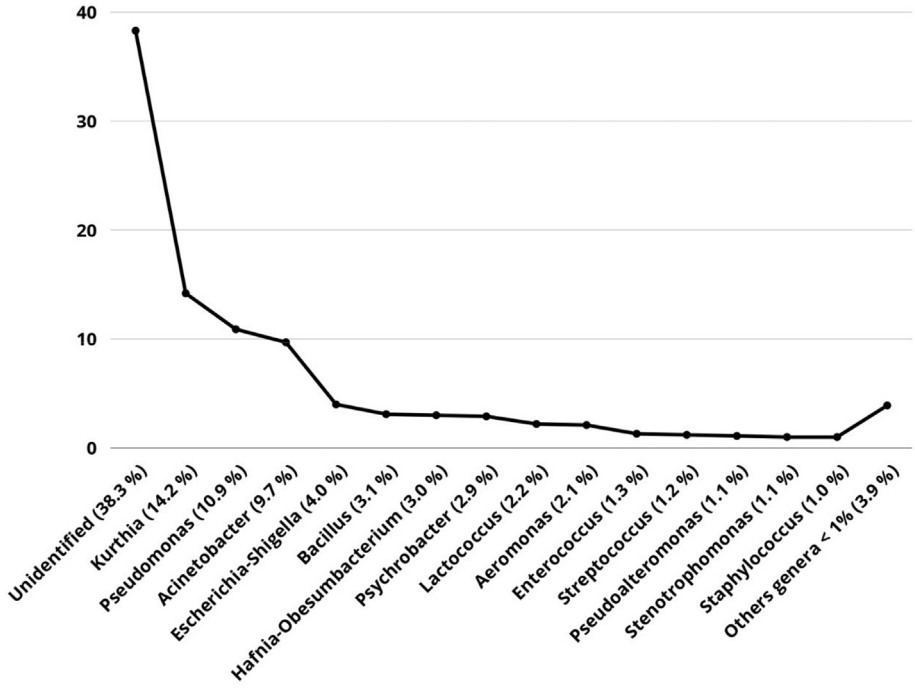

Fig. 2. Percentage of bacterial genera above 1% of ASV, found in all of the samples.

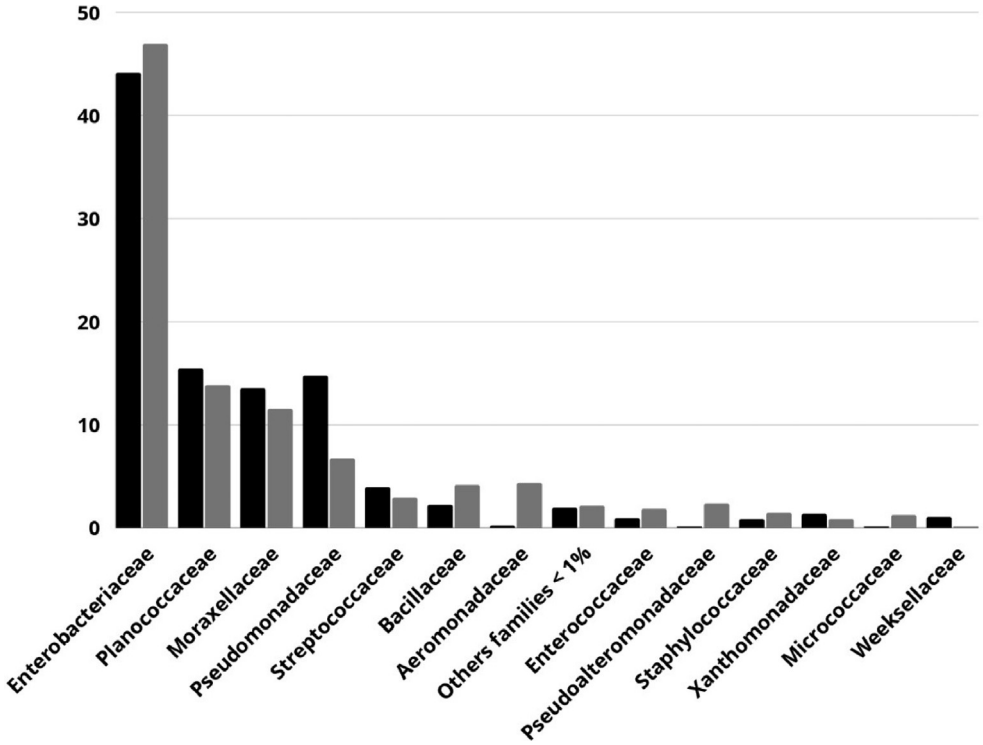

Fig. 3. Prevalence of bacterial families found in organic (■) and conventional (■) dairy products.

The Planococcaceae family is part of the Bacillales order and comprises 14 genera, including *Kurthia* and *Solibacillus* that were found and identified in this study. The members of this family are Gram-variable, spore-forming or non-spore-forming, mobile or non-mobile. In addition, its morphology varies from trichomes, filaments, rods or rod/cocci, or spherical rods (Shivaji, Srinivas, & Reddy, 2014). One of the most prominent genera identified in both organic (15.1%) and conventional (13.2%) products, was *Kurthia*, a psychrotrophic genus (Cardinali et al., 2017; Ribeiro Júnior et al., 2019a; Zheng et al., 2018); although some species were associated with food deterioration at high storage temperatures (Holzapfel, 1992).

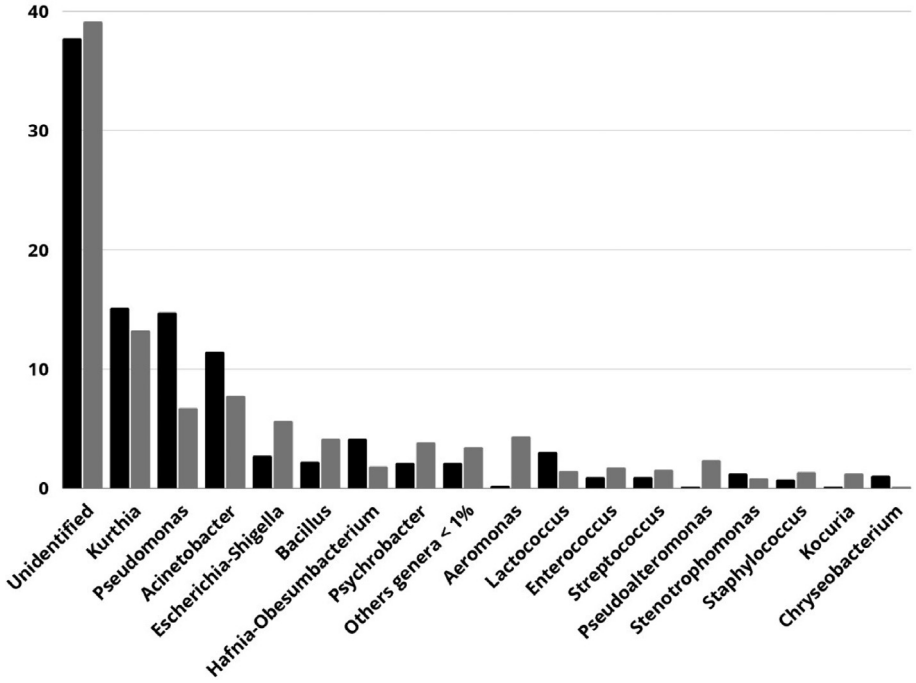

Fig. 4. Prevalence of bacterial genera found in organic (■) and conventional (■) products.

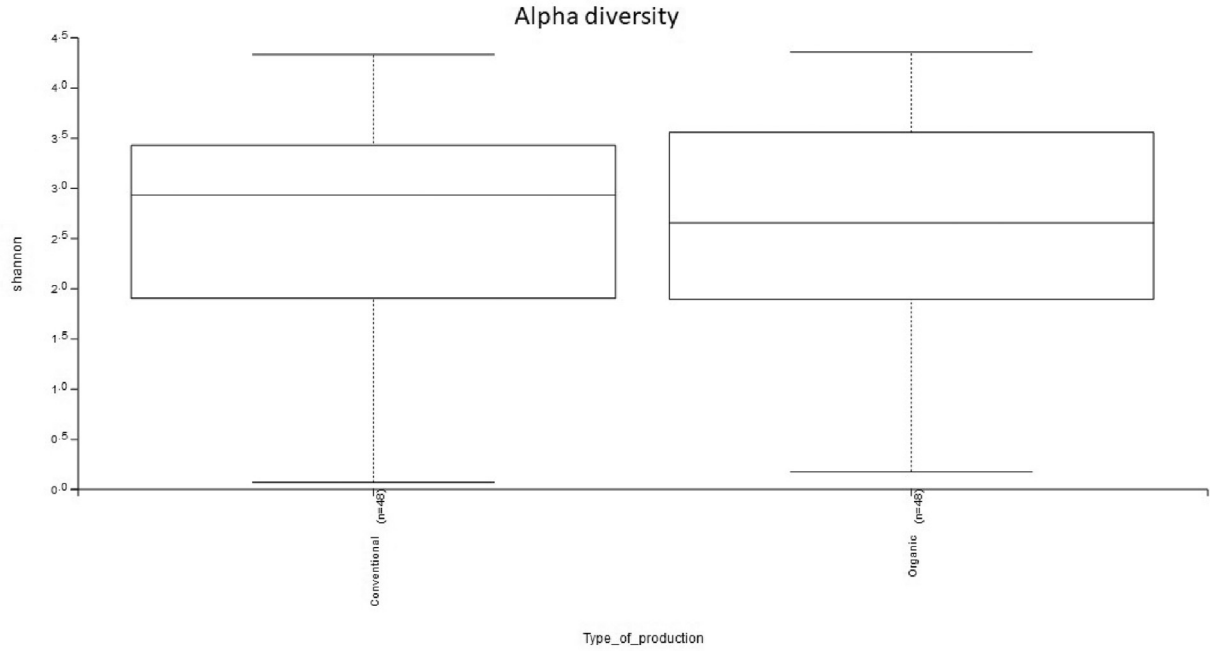

Fig. 5. Alpha diversity (Shannon index) comparing conventional and organic environments. There is no statistically significant difference in the amount of bacteria identified in the two environments.

In this study, Moraxellaceae was the third most prevalent family found in the total samples (12.6%), and when analysing organic and conventional dairy products, this family was present in 13.5% and 11.5%, respectively. Moraxellaceae is a family of microorganisms belonging to the Pseudomonadales order, Gammaproteobacteria class and Proteobacteria phylum. This family includes the genera *Moraxella*, *Acinetobacter* and *Psychrobacter*. In addition to the species *Perlucidibaca piscinae* and *Paraperlucidibaca baekdonensis*, which were described and added in 2008 and 2011, respectively (Yang, 2014). In this study, the *Acinetobacter* genus was the third most prevalent, with 9.7% of presence in the samples. These results corroborate with those determined by Duarte et al. (2020), who evaluated the cheese whey microbiota of four dairy companies located in Northeast Italy in two seasons. They identified *Acinetobacter* as the fourth most abundant genus; as well as a genus highly present in whey samples collected during winter. Microorganisms

belonging to the *Acinetobacter* genus are commonly found as part of the raw milk microbiota both as contaminating agents and as deteriorators in the production of hard cheese (Alessandria et al., 2016; Duarte et al., 2020; Li et al., 2018). This genus includes 78 species (Parte, Sardà Carbasse, Meier-Kolthoff, Reimer, & Göker, 2020), and many of the recognised species have been isolated from human specimens, although some are also ubiquitous in the environment (Yang, 2014). In food, *Acinetobacter* has been isolated from vegetables, fruits, dairy products, meat, and chicken carcasses. They are present in raw milk in large numbers and some strains produce fructose polymers, such as capsular polysaccharides known as levan; which, when accumulated, can cause milk to deteriorate as well as form slime on the surface of soft cheeses or curds (Yang, 2014).

Psychrobacter is another genus belonging to the Moraxellaceae family and was present in 2.9% of the analysed samples. These microorganisms range from psychrophilic to psychrotolerant, halotolerant, aerobic, non-mobile, Gram-negative coccobacilli. Some species are frequently found in milk and cheese and because they do not survive the pasteurisation process, they are considered as deteriorating bacteria in pasteurised milk resulting from post-processing contamination (Cogan et al., 2014; Delcenserie et al., 2014; Monnet et al., 2013; Mounier, Coton, Irlinger, Landaud, & Bonnarme, 2017). In addition, other studies reported significant contribution of these bacteria to the ripening of cheese and its sensory quality; being responsible for the production of aromatic compounds (Deetae et al., 2009; Irlinger et al., 2012; Mounier et al., 2017). Tolerance to high temperatures, high salinity, high concentrations of potassium and magnesium and growth in pH close to neutrality suggest that this genus is well adapted to dairy and cheese environments (Mounier et al., 2017; Rodrigues et al., 2009).

Pseudomonadaceae was the fourth most prevalent family found in the dairy products in this study, occurring in 14.7% of organic and 6.7% of conventional dairy product samples. In our study, the *Pseudomonas* genus belonging to this family was the second most prevalent microorganism in the total analysed samples (10.9%). In the literature, several studies have already shown the presence of *Pseudomonas* in milk and cheeses (Dogan & Boor, 2003; Hammad, 2015; Teider et al., 2019). Bacteria of this genus produce stable lipolytic and proteolytic enzymes after pasteurisation and UHT treatments, thus they can cause deterioration in pasteurised milk during storage under refrigerated conditions. For this reason, they are one of the most important agents of deterioration in dairy products (Simões, Simões, & Vieira, 2010). Contamination by this microorganism can occur through the hands of milkers, the surface of the udders, the milking equipment of cows and also through poorly sanitised refrigerated tanks (Teider et al., 2019; Vidal et al., 2017).

The fifth most prevalent genus was *Bacillus* (3.1%), present in 2.2% of organic samples and 4.1% in conventional dairy production samples. The presence of *Bacillus* spp. is extremely common in dairy products (Molva, Sudagidan, & Okuklu, 2009), and is extremely harmful to the dairy industry; they are spore-forming, can survive during pasteurisation (Molva et al., 2009; Moradi-Khatoonabadi, Ezzatpanah, Maghsoudlou, Khomeiri, & Aminafshar, 2014; Spanu et al., 2016), grow at low temperatures, and produce various enzymes and toxins (Moradi-Khatoonabadi et al., 2014). The presence of *Bacillus* is generally related to the deterioration of cheeses (Tirloni et al., 2020), due to its ability to produce proteases, lipases, and phospholipase; which can produce strange flavours (Molva et al., 2009). In both milk and its derivatives, it breaks down casein into peptides and amino acids, and milk fat into free fatty acids; degrading the quality of dairy products and shortening their shelf life (Molva et al., 2009). *Bacillus* can be pathogenic, causing food poisoning (Spanu et al., 2016; Tirloni et al., 2020) due to some species producing various toxins, including emetic toxin and diarrheal enterotoxins (Molva et al., 2009; Moradi-Khatoonabadi et al., 2014; Spanu et al., 2016).

The genus *Enterococcus* (1.3%) is among the ten most prevalent in the present study, with its presence in around 0.9% of organic dairy products and 1.8% in conventional production. Currently, more than 70 species of *Enterococcus* have been identified (Parte, SardàCarbasse, Meier-Kolthoff, Reimer, & Göker, 2020). *Enterococcus* comprises one of the main genera of the group of lactic acid bacteria (LAB), having an important role in some foods as starter cultures. This is due to the production capacity of proteolytic and lipolytic enzymes and to the production of diacetyl from citrate; positively influencing the ripening of cheeses (İspirli, Demirbaş, & Dertli, 2017; Margalho, van Schalkwijk, Bachmann, & Sant'Ana, 2020; Özkan, Demirci, & Akın, 2021). In addition, these microorganisms may have antimicrobial properties called bacteriocins (enterokinase), which are effective against harmful microorganisms in cheese (Margalho et al., 2020; Vandera et al., 2020; İspirli et al., 2017). The role of *Enterococcus* in cheese and other foods remains controversial because several species comprise of opportunistic pathogenic strains, including several clinical strains (Vandera et al., 2020). Reports of hospital infections (such as endocarditis, bacteraemia and urinary tract infections) due to increased resistance to antibiotics, especially vancomycin and ampicillin (Amarnani & Rapose, 2017; Bartash & Nori, 2017), calls into question the application of *Enterococcus* in the food industry. Therefore, it is extremely important to characterise the *Enterococcus* in organic and conventional dairy products, as they may be bacteria with potential as an emerging pathogen in food.

One of the most important bacteria studied in dairy products is the *Staphylococcus* genus, known to be an important pathogen in dairy products (Bonsaglia et al., 2018; Papadopoulos et al., 2018; Silveira-Filho et al., 2014), in particular in Minas Frescal cheese (Komatsu, Rodrigues, Loreno, & Santos, 2010; Nunes, Souza, Pereira, Aguila, & Paschoalin, 2016; Rodrigues et al., 2017). In the present study, from the total microbiome of the samples there was 1% *Staphylococcus*; 0.8% in organic samples and 1.4% in conventional production samples. In another study published by our research group using the same samples, the search for *Staphylococcus* species present in the samples was carried out, and it was possible to identify 14 different species (Cândido et al., 2020). It was also possible to identify countless genes for the production of enterotoxins, biofilm formation, and resistance to antibiotics and sanitisers (Abreu et al., 2021; Cândido et al., 2020). Indicating that the genus identified in the present research has virulent potential.

Staphylococcus spp. can be pathogens capable of causing various diseases (Lin et al., 2016; Nyman, Fasth, & Waller, 2018; Silva, Rodrigues, & Silva, 2020), mainly food poisoning in humans (Krukowski et al., 2020; Schmid et al., 2009; Solano et al., 2013); and their presence in dairy products can often arise either from raw material or from cross-contamination with food handlers (Alhashimi, Ahmed, & Mustafa, 2017; André et al., 2008) and are present regardless of whether the product is organic or not (Mullen, Sparks, Lyman, Washburn, & Anderson, 2013; Tenhagen et al., 2018). Emphasising that in both production systems hygiene measures must be taken at all stages of production, to avoid contamination by pathogenic microorganisms.

3.2. Significant statistical difference observed between samples

We compared the results of the 48 organic dairy samples with the 48 conventional dairy samples; to determine whether the products and microbiota of organic and conventional products differ significantly. Stages of Minas Frescal cheese production were also compared to find a possible point of contamination; therefore,

we compared raw milk with pasteurised milk, then pasteurised milk to the final Minas Frescal cheese to determine whether there was a difference in bacterial diversity between stages. We also compared swabs (manipulators, utensils and surfaces) with the final Minas Frescal cheese to determine if contamination occurred during the preparation process. This type of comparison was performed to verify which process stage would have a greater bacterial diversity indicating significant potential for contamination.

Table 3 shows the bacteria with statistically significant difference for both families and genus. For a better explanation of Table 3 and its statistical results, we can evaluate the condition "Organic Dairy versus Conventional Dairy"; in which the family Pseudoalteromonadaceae had a negative value (−3.86), which means that a smaller amount of this family is found in the first condition (Organic Dairy) compared with the second condition (Conventional Dairy). Another example is the condition "Cheese versus Pasteurised Milk"; in which the *Pantoea* genus had the positive value (22.58), which means that a greater amount of this genus was found in the first condition (Cheese) compared with the second condition (Pasteurised Milk).

The only genus resulting in a statistically significant difference under the "Organic Dairy versus Conventional Dairy" comparison was *Pseudoalteromonas*; a bacteria previously classified as *Alteromonas*, and often associated with eukaryotic hosts (Alikunhi, Batang, AlJahdali, Aziz, & Al-Suwailem, 2017; Sánchez-Díaz et al., 2019). This species is characterised by a flagella, Gram-negative, heterotrophic, aerobic, and non-spore-forming. It can produce antibacterial compounds and antibiotics in addition to a lethal toxin, tetrodotoxin; however, no species of *Pseudoalteromonas* has been described as a human pathogen (Alikunhi et al., 2017; Sánchez-Díaz et al., 2019).

Regarding the statistical analysis between "Organic dairy versus Conventional dairy", as far as we know, the consulted literature presents few studies comparing the microbiological characteristics between organic versus conventional food (Cicconi-Hogan et al, 2013, 2014; Mullen et al., 2013; Tenhagen et al., 2018). Despite this, it is difficult to relate bacterial differences between these products, as well as the variables involved in many processes that will determine their microbiological characteristics; such as bacterial contamination, which may come from milking equipment, bulk tank milk (Latorre et al., 2010), water (Leriche et al., 2004) or raw milk (Metz et al., 2020). Due to these variables, it is very difficult to determine if the microbiological difference is a result of the food being organic or not.

The comparison between "Pasteurised milk versus Raw milk", identified a greater amount of the genus *Bacillus*, and its respective family Bacillaceae stands out in pasteurised milk more than in raw milk. The genus *Bacillus* consists of Gram-positive, rod-shaped, motile and sporulated bacteria, present in several environments but mainly in the soil (Foysal & Lisa, 2018). According to Ostrov, Sela, Belausov, Steinberg, and Shemesh (2019), the *Bacillus* genus is often related to the contamination of dairy products and because they are biofilm producers, they represent a great challenge in the area of food quality and safety. According to the Centers for Disease Control and Prevention (CDC, 2019), this genus was responsible for 763 food poisoning outbreaks in the US; leading to 133 hospitalisations and 5 deaths in the past 20 years. *Bacillus cereus* is among the eight most identified species causing outbreaks in Brazil between 2009 and 2018 according to Health Surveillance Secretariat (SVS, 2019).

As shown, a higher amount of *Bacillus* was found in pasteurised milk compared with raw milk; theoretically the milk that undergoes pasteurisation should have a smaller amount of bacteria, but according to Kmiha et al. (2017), the *Bacillus* genus is an important contaminant in milk due to heat resistant spores being able to survive the thermal treatment of milk and deteriorate the final product. This may explain why we had more bacilli in pasteurised milk than in raw milk, it is possible that they germinated and subsequently multiplied after pasteurisation.

The cheeses were also compared with pasteurised milk, and we observed a higher concentration of the *Pantoea*, *Pseudomonas* and *Aeromonas* genera and the families Pseudomonadaceae and

Table 3
Statistically significant differences (*padj* ≤ 0.05) of families and bacterial genera identified in the types of production and samples.[a]

Condition	Family			Genera		
	(log2FoldChange)	*p*-value (≤0.05	*padj* (≤0.05)	(log2FoldChange)	*p*-value (≤0.05)	*padj* (≤0.05)
Organic dairy versus Conventional dairy	Pseudoalteromonadaceae (−3.86)	6.15×10^{-4}	2.58×10^{-2}	*Pseudoalteromonas* (−3.44)	5.30×10^{-3}	3.29×10^{-1}
Pasteurised milk versus Raw milk	Bacillaceae (22.09)	6.51×10^{-14}	2.41×10^{-12}	*Bacillus* (22.37)	3.10×10^{-14}	1.64×10^{-12}
Cheese versus Pasteurised milk	*	*	*	*Pantoea* (22.58)	1.84×10^{-14}	8.45×10^{-13}
	Pseudomonadaceae (4.55)	2.31×10^{-4}	7.39×10^{-3}	*Pseudomonas* (4.15)	9.85×10^{-4}	2.27×10^{-2}
	Aeromonadaceae (5.96)	3.37×10^{-3}	5.39×10^{-2}	*Aeromonas* (5.56)	7.24×10^{-3}	8.33×10^{-2}
Cheeses versus Swabs	*	*	*	*Macrococcus* (4.90)	3.48×10^{-16}	2.36×10^{-14}
				Staphylococcus (−3.74)	2.96×10^{-5}	4.02×10^{-4}
				Sphingobacterium (1.40)	2.89×10^{-4}	2.62×10^{-3}
				Pantoea (2.67)	3.08×10^{-4}	2.62×10^{-3}
				Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium (1.31)	6.71×10^{-4}	5.07×10^{-3}
				Morganella (−2.54)	1.14×10^{-3}	7.75×10^{-3}
				Propioniciclava (1.16)	2.58×10^{-3}	1.35×10^{-2}
				Enhydrobacter (1.05)	6.53×10^{-3}	2.96×10^{-2}
				Solibacillus (−1.87)	9.63×10^{-3}	4.09×10^{-2}
	Vibrionaceae (2.90)	2.22×10^{-10}	1.22×10^{-8}	*Vibrio* (2.90)	7.01×10^{-10}	1.59×10^{-8}
	Weeksellaceae (3.45)	3.22×10^{-8}	8.86×10^{-7}	*Chryseobacterium* (3.83)	1.51×10^{-13}	5.15×10^{-12}
				Elizabethkingia (1.53)	4.49×10^{-5}	5.09×10^{-4}
	Pseudoalteromonadaceae (3.50)	1.11×10^{-5}	2.04×10^{-4}	*Pseudoalteromonas* (3.49)	1.35×10^{-5}	2.29×10^{-4}
	Dermacoccaceae (1.14)	1.37×10^{-3}	1.88×10^{-2}	*Dermacoccus* (1.16)	2.53×10^{-3}	1.35×10^{-2}
	Aeromonadaceae (2.60)	2.32×10^{-3}	2.56×10^{-2}	*Aeromonas* (2.63)	2.04×10^{-3}	1.26×10^{-2}

[a] The log2FoldChange: when positive, indicates a greater amount of that family and/or genus of bacteria in the first versus the second condition. When the value of log2FoldChange is negative (−), it represents less quantity in the first versus the second condition. An asterisk (*) indicates that there are no families statistically different to the corresponding genera.

Aeromonadaceae in the Minas Frescal cheese. *Pseudomonas* spp. are environmental Gram-negative rods, belonging to the Pseudomonadaceae family; and many species are opportunistic human pathogens (Kačániová et al., 2017). The genus *Pantoea* consists of species from different geographical and ecological origins, which are abundantly distributed in nature and have been found in soil, water, and different types of food (Cooney, O'Brien, Iversen, & Fanning, 2014).

Regarding *Aeromonas*, it is a facultative oxidase positive anaerobic bacteria, depending on the physiological and host properties, they are divided into two large groups. The first includes mobile *Aeromonas*, with some species capable of causing infections in humans. The other group consists of non-motile species, causing some diseases in aquatic animals. *Aeromonas* has been isolated from a wide variety of foods, such as fish, mussels, shrimps, meat, meat products, vegetables, and also milk, and it is present in 4% of dairy products (Stratev & Odeyemi, 2016).

Finally, a comparison between cheeses and swabs identified a greater presence of the genera *Macrococcus*, *Chryseobacterium*, *Vibrio*, *Pseudoalteromonas*, *Elizabethkingia*, *Sphingobacterium*, *Pantoea*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Aeromonas*, *Dermacoccus*, *Propioniciclava* and *Enhydrobacter* in cheeses compared with swabs; and a smaller amount of the genera *Staphylococcus*, *Morganella*, *Solibacillus* in cheeses compared with swabs. Regarding families, we found a higher amount of Vibrionaceae, Weeksellaceae, Pseudoalteromonadaceae, Dermacoccaceae and Aeromonadaceae present in cheeses.

The swabs analysed come from food handlers, equipment and surfaces in the industry; showing a great bacterial diversity with statistical difference compared with the cheeses produced. According to André et al. (2008), food handlers, milking equipment, and the environment are possible sources of contamination. Food handlers have an important role in the contamination of processed foods and can be asymptomatic carriers of some bacteria, causing foodborne diseases (Walker, Pritchard, & Forsythe, 2003). Therefore, it is extremely important to characterise the bacterial diversity found in swab samples in the food industry.

In this study, the Vibrionaceae family and the respective *Vibrio* genus were found in greater quantity in cheeses than in swabs. In the study conducted by Quigley et al. (2013) they also used the V4 region of the 16S rRNA gene in samples of raw and pasteurised cows' milk, and found the Vibrionaceae family in their samples. This family is composed of Gram-negative bacteria, most species prefer coastal environments and grow in the presence of salt, thus they might be found in Minas Frescal cheese, which usually has salt in its composition (Prezzi et al., 2020; Rocha et al., 2020). Some species of *Vibrio* can cause several infections, epidemics and pandemics of acute diarrhoea (Harris, LaRocque, Qadri, Ryan, & Calderwood, 2012).

The Weeksellaceae family was identified in greater quantities in cheeses than in swabs; this type of bacterial family present in the dairy sector corroborates data from Ramirez−Ramirez et al. (2016) who used the sequencing of the 16S rRNA gene in samples of dairy cows and identified the family. Gil-Pulido et al. (2018) also found the same family by sequencing samples of wastewater from dairy plants, indicating that it may become a frequent family in the dairy sector.

The *Macrococcus* genus was also present in greater quantity in cheeses compared with swabs, with 4.90 log 2. FoldChange. This genus is composed of 11 Gram-positive species (Parte et al., 2020). Despite being considered an avirulent, species of this genus have recently showed resistance genes to methicillin (Brawand et al., 2017; Mazhar, Hill, & McAuliffe, 2018). *M. caseolyticus* has been documented as a microorganism present in the cheese microbiota, in addition, its role in ripening and flavour development has also been described (Bhowmik & Marth, 1990; Mazhar et al., 2018). Moreno and Kosikowski (1973) described the protease and peptidase activity of *M. caseolyticus* isolates, leading to the production of short amino acids, and suggests that this microorganism could produce compounds responsible for the flavour of the cheeses during their ripening (Mazhar et al., 2018).

The *Staphylococcus* genus is part of the surface of human skin, hands, nasal mucosa, throat, eyes and gastrointestinal tract, so it can be found on poorly sanitised surfaces and hands from manipulators (Cândido et al., 2020; Hennekinne, De Buyser, & Dragacci, 2011). This explains a greater number of *Staphylococcus* in manipulators, utensils and surfaces analysed in this study when compared with cheese.

The genus *Solibacillus* is considered a faecal contaminant and recent studies have suggested that contamination of the skin of the teat or of environmental sources may occur, and that this tends to interfere in the microbiological analysis and bacteriological results based on PCR (Catozzi et al., 2017; Hiitiö et al., 2016). The fact that this microorganism is present in greater quantities in the swabs compared with cheese can be justified by contamination coming from the raw material, through the manipulators and by the failure in hygiene. Furthermore, as already mentioned, the presence of microorganisms of the Planococcaceae family, in which *Solibacillus* belongs, may suggest that these microorganisms are strongly associated with the environment and the management of cheese; because the processing environment forms different functional niches and selects the species that perform best in that environment.

Similar to our results, Dugat-Bony et al. (2016) analysed the microbial diversity of 12 popular French cheese variations and also found species of the genera *Morganella*, *Sphingobacterium* and *Pantoea*. The occurrence analysis revealed the presence of generalised taxa as well as operational taxonomic units (OTUs) specific to one or more varieties of cheese. The technological processes associated with the manufacturing environment form the microbiological and chemical composition of the cheeses and, as a consequence, provide the sensory characteristics that express the personality and the unique identity of each cheese (Donnelly, 2013; Dugat-Bony et al., 2016).

The *Dermacoccus* genus was present in greater quantity in Minas Frescal cheese when compared with swabs. The Micrococcaceae and Dermacoccaceae were classified in the suborder Micrococcineae, order Actinomycetales and class Actinobacteria. *Dermacoccus* species are universally found in the environment, and in the skin and mucous membranes of humans and animals. In addition, Micrococcineae species can be found in food, and the performance of these species as flavour and odour enhancers in cheese production has been described (Becker & Peters, 2009).

The characterisation of bacterial diversity by the 16S rRNA NGS data is still a new field to be researched and explored around food studies. However, its reputation has increased and its use has become accessible not only for research, but for industry (Cocolin et al., 2018; Jagadeesan et al., 2019; Kergourlay, Taminiau, Daube, & Champomier Vergès, 2015).

In the sequencing of the V4 region of the 16S rRNA gene conducted by Quigley et al. (2013) the samples of raw and pasteurised milk contained bacteria that are not normally considered common in those samples. Indicating the importance of bacteria present at sub-dominant levels, or that cannot be easily grown in the laboratory. That is why analyses of bacterial diversity result in such a different amount of bacteria in a given food sample (Quigley et al., 2013).

Due to the fact that conventional microbiology methods cannot identify all microorganisms present in a sample, the metagenomics of the 16S gene ends up presenting more complete data regarding

the microbiological characteristics of a given food (Ercolini, 2013; Fuka et al., 2013; O'Sullivan et al., 2015; Parente, Ricciardi, & Zotta, 2020) But still this classic method of sequencing the 16S gene is not possible to identify the DNA difference of living or dead bacteria (Bengtsson-Palme, 2017; Parente et al., 2020). DNA can persist in the environment, resulting in extracellular DNA and dead cell DNA that is indistinguishable from DNA that represents living cells (Emerson et al., 2017).

But the ability of metagenomics to identify many microorganisms in several samples at the same time is very important for scientific research, helping to fill in gaps regarding microbiological characteristics, from which it can provide guidance regarding food security (Bengtsson-Palme, 2017). Remembering also that there are microorganisms that may be viable in food, but it does not mean that it is cultivable by conventional method of classical microbiology, it is estimated that around 99% of microorganisms are not cultivable (Emerson et al., 2017).

Recently, complementary sequencing methods have been sought to distinguish living and dead cells, sequencing compatibility such as: additional live/dead techniques, including many dyes and stains, cellular energy (adenosine 5′-triphosphate, ATP), metaproteomics, isotope probing, measurements of membrane potential and respiratory activity, measurements of heat flow, flow cytometry, quantitative PCR (qPCR) and digital PCR (Emerson et al., 2017) and Viability PCR method (vPCR) (Agustí, Fittipaldi, & Codony, 2017).

Therefore, despite the advantages and limitations of the method, in general the metagenomics of the 16S rRNA gene is a promising analysis under development in the area of food microbiology (Gill, 2017), especially in the area of dairy products. The data collected here are extremely important to direct which microorganisms we can find in Minas Frescal cheese and finally come to study the best tools to prevent possible contamination. It is relevant to think that the DNA found in the samples are from bacteria that at some point were already in that food, producing toxins and enzymes, which can cause disease or deterioration in the products. These data are of extraordinary relevance to understand the characteristics of the product and sometimes even track any problem or defect in it.

4. Conclusion

It was possible to identify a large taxonomic variety of families and genera in samples of organic and conventional dairy products, from raw milk to the final product, Minas Frescal cheese. This study provided a broader view of the bacterial diversity of dairy products. Thus, the NGS analysis of the 16S rRNA gene allows a complex and complete analysis of the bacterial diversity of the sample. It can help to point out new pathogenic or deteriorating bacteria in products due to its ability to identify many bacteria that are considered non-cultivable by traditional methods. The NGS 16S rRNA tool proved to be very useful for bacterial characterisation, but it is still not possible to say whether a product has more or less bacteria just because it is organic; given that there are several factors that contribute to food contamination.

Funding

This study was funded by the São Paulo Research Foundation (FAPESP) [grant numbers 2016/02118-7 and 2018/00986-7]; Programa de Excelência Acadêmica (PROEX) [grant numbers 23038.000795/2018−61]; and Coordenação de Aperfeiçoamento Pessoal de Nível Superior − Brasil (CAPES) - Finance Code 001. The authors thank Espaço da Escrita − Pró-Reitoria de Pesquisa − UNICAMP - for the language services provided. This research used resources from Brazilian Biorenewables National Laboratory (LNBR), an open national facility operated by the Brazilian Center for Research in Energy and Materials (CNPEM) for the Brazilian Ministry of Science, Technology, Innovations and Communications (MCTIC). The LNBR staff is acknowledged for the assistance during the experiments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2021.105139.

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CAPÍTULO 3

Antimicrobial resistance of *Staphylococcus* spp. isolated from organic and conventional Minas Frescal cheese producers in the state of São Paulo – Brazil

Artigo publicado no periódico “Journal of Dairy Science”: Abreu, A. C. S.; Matos, L. G.; Cândido, T. J. S.; Barboza, G. R.; Souza, V. V. M. A.; Nuñez, K. V. M.; Silva, N. C. C. Antimicrobial resistance of *Staphylococcus* spp. isolated from organic and conventional Minas Frescal cheese producers in the state of São Paulo – Brazil. Journal of Dairy Science. 2021.

J. Dairy Sci. 104:4012–4022
https://doi.org/10.3168/jds.2020-19338

Antimicrobial resistance of *Staphylococcus* spp. isolated from organic and conventional Minas Frescal cheese producers in São Paulo, Brazil

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ABSTRACT

The genus *Staphylococcus* is recognized worldwide as a cause of bacterial infections in humans and animals. Antibiotics used in dairy cattle combined with ineffective control can increase antimicrobial resistance. The objective of this study was to characterize 95 *Staphylococcus* strains isolated from organic and conventional Minas Frescal cheese production regarding antibiotic resistance (phenotype and genotype), presence of sanitizer-resistant genes and biofilm-formation genes, and SCC*mec* typing. Most strains (25.3%) showed higher resistance to penicillin, followed by oxacillin (21.1%) and clindamycin (11.6%). Among antibiotic resistance genes, the most prevalent were *blaZ* (25.3%), *mecA* (13.7%), *lsaB* (6.3%), *msrA* (4.2%), *ant4* (3.2%), and *tetM* (2.1%); among sanitizer-resistance genes they were *qacA/B* (5.3%) and *qacC* (6.3%); and among biofilm, *bap* (4.2%), *icaA* (29.5%), *icaD* (41.1%). However, there was no statistically significant difference between organic and conventional dairy products, possibly due to the lack of synthetic antibiotic use on conventional farms during the sample collection period. Methicillin-resistant *Staphylococcus aureus* (MRSA) had their SCC*mec* identified as types I and IVc, and the methicillin-resistant coagulase-negative staphylococci had nontypeable SCC*mec*. These results suggest that there are antibiotic-resistant strains in both organic and conventional Minas Frescal cheese production in the state of São Paulo, Brazil. This supports the idea that improved quality control is needed from the milking stage up to the final product.

Key words: antibiotic, biofilm, sanitizer, quaternary ammonium compound

INTRODUCTION

Mastitis in dairy cattle is one of the most important diseases worldwide, generating the most economic damage to the milk production chain due to reduced production and disposal, premature slaughter, veterinary treatment costs, use of antimicrobials, and altered milk quality (Halasa et al., 2007; Haran et al., 2012). Among the various pathogens that cause bovine mastitis, *Staphylococcus aureus* stands out for its pathogenicity, contagious profile, persistent infection, and low cure rates associated with current therapies (Rainard et al., 2018). In addition, *S. aureus* is one of the most frequently isolated pathogens from mastitis cases worldwide (Rainard et al., 2018), and it is associated with the greatest economic losses among all pathogens that cause mastitis (Halasa et al., 2009). Currently, despite the importance of *S. aureus*, there is growing concern about CNS, as pathogenic species of this group are also frequently isolated from mastitis cases (Frey et al., 2013; De Visscher et al., 2015; Nyman et al., 2018). Among the CNS species that cause mastitis, the most common are *Staphylococcus chromogenes*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus sciuri*, and *Staphylococcus xylosus* (Frey et al., 2013; Vanderhaeghen et al., 2015; Mahmmod et al., 2018).

Staphylococcus is also a major cause of human infections. They can be found as symbionts in the skin, glands, and mucous membranes, but can become pathogenic-causing diseases such as boils, cellulite and impetigo, bacteremia, pneumonia, osteomyelitis, encephalitis, meningitis, chorioamnionitis, scalded skin syndrome, muscle abscesses, and intra-abdominal and urogenital tract infections. *Staphylococcus aureus* has long been considered the most dangerous species for expressing various mechanisms of pathogens (Fontana and Favaro, 2018); however, CNS have aroused interest in recent years as potential causes of nosocomial infections (Martínez-Meléndez et al., 2015).

Methicillin-resistant *Staphylococcus aureus* (**MRSA**) are the main human pathogens that exhibit greater

Received July 22, 2020.
Accepted November 2, 2020.
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virulence and resistance to different antibiotics. Causing numerous infections, from mild to fatal conditions (Wang et al., 2010; Barcudi et al., 2020), MRSA can be further subdivided into community-associated MRSA and healthcare-associated MRSA (Wang et al., 2010; Yanagihara et al., 2012). It is noteworthy that CNS can also become resistant to methicillin (Martínez-Meléndez et al., 2015). Therefore, all staphylococci can be of concern in both community-acquired and nosocomial infections (Fontana and Favaro, 2018).

Currently, the prevalence of antibiotic-resistant pathogens has increased and the approval of new drugs has decreased, posing a growing challenge to public health. However, the use of antibiotics in farm animals is significant and has been discussed for selecting resistant microorganisms, with the animals acting as reservoirs for resistant pathogens and the ability to transmit them through consumption and food products (Nordstrom et al., 2013; FDA, 2020).

The use of antimicrobials in food production has contributed to the emergence of resistant bacteria, indicating that these bacteria harbor resistance genes and can therefore contaminate food, animals, and humans, and pass their genes on to other bacteria. Methicillin-resistant *Staphylococcus aureus* stands out among antibiotic-resistant bacteria. It is currently endemic in many hospitals worldwide and has also emerged in the community, having been identified in food-producing animals and people in contact with them (Kluytmans, 2010).

Methicillin-resistant *Staphylococcus aureus* becomes resistant to β-lactam antibiotics due to the presence of the *mec* gene (*mecA*, *mecB*, and *mecC*) that encodes a new specific penicillin-binding protein (PBP2a; Liu et al., 2016). The *mecA* gene encodes the penicillin-binding protein (PBP2a or PBP2), thus enabling methicillin resistance (Wang et al., 2015). These binding proteins decrease the ability of β-lactam antibiotics to act on bacteria (Wan and Chou, 2014).

The SCC*mec* element carries *mecA*, *mecB*, and *mecC*, along with the genes that control its expression [*mecR1* (encoding the MecR1 protein-transducing signal) and *MecI* (encoding MecI-repressor protein)] and acts as a carrier for the exchange of genetic information among *Staphylococcus* strains (Liu et al., 2016). In addition, SCC*mec* carries site-specific recombinases called cassette chromosome recombinases (*ccr*), responsible for the mobility of each element. In *S. aureus*, 3 types of *ccr* genes (*ccrA*, *ccrB*, and *ccrC*) have already been identified (Doulgeraki et al., 2017). Currently, 11 types of SCC*mec* have been described (IWG-SCC, 2014).

In addition to the abovementioned methicillin resistance, CNS can also become resistant to various antibiotics, such as tetracycline, tobramycin, and gentamicin, among others (Silva et al., 2014). Multiresistance in CNS is a common concern (Nam et al., 2010; Virdis et al., 2010), and multiresistant CNS can act as reservoirs of antimicrobial-resistant genes that can be transferred to other microorganisms such as *S. aureus* (Archer and Climo, 1994).

Bacteria evolve different ways to avoid toxic effects. In addition to their ability to develop resistance to antibiotics, they may acquire resistance to chemicals and disinfectants (Shamsudin et al., 2012). Quaternary ammonium compounds (**QAC**) are known for their ability to disrupt the cell membranes of microorganisms and are considered to have efficient antimicrobial effects (Zhang et al., 2018). The bacteria's defense mechanism is related to the efflux pumps (Minbiole et al., 2016), which are encoded by plasmids (Shamsudin et al., 2012).

Poor hygiene in the food industry facilitates product contamination through pathogenic microorganisms, which pose a danger to consumers' health. Eliminating microorganisms in the food industry is difficult. It is extremely common in nature for bacteria to produce biofilms. They first fix their cells on a surface and then begin to multiply by secreting an array of extracellular polymeric substances (Gutiérrez et al., 2012). The ability of bacteria to form biofilms on food-contact surfaces is an aggravating factor, as they survive even after cleaning and disinfection processes due to the protection provided by the biofilm (Yang et al., 2012).

Biofilm-formation genes are responsible for the production of intercellular adhesion polysaccharide, and may also decrease the efficiency of disinfectants, increasing cases of food contamination and causing financial losses to the food industry (Kroning et al., 2016). Biofilms also have the capacity to reduce susceptibility to antibiotics due to the numerous mechanisms involved (Mah and O'Toole, 2001). *Bap*, *icaA*, and *icaD* are some of the genes found in *Staphylococcus* responsible for the formation of biofilms (Kroning et al., 2016).

Staphylococcus aureus has been considered an important foodborne pathogen because of its potential to produce enterotoxins (under specific conditions). The other members of the genus *Staphylococcus* have also drawn attention, especially due their abilities to carry resistance genes (Podkowik et al., 2013; Ruaro et al., 2013). Foodborne diseases occur worldwide and affect a large portion of the population, causing a global concern among health agencies (Kim et al., 2015). Numerous pathogenic bacteria are found in the environment, water, and soil, with potential to transmit diseases to humans through food. Annually, millions of people suffer from foodborne illnesses after consuming contaminated food (Rubab et al., 2018). Among the many foods that may become sources of food poisoning

is Minas Frescal cheese, a typical Brazilian fresh cheese highly consumed in Brazil (Casaes Nunes et al., 2016). Contamination of Minas Frescal by various staphylococci has been reported frequently (Casaes Nunes et al., 2016; Nunes and Caldas, 2017; Rodrigues et al., 2017; Cândido et al., 2020).

According to Normative Instruction No. 4 (BRASIL, 2004), Minas Frescal cheese is defined as a fresh cheese obtained through enzymatic coagulation of milk with rennet or other coagulating enzymes. In some cases, lactic bacteria are also used. Minas Frescal cheese is classified as a semifat cheese of very high humidity that is eaten fresh. The milk used in the production of this type of cheese must be sanitized by mechanical means and subjected to pasteurization (BRASIL, 2004).

In recent years, the demand for products of organic origin has increased, especially organic milk. Organic dairy products are produced without the use of antibiotics, hormones, synthetic chemicals, or genetic modification, and can have benefits for human health (Schwendel et al., 2015). Thus far, many studies of organic and conventional food have had misleading results, as there are numerous factors that can influence their composition (Schwendel et al., 2015). Regarding organic production in Brazil, according to Normative Instruction No. 46 (BRASIL, 2011), the use of products from genetically modified organisms (with the exception of mandatory vaccines), artificial chemosynthetics, and hormones for therapeutic purposes are allowed in the case of diseases or injuries in which the use of permitted substances (nonsynthetic) does not have an effect, and if the animal is suffering or is at risk of death. When using artificial chemosynthetic products, the waiting period for the animal to be milked should be twice the period determined by the chemosynthetics manufacturer, at least 96 h.

The objective of this study was to characterize *Staphylococcus* spp. isolates in organic and conventional Minas Frescal cheese production in the state of São Paulo, Brazil regarding antibiotic resistance, sanitizer-resistance genes, and biofilm production, and to perform SCC*mec* typing of the MRSA samples found to observe the microbiological differences between the 2 cheese production methods.

MATERIALS AND METHODS

Origin of Isolates and Dairy Descriptions

The 95 strains of *Staphylococcus* spp. used in this study were isolated and identified in a previous study (Cândido et al., 2020). The samples were obtained from 3 organic producers (A, B, and C) and 3 conventional producers (D, E, and F) of Minas Frescal cheese located in different cities of the state of São Paulo within a radius of up to 200 km from the city of Campinas. Two collections were performed for each dairy producer. The following collections were performed on each visit: Minas Frescal cheese samples, pasteurized milk, raw milk, and swabs (food handler, environment, equipment, and utensils).

All 6 dairies produced an average of 1,000 L of milk/day. All 3 organic dairy farms produced their own milk for the production of Minas Frescal cheese. Dairy D (conventional) also had its own production; only dairies E and F received milk from several conventional dairy farms. None of the 3 organic milk farms used synthetic antibiotics, unless in extreme cases when natural products have no effect, and the conventional producers made use of antibiotics, but only when needed.

All strains were verified through biochemical analysis (coagulase, catalase, and Gram stain), and by PCR of the *coa* and *nuc* genes for *S. aureus*, and the *sodA* gene sequencing for the remaining *Staphylococcus* that were not confirmed by *coa* and *nuc* genes. All strains were previously analyzed for classical and nonclassical enterotoxins (SEA-SEE, SHE-SEJ) and typed by pulsed-field gel electrophoresis, *spa* type, and *agr* type (Cândido et al., 2020). Table 1 shows all 95 strains of *Staphylococcus* spp. used in the research.

Phenotypic Characterization of Antibiotic Resistance

Antibiotic resistance analyses were performed for the 95 strains identified. Antibiotic sensitivity of the strains was verified by means of the agar diffusion method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2018). The disk-diffusion sensitivity test was performed with disks impregnated with penicillin (10 μg), oxacillin (1 μg), cefoxitin (30 μg), gentamicin (10 μg), tobramycin (10 μg), erythromycin (15 μg), tetracycline (30 μg), clindamycin (2 μg), and chloramphenicol (30 μg; all from Laborclin, Paraná, Brazil).

Molecular Characterization

The genes investigated were resistant to the antibiotics penicillin (*blaZ*), cefoxitin and oxacillin (*mecA* and *mecC*), gentamicin (*aac2′aph6′*), tobramycin (*ant4*), clindamycin and erythromycin (*ermA*, *ermB*, *ermC*, *ermF*, *ermT*, *msrA*, *cfr*, *vgaC*, *lnuA*/*A′*, *lnuB*, *lsaB*, *lsaE*), tetracyclin (*tetK*, *tetL*, and *tetM*), or related to resistance to sanitizer QAC (*qacA*/*B* and *qacC*), biofilm formation (*bap*, *icaA*, and *icaD*), SCC*mec* typing (type I, II, III, Iva, IVb, IVc, IVd, and type V), *ccr* (*ccrA1-ccrB*, *ccrA2-ccrB*, *ccrA3-ccrB*, and *ccrC*), and *mec* complex (*mecA-mecI*, *mecA*-IS1272, and *mecA*-IS431).

For amplification of the targeted genes, a 25-μL reaction was performed containing the following: 5 μL of buffer (5×; Promega Corp., Madison, WI), 2 to 4 μL of $MgCl_2$ (25 m*M*; Promega Corp.) according to the gene, 0.5 μL of dNTP (10 m*M*; Invitrogen, Carlsbad, CA), 1 μL of each pair of forward and reverse primers (10 pmol; Invitrogen, or Sigma, St. Louis, MO) according to the gene, 0.25 μL of hot start polymerase (Promega Corp.), 5 μL of DNA, and as much ultrapure Milli-Q water (Merck KGaA, Darmstadt, Germany) as needed to complete the 25-μL reaction.

The reactions were performed in a thermal cycler (Thermo Fisher Scientific, Waltham, MA) under the following conditions: initial denaturation of 94 to 95°C for 2 min at 25 to 40 cycles (varying according to primer), denaturation of 94 to 95°C for 30 s to 1 min, annealing temperature following the references cited in Supplemental Table S1 (http://dx.doi.org/10.17632/79frfcjzhm.2), range 72 to 74°C for 1 min, and final extension of 72 to 74°C for 5 min.

Statistical Analysis

Cochran's Q test was used to compare the relative prevalence of the genes studied between conventional and organic dairy products.

RESULTS

The results of antibiotic resistance and the presence of virulence genes are shown in Table 2. No results in this study showed resistance to chloramphenicol. Of the 95 strains of *Staphylococcus* spp., 51 showed no resistance to any of the antibiotics, and 44 showed resistance to at least 1 antibiotic. The antibiotic with the highest number of resistant isolates was penicillin with 25.3%, followed by oxacillin (21.1%), clindamycin (11.6%), erythromycin (6.3%), cefoxitin (4.2%), gentamicin (2.1%), tetracycline (1.1%), and tobramycin (1.1%). Of the 95 *Staphylococcus* spp., none showed the following antibiotic resistance genes: *mecC*, *tetK*, *tetL*, *ermA*, *ermC*, *ermF*, *ermT*, *lnuA*/*A′*, *lnuB*, or *lsaE*. The most prevalent antibiotic resistance gene was *blaZ* with 25.3%, followed by *mecA* (13.7%), *lsaB* (6.3%), *msrA* (4.2%), *ant4* (3.2%), *tetM* (2.1%), *aac2′aph6* (1.1%), *ermB* (1.1%), *cfr* (1.1%), and *vgaC* (1.1%).

The results in Table 2 show the prevalence of virulence genes and the phenotypic resistance of *Staphylococcus* spp. All data were subjected to statistical analysis by Cochran's Q test. Although most strains of conventional production had a greater amount of phenotypic resistance compared with organic production, and organic products had a higher prevalence of genes compared with conventional products, such numbers were not sufficient to demonstrate a statistically significant difference by the evaluation method. Regarding sanitizer-resistance genes, 5.3% of *Staphylococcus* had the *qacA*/*B* gene, and 6.3% had the *qacC* gene. Among biofilm-forming genes, the most prevalent was *icaD* with 41.1%, followed by *icaA* (29.5%) and *bap* (4.2%).

Of the 95 strains of *Staphylococcus* spp., at least 72 had a resistance gene or biofilm-forming gene, more than 47 had a combination of more than 2 genes, and 44 were resistant to at least 1 antibiotic. Table 3 shows the 12 strains with the greatest diversity of genes or resistance to multiple antibiotics. Of the 13 isolates that presented the *mecA* gene, all were subjected to typing analysis by SCC*mec*. Only 5 isolates presented any of them, as described in Table 4.

Table 1. Strains of *Staphylococcus* spp. isolates from 6 (A–F) organic and conventional dairy productions

	Organic				Conventional				
Staphylococcus spp.	A	B	C	Total	D	E	F	Total	Overall total
Staphylococcus aureus	3	6	9	18	10	2	8	20	38
Staphylococcus saprophyticus	1	3	1	5	3	8	3	14	19
Staphylococcus warneri	0	1	1	2	4	3	0	7	9
Staphylococcus xylosus	0	2	0	2	2	2	1	5	7
Staphylococcus epidermidis	1	0	1	2	2	2	0	4	6
Staphylococcus chromogenes	1	1	0	2	3	0	0	3	5
Staphylococcus hyicus	1	0	0	1	0	2	0	2	3
Staphylococcus haemolyticus	1	0	0	1	1	0	0	1	2
Staphylococcus agnetis	0	0	0	0	0	1	0	1	1
Staphylococcus capitis	0	0	0	0	0	1	0	1	1
Staphylococcus kloosii	1	0	0	1	0	0	0	0	1
Staphylococcus pasteuri	0	1	0	1	0	0	0	0	1
Staphylococcus sciuri	0	0	0	0	1	0	0	1	1
Staphylococcus simulans	0	1	0	1	0	0	0	0	1
Total	9	15	12	36 (37.9%)	26	21	12	59 (62.1%)	95 (100%)

Table 2. Phenotypic resistance to antibiotics[1] and genes identified in *Staphylococcus* spp. isolated from dairies

Dairy (no.)	*Staphylococcus* spp. (no.)	Antibiotic resistance (no.)	Antibiotic resistance genes (no.)	Sanitizer resistance genes (no.)	Biofilm formation genes (no.)
Organic (36)	*S. aureus* (18)	PEN (4), OXA (2)	*blaZ* (4), *ant4* (3), *mecA* (1), *tetM* (1)	*qacC* (1)	*icaD* (18), *icaA* (7), *bap* (1)
	S. saprophyticus (5)	CLI (2), PEN (1), OXA (1), CEF (1)*	*mecA* (2), *blaZ* (1), *lsaB* (1)	—	*icaA* (2), *bap* (1)
	S. warneri (2)	PEN (1), GEN (1), CEF (1)*	*blaZ* (1)	*qacA/B* (2)	—
	S. xylosus (2)	OXA (2)	*mecA* (1)	*qacA/B* (1)	*icaD* (1)
	S. epidermidis (2)	OXA (1), CEF (1)*	*mecA* (1)	—	*icaA* (1)
	S. chromogenes (2)	OXA (1)	*mecA* (1)	—	—
	S. hyicus (1)	PEN (1), OXA (1), CLI (1), ERY (1)	*blaZ* (1), *mecA* (1), *lsaB* (1)	—	—
	S. haemolyticus (1)	OXA (1), CLI (1)	—	—	—
	S. kloosii (1)	PEN (1), OXA (1), CLI (1), CEF (1)*	*mecA* (1), *blaZ* (1)	—	—
	S. pasteuri (1)	CLI (1), CEF (1)	*lsaB* (1), *cfr* (1), *vgaC* (1)	*qacC* (1)	—
	S. simulans (1)	ERY (1)	*ermB* (1)	—	—
Conventional (59)	*S. aureus* (20)	PEN (9), OXA (2), CEF (2), CLI (1), TET (1), ERY (1), ERY (2)*	*blaZ* (9), *msrA* (2), *mecA* (1), *tetM* (1)	*qacC* (2)	*icaD* (20), *icaA* (10), *bap* (1)
	S. saprophyticus (14)	PEN (2), OXA (2), CLI (1), ERY (1), CEF (1)*	*blaZ* (2), *mecA* (1), *lsaB* (1)	*qacA/B* (1), *qacC* (2)	*icaA* (2), *bap* (1)
	S. warneri (7)	OXA (2), CLI (2), PEN (1), CEF (1), GEN (1)*	*blaZ* (1), *lsaB* (1), *aac2′aph6′* (1)	—	*icaA* (1)
	S. xylosus (5)	ERY (1)	*msrA* (1)	—	*icaA* (1)
	S. epidermidis (4)	PEN (1), ERY (1)	*blaZ* (1), *msrA* (1), *mecA* (1)	—	*icaA* (1)
	S. chromogenes (3)	OXA (1), CLI (1), CEF (1)*	*mecA* (1), *lsaB* (1)	—	*icaA* (1)
	S. hyicus (2)	OXA (1)	*mecA* (1)	—	—
	S. haemolyticus (1)	PEN (1), CEF (1)*	*blaZ* (1)	—	*icaA* (1)
	S. agnetis (1)	PEN (1)	*blaZ* (1)	—	—
	S. capitis (1)	PEN (1), OXA (1), TOB (1)	*blaZ* (1)	*qacA/B* (1)	—
	S. sciuri (1)	OXA (1), GEN (1)	—	—	*icaA* (1)

[1]CEF = cefoxitin, CLI = clindamycin, ERY = erythromycin, GEN = gentamicin, PEN = penicillin, OXA = oxacillin, TET = tetracycline, TOB = tobramycin; — = gene not detected.

*Indicates intermediate resistance.

Table 3. Strains identified with greater combinations of different genes or phenotypic resistance to multiple antibiotics[1]

Type	Dairy	*Staphylococcus* spp.	Origin	Antibiotic	Antibiotic	Sanitizer	Biofilm
Organic	A	*S. kloosii*	Raw milk	PEN, OXA, CEF*, CLI	*BlaZ, mecA*	—	—
	A	*S. aureus*	Cooling chamber	OXA	*ant4*	*qacC*	*icaA, icaD*
	A	*S. aureus*	Raw milk	OXA	*mecA*	—	*bap, icaA, icaD*
	A	*S. hyicus*	Raw milk	PEN, OXA, ERY, CLI	*blaZ, mecA, lsaB*	—	—
	A	*S. saprophyticus*	Lira	PEN, OXA, CLI	*blaZ lsaB*	—	*icaA*
	B	*S. xylosus*	Raw milk	OXA	*mecA*	*qacA/B*	*icaD*
	B	*S. pasteuri*	Floor	CEF, CLI	*cfr, vgaC, lsaB*	*qacC*	—
Conventional	D	*S. warneri*	Cheese	PEN, OXA, CEF, CLI	*blaZ lsaB*	—	—
	E	*S. aureus*	Raw milk	PEN, OXA, CEF, CLI	*blaZ, mecA*	—	*icaD*
	F	*S. aureus*	Cheese	PEN, ERY	*blaZ, msrA*	—	*icaA, icaD*
	F	*S. aureus*	Cheese	PEN	*blaZ*	—	*bap, icaA, icaD*
	F	*S. aureus*	Lira	PEN, TET	*blaZ, tetM*	*qacC*	*icaD*

[1]CEF = cefoxitin, CLI = clindamycin, ERY = erythromycin, PEN = penicillin, OXA = oxacillin, TET = tetracycline; — = gene not detected.

DISCUSSION

This study investigated the distribution of *Staphylococcus* spp. in organic and conventional dairy producers in the state of São Paulo, Brazil to characterize their differences. The results showed that both organic and conventional dairy producers presented strains with antibiotic resistance and presence of virulence genes, with no statistically significant difference between them using Cochran's Q test.

As shown in Table 3, several strains of *Staphylococcus* spp. simultaneously contained biofilm-forming genes and resistance to sanitizing agents and antibiotics. Such a combination of genes can prove to be extremely dangerous, as it increases their persistence in environments and makes it difficult to eliminate them, which may become a health risk. Regarding antibiotic resistance, chloramphenicol was 100% efficient in inhibiting *Staphylococcus* spp. It is noteworthy that MAPA Normative Instruction No. 9, dated June 27, 2003, prohibits the veterinary use of chloramphenicol (BRASIL, 2003). This may explain the high susceptibility, and thus the reason animals should normally not be exposed to this antibiotic.

The antibiotic that showed the lowest inhibition efficiency was penicillin (25.5% resistant), which is part of the group of β-lactams. The use of β-lactam antibiotics is allowed by the Brazilian Health Surveillance Agency (ANVISA); however, the presence of residues of β-lactam antibiotics in milk poses risks to consumer health, as they may cause allergic reactions and trigger anaphylactic shock in individuals sensitive to this group of antibiotics, which is composed of 4 subclasses: penicillins, cephalosporins, carbapenems, and monobactam (ANVISA, 2009). The extensive use of β-lactam may be responsible for the lower efficiency of penicillin against *Staphylococcus* spp. isolates.

The *S. aureus* that presented simultaneous resistance to oxacillin and cefoxitin antibiotics were considered to be phenotypic MRSA in this paper, and as such, this study included 2 isolates with phenotypic MRSA characteristics. Among these isolates, we have an isolate from raw milk from conventional dairy E and an isolate from the brine sample from conventional dairy F; the latter did not show the MRSA character genotype of the *mecA* and *mecC* resistance genes. Its resistance may come from newer genes discovered, such as *mecB* (Becker et al., 2018) or *mecD* (Schwendener et al., 2017). According to the Brazilian Health Surveillance Agency, some strains of *S. aureus* have a less common type of oxacillin resistance, a borderline resistance in which the resistance mechanism is possibly due to β-lactamase overproduction or modifications to penicillin-binding proteins PBPs 1, 2 and 4 (ANVISA,

2007a). In addition to the 2 strains of MRSA identified phenotypically, 1 strain of *Staphylococcus warneri* from conventional dairy production was identified with simultaneous resistance to oxacillin and cefoxitin, in addition to resistance to penicillin and clindamycin. This strain was considered our strain of methicillin-resistant CNS phenotype because it did not have the *mecA* and *mecC* genes, indicating that it possibly would have other genes or mechanisms responsible for its resistance, as already mentioned for *S. aureus*. We also identified a strain of *Staphylococcus pasteuri* from organic production with resistance to cefoxitin and sensitivity to oxacillin, and with the absence of the *mecA* and *mecC* genes. Such characteristics suggest that the multiple resistance mechanisms should be better explored in *Staphylococcus*, mainly in the CNS.

Of the 95 strains tested, 11 were resistant to clindamycin, 6 were resistant to erythromycin, and 2 had intermediate resistance to erythromycin. Of these strains, only a single isolate showed resistance to both antibiotics, a *Staphylococcus hyicus* from organic dairy products. Both the antibiotics clindamycin and erythromycin have bacteriostatic action, even though they belong to different classes of antibiotics (macrolides and lincosamides). Macrolides are capable of inhibiting RNA-dependent protein synthesis by binding to receptors located in the 50S portion of the ribosome, particularly in the 23S RNA molecule, preventing transpeptidation and translocation reactions. Lincosamides also inhibit protein synthesis in ribosomes, binding to the 50S subunit, changing the bacterial surface, and facilitating opsonization, phagocytosis and intracellular destruction of microorganisms (ANVISA, 2007b).

Regarding the characterization of resistance phenotypes in products similar to the ones we researched, we found that Rodrigues et al. (2017) also had a high number of penicillin-resistant strains, followed by cefoxitin, oxacillin, clindamycin, erythromycin, tetracycline, tobramycin and gentamicin, ciprofloxacin, and chloramphenicol in samples from dairy products in Brazil. The distribution of resistant isolates in our study was similar to that found in the Rodrigues et al. (2017) study. Ferreira et al. (2016) evaluated the presence of 29 *S. aureus* isolates in artisanal and industrialized Minas Frescal cheese in Goiânia, Goiás, and verified antimicrobial susceptibility (penicillin presented the highest resistance), where 13 isolates (44.8%) were resistant to penicillin and 3 (10.3%) to tetracycline. Their results corroborate ours; we also obtained a greater number of isolates resistant to penicillin. In Turkey, Kürekci (2016) studied 17 *Staphylococcus* spp. isolated from cheese samples. The disk-diffusion test was performed and revealed that the greatest resistance was to penicillin (76.5%), erythromycin (35.3%), and tetracycline (29.4%), and all were susceptible to gentamicin; these results are similar to those found in our study. When comparing only the same antibiotics used, we also found greater resistance to penicillin, followed by erythromycin, tetracycline, and 2 isolates resistant to gentamicin.

Regarding molecular characterization, *blaZ* was the most prevalent gene, found in 24 (25.3%) isolates in total. It is responsible for the penicillin-resistance mechanism and the antibiotic that presented the highest number of resistant isolates, which is corroborated by the phenotypic and genotypic results, according to Table 3. Regarding the prevalence of the *mecA* gene, it was found in 13 (13.7%) *Staphylococcus* spp. strains, with highest prevalence in 8 (22.2%) strains of organic dairy products. The presence of the *mecA* gene, responsible for methicillin resistance in MRSA, should make these groups of microorganisms considered to be resistant to all β-lactam antibiotics (Börjesson et al., 2009). Of these 13 isolates, only 2 were considered as genotypic MRSA, both isolated from raw milk (1 conventional and 1 organic). The other 11 *Staphylococcus* spp. with *mecA* genes were *Staphylococcus saprophyticus* (n = 3), *S. chromogenes* (2), *S. epidermidis* (2), *S. hyicus* (2), *S. kloosii* (1), and *S. xylosus* (1).

Regarding the *ant4* gene, 3 strains of *S. aureus* tested positive, but none of them were resistant to tobramycin. All evidence indicated that such strains had the genotype, but were not capable of presenting the resistance phenotype. On the other hand, a single strain of *Staphylococcus capitis* was resistant to tobramycin, but did not present the resistance genotype (*ant4* gene). Only 1 strain of *S. warneri* was positive for the *aac2′aph6* gene, responsible for resistance to

Table 4. Typing results for SCC*mec*[1]

Type	Dairy	*Staphylococcus* spp.	Origin	*ccr*	Type
Organic	A	*S. aureus*	Raw milk	*ccr*A1-*ccr*B	I
	B	*S. chromogenes*	Pasteurized milk	*ccr*A3-*ccr*B	—
	C	*S. epidermidis*	Raw milk	*ccr*A2-*ccr*B	—
Conventional	E	*S. aureus*	Raw milk	—	IVc
	E	*S. hyicus*	Chamber floor swab	*ccr*A1-*ccr*B	—

[1]— = gene not detected.

gentamicin. The same strain also showed intermediate resistance in the disk-diffusion test. In this study, the *tetM* gene, responsible for tetracycline resistance, was also present in 2 *S. aureus* isolates. Only 1 of them, stemming from the lira of a conventional dairy product, presented the resistance phenotype. The other isolate with the *tetM* gene was isolated from organic Minas Frescal cheese, but did not show a tetracycline-resistant phenotype profile. No isolates were found with the presence of the *tetK* or *tetL* gene, which are responsible for tetracycline resistance.

The antibiotics erythromycin and clindamycin can often act in synergy, and there are numerous genes that can be responsible for bacterial resistance. In this study, however, only *lsaB*, *msrA*, *ermB*, *cfr*, and *vgaC* were detected. Regarding sanitizing-resistant genes, the *qacA/B* gene was found in 5 isolates; all of them were CNS. Six isolates contained the *qacC* gene, of which 3 were *S. aureus* and 3 were CNS, according to Table 3. The QAC are generally more effective against gram-positive bacteria than gram-negative bacteria because they have very complex multiple layers (Minbiole et al., 2016). Although *S. aureus* is a gram-positive bacterium, it can acquire resistance to QAC (Shamsudin et al., 2012; Lin et al., 2016; Iñiguez-Moreno et al., 2018).

As for biofilm production genes, as shown in Table 2, the most prevalent was *icaD* (41.1%), followed by *icaA* (29.5%). Only 4.2% presented the *bap* gene, which was the least prevalent biofilm gene in the entire study. The *icaA* gene was found to be more present in *Staphylococcus* spp. isolated in conventional dairy products (30.5%), whereas *icaD* (52.8%) and *bap* (5.6%) were more present in organic dairy products. However, none of them presented significant statistical results. In this study, 4 strains presented the *bap* gene, 2 *S. aureus*, and 2 *S. saprophyticus*. The importance of controlling sanitizer-resistant and biofilm-forming bacteria is emphasized because their persistence in the industrial environment may cause contamination in the final product for consumption.

Several studies on the molecular characterization of different types of *Staphylococcus* spp. have reported the prevalence of biofilm-forming and antimicrobial-resistant genes in food samples, mainly of animal origin. These studies are of great importance to demonstrate how strains isolated from food are as virulent as clinical isolates. As an example, a study by Rodrigues et al. (2017) on samples from Minas Frescal cheese production found the *tetM* and *mecA* genes in different strains of *Staphylococcus* spp., similar to our research. Johler et al. (2018), in a study involving isolated *S. aureus* from artisanal cheese production, found no *mecC* isolates, which corroborates this study because no strain carrying the *mecC* gene was identified herein. Kürekci (2016) also observed the *mecA*, *tetM*, *ermB*, *blaZ*, and *ant4* genes in 17 strains of *Staphylococcus* spp. from cheese in Turkey, as in our study and described in Table 3.

All 13 strains that presented the *mecA* gene were subjected to the SCC*mec* typing technique. However, not all isolates could be classified, as some did not amplify the type of SCC*mec*, *ccr*, or *mec* complex, as observed in other studies (Cuny and Witte, 2005; Vanderhaeghen et al., 2012; Taniguchi et al., 2020). There are currently 11 officially recognized main types of SCC*mec*, with their various exclusion variants, composite elements, and irregular elements (IWG-SCC, 2014). According to Martínez-Meléndez et al., (2015), in addition to the types and subtypes of SCC*mec*, there are nontypeable SCC*mec* observed in several studies, mainly in CNS. The *mec* and *ccr* complexes undergo complex recombination and reordering processes in CNS genomes, through which the generation of new SCC*mec* types occurs. Most likely, only a small fraction of SCC*mec* diversity is present in *S. aureus*. It is not possible to detect it with the methodology established for *S. aureus* (Martínez-Meléndez et al., 2015).

Basanisi et al. (2017) isolated *S. aureus* from dairy products, 92.5% with SCC*mec* type V, 25% with IVa, and 1 strain unidentified SCC*mec*. Rodrigues et al. (2017) identified SCC*mec* type III, IVa, and IVd in MRSA of dairy samples. Some SCC*mec* could not be identified, as it was not possible to amplify the tested gene. These studies demonstrate that it is not always possible to identify the type of SCC*mec*. In the literature, CNS positive for *mecA* are less studied compared with MRSA.

Herrera et al. (2016) identified only SCC*mec* type IV in MRSA from cheese. The risk of human infection by MRSA through contaminated food is considered low, but contaminated food products can contribute to the worldwide spread of community-associated MRSA clones. Until a few years ago it was said that traditionally, types I to III have been associated with MRSA strains of clinical isolates, which are acquired in hospital settings, and community or animal-associated strains tend to harbor smaller, and supposedly more mobile, SCC*mec* types IV and V (Deurenberg et al., 2007; Monecke et al., 2018). Perhaps due to evolution, spread, and genetic exchange, it is possible to find different types of *Staphylococcus* with different types of SCC*mec* in foods today.

Regarding the characteristics of organic versus conventional dairy products in this study, no statistically significant difference was found with Cochran's Q test. This corroborates several studies that did not find great differences either, or that stated that organic versus conventional production is not enough to predict the characteristics of *Staphylococcus* spp. because there

are several factors that influence milk quality, such as demographic factors, types of milking, and others (Cicconi-Hogan et al., 2013, 2014; Mullen et al., 2013; Tenhagen et al., 2018).

Theoretically, a greater amount of resistant strains is expected to be found in conventional rather than organic production due to the use of antibiotics in the former. First, the sampling of dairy products in this study was carried out randomly, without requiring details of the health conditions of the milked animals. In Brazil, the use of synthetic antibiotics in organic milk production cows is only allowed when the animal is suffering and natural medicines have already been administered to no effect. The milk samples were collected from tank milk, and thus the samples could contain milk from cows that may have previously been treated with synthetic antibiotics. However, the opposite is also possible on conventional farms: even though the use of antibiotics is allowed, it is possible that the animals were not treated with synthetic antibiotics. This could explain why there was no statistically significant difference between them. It is also worth mentioning the possibility of contamination from food handlers, both in milking and in the preparation of Minas Frescal cheese, as it is known that such handlers can be sources of contamination in food production (Kousta et al., 2010; Sospedra et al., 2012). Especially in Minas Frescal cheese production, strains that are transmitted to food through handlers may or may not be resistant to antibiotics (André et al., 2008).

CONCLUSIONS

Staphylococcus with antibiotic resistance, resistance genes and biofilm-forming genes have been found in both organic and conventional dairy products. There was no statistically significant difference in the presence of the researched genes between types of production. Regarding the typing methodology, we observed that it is necessary to explore better the techniques used, mainly in relation to CNS, the available methodologies for *SCCmec* are aimed at *S. aureus*. The genes and phenotypes found in this study are extremely important for the survival of bacterial strains in the industry, posing a danger to consumer health. As such, improvements in quality control of dairy products are required to avoid the contamination and spread of resistant *Staphylococcus*.

ACKNOWLEDGMENTS

This study was funded by the São Paulo Research Foundation (FAPESP, São Paulo, Brazil; grant numbers 2016/02118-7 and 2018/00986-7), Programa de Excelência Acadêmica (PROEX, Brasília, Brazil; grant numbers 23038.000795/2018-61), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES, Brasília, Brazil; grant numbers 001). The authors thank Espaço da Escrita – Pró-Reitoria de Pesquisa – UNICAMP (Campinas, Brazil) for the language services provided. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CAPÍTULO 4

Assessment of sanitizer efficacy against *Staphylococcus* spp. isolated from Minas Frescal cheese producers in São Paulo, Brazil

Artigo publicado no periódico "International Dairy Journal": Abreu, A. C. S.; Crippa, B. L.; Souza, V. V. M. A.; Nuñez, K. V. M.; Almeida, J. M.; Rodrigues, M. X.; Silva, N. C. C. Assessment of sanitizer efficacy against *Staphylococcus* spp. isolated from dairy products of organic and conventional Minas Frescal cheese. International Dairy Journal. 2021.

International Dairy Journal 123 (2021) 105171

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Assessment of sanitiser efficacy against *Staphylococcus* spp. isolated from Minas Frescal cheese producers in São Paulo, Brazil

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ARTICLE INFO

Article history:
Received 4 April 2021
Received in revised form
27 June 2021
Accepted 19 July 2021
Available online 4 August 2021

ABSTRACT

This study evaluated bactericidal efficacy test of sanitisers for benzalkonium chloride, sodium hypochlorite and peracetic acid, determined minimum inhibitory concentration (MIC) of sanitisers and assessed their effect on the reduction of biofilms produced by *Staphylococcus* spp. strains isolated from organic and conventionally produced Minas Frescal cheese dairy products. The MIC was determined at three different contact times (5, 10, and 15 min). Biofilms formed on stainless steel and the efficiency of sanitisers in removing the biofilms were investigated at contact times of 5 and 10 min and incubated for 24 and 48 h. All strains tested were able to form biofilm within 24 and 48 h for all evaluated sanitisers, indicating the MIC used in this study was not sufficient to inactivate biofilm formation. Therefore, the MICs obtained through the analysis of the microorganism in its planktonic form were not efficient to eliminate the biofilms formed.

1. Introduction

In Brazil, demand for organic food has grown (Feil, Cyrne, Sindelar, Barden, & Dalmoro, 2020), as it has worldwide (Britwum, Bernard, & Albrecht, 2020; González, Marquès, Nadal, & Domingo, 2019; Katt & Meixner, 2020; Kushwah, Dhir, Sagar, & Gupta, 2019); included in this is organic milk (Brito & Silva, 2020; Schwendel et al., 2015). Organic and conventional milk may both be used for Minas Frescal cheese production in Brazil (Cândido et al., 2020); this type of cheese is extremely popular and widely consumed in Brazil (Lollo et al., 2015). According to the Normative Instruction No. 4 of March 1, 2004, Minas Frescal Cheese is defined as a fresh cheese obtained by the enzymatic coagulation of milk with rennet and/or other appropriate coagulating enzymes; complemented or not with the action of specific lactic bacteria. It is a semi-fat cheese, with a very high humidity, intended to be consumed fresh, and storage of this product should not exceed 8.0 °C (BRASIL, 2004).

Milk and its subsequent derivatives are extremely nutrient-rich products, making them excellent culture media for numerous microorganisms (Delorme et al., 2020) such as *Staphylococcus* spp. (Prezzi et al., 2020). *Staphylococcus aureus* is an infectious pathogen that has become a major worldwide health threat; recently, it has been classified by the World Health Organisation (WHO) as a priority 2 pathogen (Gatadi, Madhavi, Chopra, & Nanduri, 2019). In addition, methicillin-resistant *S. aureus* (MRSA) has been described as one of the main pathogens involved in localised skin infections and systemic diseases, including toxic shock syndrome (Shankar, Soe, & Tam, 2020) and food poisoning (Silva, Rodrigues, & Silva, 2020). The inappropriate use of antibiotics has contributed to increasing MRSA, which has also affected the food production chain; particularly animal-based food production (Doulgeraki, Di Ciccio, Ianieri, & Nychas, 2017; Oniciuc, Nicolau, Hernández, & Rodríguez-Lázaro, 2017; Silva et al., 2020).

Many studies have described the prevalence of resistant and/or multiresistant staphylococci to antimicrobials in food, the food processing environment, and food handlers (hands and nose) (Bencardino & Vitali, 2019; Castro, Santos, Meireles, Silva, & Teixeira, 2016; Chung et al., 2021; Ferreira et al., 2014; Li et al.,

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https://doi.org/10.1016/j.idairyj.2021.105171
0958-6946/

2021; Li, Stegger, Dalsgaard, & Leisner, 2019; Ou et al., 2020; Venkatvasan et al., 2020; Wang et al., 2017). Dairy products have repeatedly been detected with significant incidences of antimicrobial-resistant *Staphylococcus* spp. (Angelidis et al., 2020; Basanisi, La Bella, Nobili, Franconieri, & La Salandra, 2017; Krukowski et al., 2020; Papadopoulos et al., 2018; Schnitt, Lienen, Wichmann-Schauer, Cuny, & Tenhagen, 2020; Titouche et al., 2019; Tomao et al., 2020); furthermore, organic dairy products may be more susceptible to MRSA contamination (Tenhagen et al., 2018) or coagulase-negative staphylococci (Cicconi-Hogan et al., 2014).

It is also important to note that strains of staphylococci may have the ability to produce biofilms on different surfaces used in food manufacturing (Doulgeraki et al., 2017). These biofilms can promote bacterial cell resistance when exposed to shear forces; which is when part of the biofilm detaches and ends up in the final product or even affix itself to other locations, recreating a new biofilm niche (Cao et al., 2019). In addition, biofilms can also favour bacterial cell resistance to external environmental factors, such as antimicrobials (Mohammed et al., 2018) and disinfectants (Chan et al., 2018; Ho, Boost, & O'Donoghue, 2015; Htun, Hon, Holden, Ang, & Chow, 2019; Marchi, Otto, Reppschläger, Iqbal, & Holtfreter, 2015; Worthing, Marcus, Abraham, Trott, & Norris, 2018). Biofilms are microbial communities of sessile cells grouped in an extracellular matrix (Suresh, Biswas, & Biswas, 2019), becoming a self-sustaining community of bacteria formed on a surface (Cao et al., 2019), in a state of reduced metabolism (Raafat, Otto, Reppschläger, Iqbal, & Holtfreter, 2019). The biofilm matrix that unites the bacteria facilitates the transfer of genes between them (Suresh et al., 2019).

Numerous mechanisms are involved in staphylococcal biofilm formation, including polysaccharide intercellular adhesin (PIA) encoded by the *ica*ADC locus, with the *ica*A and *ica*D genes being important in biofilm expression; and the biofilm-associated protein (BAP) that can promote *ica*-independent biofilm formation (Arciola, Campoccia, Speziale, Montanaro, & Costerton, 2012; Montanaro, Campoccia, & Arciola, 2007).

Resistance to sanitisers is another concern for the food industry, as sanitisers are commonly used in the decontamination of food or food processing equipment (Liao et al., 2020). Bacteria acquire resistance to sanitisers due to long-term sublethal exposure, and subsequently transfer the acquired resistance genes (Oniciuc et al., 2019). The main genes identified in *Staphylococcus* are the quaternary ammonium compound (QAC) resistance genes, which are transported by plasmids and code for efflux pumps (Chan et al., 2018; Ho et al., 2015; Htun et al., 2019; Marchi et al., 2015; Worthing et al., 2018).

Therefore, the aims of this study were to evaluate the bactericidal efficacy of sanitisers (comprising of benzalkonium chloride, sodium hypochlorite, and peracetic acid), determine the minimum inhibitory concentration and assess their ability to reduce biofilm production of against Staphylococcus isolates from Minas Frescal cheese production organic and conventional.

2. Materials and methods

2.1. Staphylococcus isolates

The *Staphylococcus* spp. used in this study were isolated and identified in a previous study conducted by Cândido et al. (2020). From the culture collection, fourteen *Staphylococcus* spp. strains were selected based on the genes of interest (Table 1); seven from organic dairy products and seven from conventionally produced dairy products. The genes of interest (biofilm formation, resistance to sanitiser and antibiotics) were previously evaluated by Abreu et al. (2021). The isolates used and the respective genes identified are shown in Table 1.

2.2. Sanitisers used in this study

Three commonly used food industry sanitisers were selected: benzalkonium chloride (0.004 M), sodium hypochlorite (74.50%), peracetic acid (76.05%) (Dinâmica Química Contemporânea Ltda, São Paulo, Brazil). Sterile distilled water was used as the control. All experiments were conducted in triplicate. For the Bactericidal Efficacy Test, concentrations commonly used in the industry were chosen for each sanitiser, while for the Minimum Inhibitory Concentration (MIC) test, a range of 1000 to 0.49 ppm was evaluated, in periods of 5, 10 and 15 min to simulate an adequate time for use in the food industry.

2.3. Bactericidal efficacy test of sanitisers

Bactericidal efficacy assays were performed according to the official AOAC method (AOAC, 2016), with modifications (Iñiguez-Moreno, Avila-Novoa, Iñiguez-Moreno, Guerrero-Medina, & Gutiérrez-Lomelí, 2017). The sanitisers evaluated had the following working concentrations and were diluted using sterile distilled water: benzalkonium chloride (400 ppm), sodium hypochlorite (200 ppm), peracetic acid (150 ppm), and water as the control.

All strains were inoculated into a brain heart infusion (BHI) broth and incubated at 37 °C for 24 h. Then, bacterial cultures were standardised using the McFarland 0.5 scale (1×10^8 cfu mL^{-1}) and diluted to 10^6 cfu mL^{-1} with a 0.85% saline solution. A 100 μL aliquot of the diluted culture was transferred to 9.9 mL of the sanitiser solution and homogenised for 15 s, the same procedure was performed with sterile distilled water (control). After 30 s, 100 μL of the mixture was transferred to a microtube with 900 μL of Letheen neutralising broth with 0.5% Tween 80 (for benzalkonium chloride sanitiser) or Tryptic Soy Broth (TSB) + 1% sodium thiosulfate (for sodium hypochlorite and peracetic acid sanitisers) to neutralise the disinfectant activity. After 30 min of contact with the neutralising broth, total bacteria count was performed using standard plate count agar (PCA), plates were incubated at 37 °C for 24–30 h, under aerobic conditions. The reduction percentage was calculated using the following formula:

$$\text{Reduction percentage}(\%) = \frac{100 - S(100)}{CC}$$

where S is the surviving bacteria after treatments with sanitisers (cfu mL^{-1}) and CC is the control count, i.e., water (cfu mL^{-1}). Disinfectants were considered effective when 99.999% bacterial reduction was observed.

2.4. Minimum inhibitory concentration

Broth microdilution in 96-well plates was used to determine the minimum inhibitory concentration (MIC) of sanitisers, according to modified methods of El-azizi, Farag, and Khardori (2016) and Iñiguez-Moreno et al., (2017). All strains were inoculated in BHI broth and incubated at 37 °C for 24 h. After, cultures were standardised using the McFarland 0.5 scale (1×10^8 cfu mL^{-1}). Then, the 96-well plates were coated with 90 μL serial dilutions of sanitiser, starting with 1000 ppm, in total 12 concentrations of sanitisers were tested (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, and 0.49 ppm). A 1000 ppm stock solution in sterile distilled water was prepared and used for dilutions up to 0.49 ppm. Following dilutions, an aliquot of 10 μL of standardised bacterial

Table 1
Genotypic characteristics of *Staphylococcus* spp. selected for analyses in this study.[a]

Type	Strains	Species	Origin	Genes		
				Biofilm formation	Sanitizer resistance	Antibiotic resistance
Positive control	ATCC 6538	*S. aureus*	Standard	*ica*A, *ica*D	–	–
Organic dairy products	C01A05	*S. aureus*	Cold chamber	*ica*A, *ica*D	*qac*C	*ant*4
	C08A25	*S. aureus*	Raw milk	*bap*, *ica*A, *ica*D		*mec*A
	C08A26	*S. hyicus*	Raw milk	–	–	*bla*Z, *mec*A, *lsa*B
	C02B11	*S. saprophyticus*	Raw milk	*bap*		*mec*A
	C02B13	*S. pasteuri*	Floor		*qac*C	*cfr*, *vga*C, *lsa*B
	C06B05	*S. xylosus*	Raw milk	*ica*D	*qac*A/B	*mec*A
	C03C01	*S. warneri*	Raw milk	–	*qac*A/B	–
Conventional dairy products	C04D06	*S. saprophyticus*	Brine	–	*qac*C	–
	C04D16	*S. saprophyticus*	Brine	–	*qac*A/B	–
	C09E45	*S. epidermidis*	Chamber floor	*ica*A	–	*mec*A
	C010E04	*S. aureus*	Raw milk	*ica*D	–	*bla*Z, *mec*A
	C10E15	*S. saprophyticus*	Chamber floor	*bap*	–	
	C11F21	*S. aureus*	Cheese	*bap*, *ica*A, *ica*D	–	*bla*Z
	C12F08	*S. aureus*	Lira	*ica*D	*qac*C	*bla*Z, *tet*M

[a] Gene not detected.

suspension (1×10^8 cfu mL^{-1}) was inoculated resulting in a final bacterial load of 1×10^7 cfu mL^{-1} per well and a final volume of 100 μL. After incubation at 20 °C and different time periods (5, 10, 15 min), sanitisers were deactivated. Deactivation was done by transferring 10 μL from each well to the second set of 96-well microplates containing 90 μL Letheen medium, supplemented with 0.5% Tween 80 for benzalkonium chloride or TSB supplemented with 1% sodium thiosulfate for sodium hypochlorite and peracetic acid sanitiser. These plates were incubated at 37 °C for 24 h. Negative controls of sanitisers and medium culture were carried out as well as a positive control of each isolate. Finally, 50 μL of resazurin solution at 0.01% was added and plates were incubated at room temperature for 5–10 min. The results were collected visually; where the wells with the lowest concentration of antimicrobial agent remain a blue colour and considered the minimum inhibitory concentration, and the wells that change to pink indicate that the bacteria were not inactivated.

2.5. Efficacy of chemical sanitisers against preformed biofilms

The effect of sanitisers on biofilm removal was evaluated using the assay efficacy of chemical sanitisers against preformed biofilms, according to a modified protocol of Campana, Ciandrini, and Baffone (2018). First, coupons of stainless steel type 304 ($10 \times 10 \times 1$ mm) were properly cleaned (acetone rinse, treatment with 5 N HCl, wash with detergent solution, and disinfection with 70% ethanol) and sterilised (15 min at 121 °C) Next, biofilm formation was induced using 15 *Staphylococcus* isolates, which were grown in TSB at 37 °C for 24 h, then cultures were standardised at 10^8 cfu mL^{-1} with a 0.85% saline solution. For this assay, *S. aureus* ATCC 6538 was the positive control for *ica*A and *ica*D genes. The bacterial suspensions and TSB were transferred to 24-well plates obtaining a final bacterial load of 10^7 cfu mL^{-1}. With tweezers, one stainless steel coupon was deposited into the wells for each treatment; in total 16 coupons per strain (8 coupons per incubation time). The plates were incubated at 37 °C for 24 and 48 h, and following incubation, the coupons were rinsed with distilled water. Rinsed coupons were dried at room temperature in a laminar flow for 10 min. For the 48-h biofilm growth, the broth was removed and 1 mL of new TSB was added 24 h post-incubation. The dried coupons were immersed in 1 mL sanitiser at the minimum inhibitory concentrations determined or in sterile distilled water (control) for 5 and 15 min. After sanitisation, the coupons were immersed in 1 mL neutralisation solution (Letheen for benzalkonium chloride or 1% sodium thiosulfate for sodium hypochlorite and peracetic acid) for 5 or 15 min. Coupons were added to sterile tubes with 9 mL saline solution and stirred for 1 min at full speed (4000 rpm) and then immersed in an ultrasound bath for 10 min (40 kHz frequency and 81 W power) to detach cells from coupon surface. After stirring, 1 mL aliquots of each sample were submitted to 10-fold serial dilution and plated in tryptic soy agar (TSA), in duplicate, using the microdroplet technique.

Plates were incubated at 37 °C for 24 h followed by counting of bacterial colony-forming units. The average cfu mL^{-1} were converted to $\log_{10}$ cfu mL^{-1}. The control treatment with water was considered equivalent to the initial count of formed biofilms. The efficacy of sanitisers for removing biofilm was calculated using the following formula:

$$\text{Reduction } \log_{10} \text{ cfu mL}^{-1} = \text{CC} - \text{TC}$$

where TC is the test count, i.e., treatments with sanitisers (cfu mL^{-1}) and CC is the control count, i.e., water or starting count (cfu mL^{-1}).

Disinfectants were considered effective when they were able to reduce more than 2 logs (based on minimal growth of positive controls in water) and present statistically significant difference between the control treatment (water) and the sanitiser treatment.

2.6. Statistical analysis

Sisvar 5.6 software (Universidade de Lavras, Lavras, Brazil) was used to calculate mean values and standard deviations; analysis of variance (ANOVA) and the Tukey method ($p \leq 0.05$) was used for multiple comparisons.

3. Results and discussion

3.1. Bactericidal efficacy of sanitisers

The bactericidal efficacy of three sanitisers commonly used by the food industry were evaluated, using their recommended concentrations. Fig. 1 shows comparisons for each sanitiser against *Staphylococcus* spp. strains. For a sanitiser to be considered effective, it must eliminate 99.999% of bacteria with a contact time of 30 s.

Benzalkonium chloride and sodium hypochlorite were not significantly different when tested against the fourteen strains

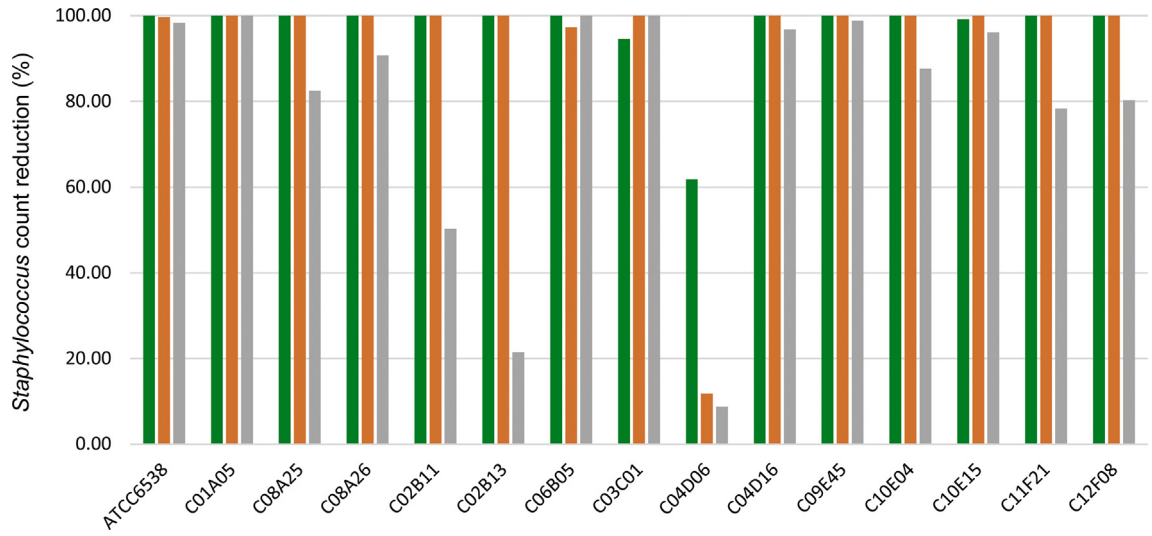

Fig. 1. Reduction of *Staphylococcus* spp. count after exposure to sanitising treatments: ■, 400 ppm benzalkonium chloride; ■, 200 ppm sodium hypochlorite; ■, 150 ppm peracetic acid.

isolated from dairy products. However, both were significantly more effective when compared with peracetic acid against the C02B11 and C02B13 strains. Both of these strains were isolated from the same organic dairy product, and identified as *Staphylococcus saprophyticus* (gene *bap*) and *Staphylococcus pasteuri* (gene *qac*C), respectively; both are NCS (Ramnarain, Yoon, & Runnegar, 2019; Souza et al., 2019) and are related to cases of subclinical mastitis (Ndahetuye et al., 2020). *S. saprophyticus* may have a high prevalence in dairy products (Soares et al., 2011) and resistance to antibiotics (Srednik, Archambault, Jacques, & Gentilini, 2017), it is also the second most common microorganism identified in acute urinary tract infections (Souza et al., 2019). *S. pasteuri* is often related to cases of endocarditis (Ramnarain et al., 2019). Bjorland, Bratlie, and Steinum (2007) recovered an isolate of *S. pasteuri* from bulk milk in a dairy herd in Norway that was resistant to QAC; the isolate had an smr gene, also known as *qac*C (Bjorland, Sunde, & Waage, 2001), the same gene present in our isolate of QAC resistant *S. pasteuri* in the current study.

Interestingly, no significant difference was found among all sanitisers and control treatments against the C04D06 strain; the highest reduction percentage observed was 61.8% for benzalkonium chloride, which is not considered effective. Indicating that this must be the most resistant strain.

Furthermore, peracetic acid was not significantly different from the control treatment, regarding the C02B13 and C04D06 strains. These results suggest that these strains are resistance to peracetic acid at the evaluated concentration in this study.

Additionally, peracetic acid was found to be the sanitiser with the most reduced bactericidal effect, ranging from 8.8% to 100%; the reduction percentage of ≥99.999% was not observed against eleven of the fourteen isolates tested.

The ineffectiveness of peracetic acid when compared with other sanitisers can be attributed to the concentration evaluated in this study. According to the Food and Drug Administration (FDA, 2019), the allowed concentration of peracetic acid for surface sanitisation in the food industry is from 100 to 200 ppm.

In the present study, the strain C04D06 (*S. saprophyticus*) which carried the *qac*C gene, can be considered multiresistant to sanitisers because it was resistant to all evaluated sanitisers. The *qac*C gene is related to the ability to resist QACs, a group that includes the disinfectant BAC (Zaki, Bastawy, & Montasser, 2019). This can lead to survival, growth, and spread of enterotoxigenic staphylococci (Heir, Sundheim, & Holck, 1999). Due to the production of a single UafA protein, this bacterium also encodes several transport proteins, allowing for quick adjustment to osmotic and pH changes (Rogers, Fey, & Rupp, 2009), and may become an issue in cheese production (Bockelmann, Willems, Neve, & Heller, 2005).

3.2. Minimum inhibitory concentration

The minimum inhibitory concentrations identified are presented in Fig. 2, according to sanitisers and contact time. For the vast majority of isolates, the BAC sanitiser showed the highest MICs under the contact times tested, compared with other sanitisers. Regarding isolated strains of organic versus conventional production, there was no statistically significant difference ($p \leq 0.05$).

In general, the lowest sanitiser concentration was observed for sodium hypochlorite at 15 min of exposure, followed by 10 and 5 min; no statistically significant differences were identified among contact times using this sanitiser. In addition, sodium hypochlorite was not significantly different from peracetic acid when compared with 10 and 15 min contact times.

From an industrial point of view, it is extremely interesting that there is a small difference in the MIC regarding the time range of 5–15 min, as the industry may choose to use a particular sanitiser for less time (5 min) at higher concentrations. This saves time during sanitation processes, consequently, increasing food production. The industry can also choose to use a lower concentration for a longer time (15 min), to save on sanitiser costs. These management options provide two good alternatives for the industry. In addition, sodium hypochlorite or peracetic acid (for 10 and 15 min) can be used, without much difference, since both have shown similar results, and can be an alternative in sanitiser changes.

Benzalkonium chloride (BAC), a quaternary ammonium compound (QAC), is widely used in disinfectants in the food industry and healthcare facilities (Tezel & Pavlostathis, 2015). In our study, the MIC of BAC ranged from 15.5 ppm to 250 ppm. In a previous study, the MICs of *Staphylococcus* expressing the QacC protein ranged from 4 to 12 ppm, when compared with MIC ≤2 ppm for sensitive strains (Fagerlund, Langsrud, Heir, Mikkelsen, & Møretrø, 2016; Heir, Sundheim, & Holck, 1995).

Differences in BAC tolerance in staphylococci have traditionally been explained by the presence of *qac* genes that encode efflux pumps. However, in our study, the C11F21 – *S. aureus* strain

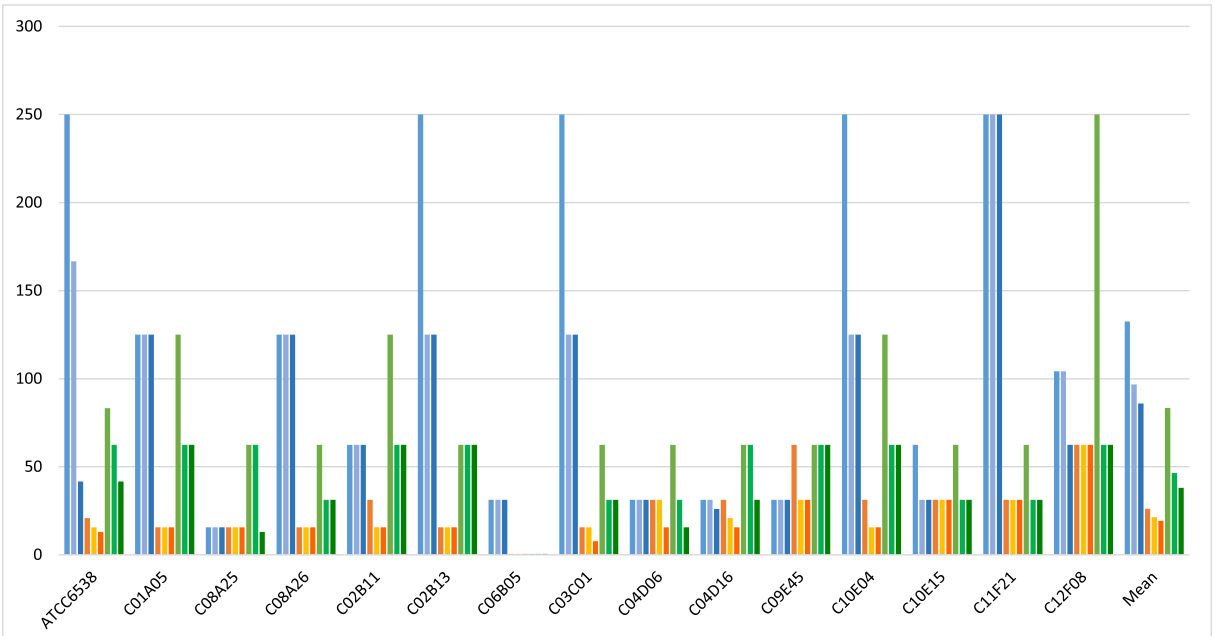

Fig. 2. Mean values for minimum inhibitory concentrations of three sanitisers evaluated for 16 strains of *Staphylococcus* spp: ■,■,■, benzalkonium chloride for 5, 10 and 15 min, respectively; ■,■,■, sodium hypochlorite for 5, 10 and 15 min, respectively; ■,■,■, peracetic acid for 5, 10 and 15 min, respectively.

presenting the highest MIC values (250 ppm in the three exposure times tested) did not present any *qac* gene in its genotypic profile. Both C06B05 – *Staphylococcus xylosus* and C04D06 – *S. saprophyticus* had a MIC of 31.3 ppm and harbour *qacA/B* and *qacC* genes, respectively. The *qac* genes are known to provide resistance to more than 30 different cationic compounds belonging to at least 12 different chemical classes (Cervinkova, Babak, Marosevic, Kubikova, & Jaglic, 2012; Hassan et al., 2006).

Peracetic acid had a similar MIC in most strains tested, with no significant differences when comparing contact times of 10 and 15 min. Several studies (Briñez, Roig-Sagués, Hernández Herrero, López-Pedemonte, & Guamis, 2006; Iñiguez-Moreno, Gutiérrez-Lomelí, Guerrero-Medina, & Avila-Novoa, 2018; Lee, Cappato, Corassin, Cruz, & Oliveira, 2016) in the literature use higher MICs for peracetic acid than the concentrations used in our study; for instance, Oxaran et al. (2018), used a concentration of 0.3% (3000 ppm). In the present study, using even lower values, we managed to obtain good results, indicating that there is no need to use very high concentrations of peracetic acid to inhibit *Staphylococcus* spp.

Sodium hypochlorite is the most common chlorine-based sanitiser, as it has hypochlorous acid (HOCl), and an active component of low molecular weight and no electrical charge. These characteristics facilitate its penetration into the bacterial cell wall, causing lysis and subsequent death of the microorganism (Martin et al., 2016). In our study, it was the most efficient sanitiser, presenting the lowest MIC.

3.3. Evaluation of the effect of sanitisers on biofilm removal

All *Staphylococcus* spp. strains studied were able to form biofilm at 24 and 48 h, according to the results presented in Figs. 3 and 4, respectively.

No significant difference was observed ($p \leq 0.05$) for the control treatment when comparing between 24 h and 48 h, the doubled incubation time was not enough to provide greater biofilm formation. Several studies used longer incubation times for biofilm formation assays, such as Fernandes, Kabuki, and Kuaye (2015) (8-day incubation), and Iñiguez-Moreno et al. (2018) and Xu, Zou, Lee, and Ahn (2010) (10-day incubation).

On the capacity of biofilm formation and its subsequent elimination when dealing with the difference of organic versus conventional dairy products, no statistically significant difference was observed. The fact that the product is organic or not is a very difficult factor to be applied to the formation of biofilms and even includes the proliferation of microorganisms in milk; since countless factors are related to the contamination of dairy products, such as handling, milking, good manufacturing practices (Cicconi-Hogan et al, 2013, 2014; Kousta, Mataragas, Skandamis, & Drosinos, 2010; Latorre et al., 2010; Leriche et al., 2004; Metz, Sheehan, & Feng, 2020; Mullen, Sparks, Lyman, Washburn, & Anderson, 2013; Sospedra, Mañes, & Soriano, 2012; Tenhagen et al., 2018).

In the present study, the minimum inhibitory concentrations of sanitisers used to eliminate planktonic cells from *Staphylococcus* spp. were applied to biofilms formed in 24 and 48 h; only using the times of 5 and 15 min, considering the minimum and maximum time of the previous analysis. As there is no official test for the elimination of biofilms, it was determined for this study that a difference of more than 2 logs and a significant difference, would be considered efficient for the reduction of biofilm; a criterion established based on the minimum growth of controls (water).

For both the 24 and 48 h incubation periods, applying sanitisers for 5 and 15 min, peracetic acid showed no elimination above 2 logs and no statistical difference; indicating that the concentrations used to eliminate *Staphylcoccus* planktonic cells, are not applicable for the removal of biofilms formed. As shown in Figs. 3 and 4, we observe that only a few strains showed significant reductions using the BAC and NaClO sanitisers for 5 and 15 min, and therefore not showing much effectiveness in general.

Biofilm resistance may be due to several factors, including: inhibition of disinfectant agent diffusion by extracellular polymeric substances (EPS), physiological heterogeneity caused by nutrients, and oxygen gradients generated in the biofilm (Iñiguez-Moreno et al., 2018; Mah & O'Toole, 2001). The EPS limits diffusion

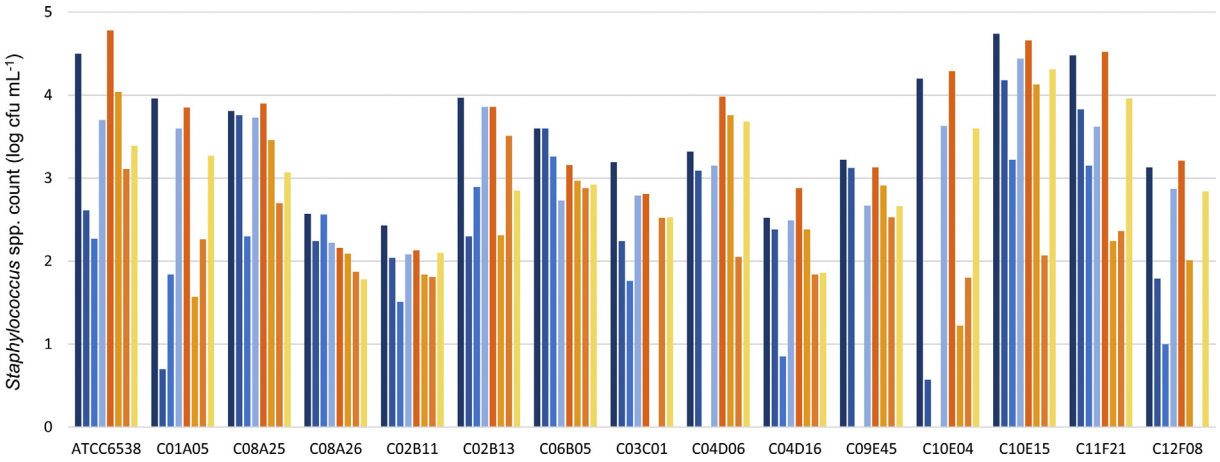

Fig. 3. Efficacy of three tested sanitisers using the proposed protocol on microbial biofilms developed for 24 h on stainless steel coupons: ■,■,■,■, control, benzalkonium chloride, sodium hypochlorite, peracetic acid, respectively for 5 min; ■,■,■,■, control, benzalkonium chloride, sodium hypochlorite, peracetic acid, respectively for 15 min. Data are expressed as log cfu mL^{-1}.

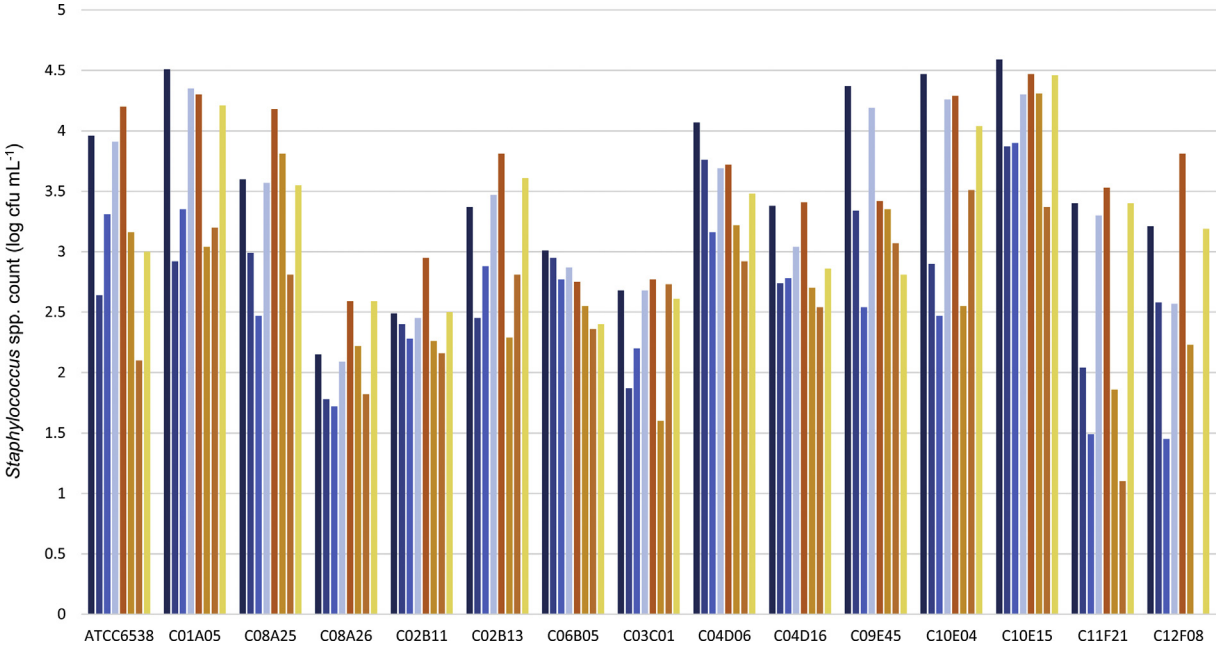

Fig. 4. Efficacy of the three tested sanitisers using the proposed protocol on microbial biofilms developed for 48 h on stainless steel coupons:: ■,■,■,■, control, benzalkonium chloride, sodium hypochlorite, peracetic acid, respectively for 5 min; ■,■,■,■, control, benzalkonium chloride, sodium hypochlorite, peracetic acid, respectively for 15 min. Data are expressed as log cfu mL^{-1}.

of the disinfectant through the biofilm matrix (Iñiguez-Moreno et al., 2018; Mah & O'Toole, 2001). The C11F21 strain (*S. aureus*), which presented the highest MIC for BAC in the three contact times, did not have the main sanitising resistance genes (*qac*A/B and *qac*C) associated with *Staphylococcus*, but it presented the combination of biofilm formation genes *bap*, *ica*A and *ica*D. The presence of these genes may explain biofilm persistence and protection against BAC after 24 and 48 h. Of all 15 strains tested, 10 harbour at least one of the genes involved in biofilm formation. Another explanation for biofilm protection is that the bactericidal agent does not reach the target cells due to reduced diffusion and/or neutralisation of compounds by the biofilm matrix (Bridier, Dubois-Brissonnet, Greub, Thomas, & Briandet, 2011; Fagerlund et al., 2016).

Finally, when evaluating the effectiveness of the 3 sanitisers in two stages (5 and 15 min) for the 24-h incubation period, we can generally say that the best treatments would be BAC or sodium hypochlorite, for both 5 and 15 min time periods; because at least 6 strains showed a significant difference and a reduction greater than 2 logs. The most effective concentrations of BAC would be between 125 ppm and 250 ppm, while sodium hypochlorite would be between 15.6 ppm and 62.5 ppm.

However, for 48 h incubation periods, using the same times and concentrations, biofilms were more persistent to sanitisation; only sodium hypochlorite in 5 and 15 min periods had a significant effect for 3 strains, at the concentrations of 31.3 and 62.5 ppm. Indicating that the longer the biofilm was incubated, the more difficult it was to eliminate or reduce.

In general, the minimum inhibitory concentrations determined by the previous analysis using planktonic cells (free in aqueous media) are not applicable for the removal of sessile cells (adhered to solid stainless steel surfaces). Countless studies corroborate our research (Burgos, López, Aguayo, Pulido, & Gálvez, 2013; Campana & Baffone, 2017; Campana et al., 2018; Chavant, Gaillard-Martinie, & Hébraud, 2004; Gómez, Abriouel, Grande, Pulido, & Gálvez, 2013), and several authors have reported that for different microorganisms and antimicrobials, the inhibition of sessile cells (biofilm) is more difficult to eliminate when compared with its planktonic form. Indicating a need for better cleaning protocols for the food industry.

Although the tests have been developed on static TBS cultures, and this favours the formation of biofilm due to the availability of nutrients, biofilms from strains of different dairy origin can be formed at different stages of processing and surfaces; they can also shear off, contaminating other processes and the final product (Anand, Singh, Avadhanula, & Marka, 2014; Marchand et al., 2012; Sharma & Anand, 2002). Furthermore, the behaviour of bacteria in dairy biofilms is controversial in the presence of dairy fluids, milk or whey. Some studies report a reduction in biofilm formation in the presence of milk protein components, while other studies show an increase (Alonso & Kabuki, 2019; Speers & Gilmour, 1985). In a recently published study, Alonso and Kabuki (2019) demonstrated that biofilms formed by different pathogens (including *S. aureus*) in the presence of different dairy substrates, can show various degrees of shedding over time, especially in the presence of skimmed milk.

It was also demonstrated that even strains that did not contain the main biofilm-forming genes, but contained multiple antibiotic resistance genes, were able to form biofilms and show some persistence to sanitisers. Emphasising the importance of strains with antibiotic resistance genes, they may become an additional adjuvant to hinder the elimination of this microorganism.

4. Conclusion

We observed that the general bactericidal efficacy test of sanitisers was effective for most strains of *Staphylococcus* isolated from both organic and conventional dairy products, except for the peracetic acid which showed lower effectiveness in this test. The minimum inhibitory concentrations (MIC) of the sanitisers tested in this study were efficient to eliminate the bacteria in their planktonic form. However, the same MICs were not efficient to eliminate biofilm formations, suggesting that higher concentrations and/or time are required to eliminate these microorganisms in their sessile form; since resistance to sanitisers is much higher in biofilm microorganisms, than the resistance in free cells. It was not possible to identify a significant difference in the inhibition of strains isolated from organic and conventional dairy products; due to the fact that organic products are a difficult factor to predict resistance or sensitivity to sanitisers and the ability to form biofilms.

Declaration of competing interest

None.

Acknowledgments

The authors thank Espaço da Escrita – Pró-Reitoria de Pesquisa – UNICAMP (Campinas, Brazil) for the language services provided. This work was supported by the São Paulo Research Foundation (FAPESP), São Paulo, Brazil (grant numbers 2016/02118-7 and 2018/00986-7), Programa de Excelência Acadêmica (PROEX), Brazil (grant number 23038.000795/2018-61), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil (grant number 001).

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DISCUSSÃO GERAL

O presente estudo foi dividido em duas pesquisas (separadas em 3 artigos de pesquisa): consistiu em determinar a diversidade bacteriana de laticínios de produção de queijo Minas Frescal orgânico e convencional do estado de São Paulo, por meio de Sequenciamento de Nova Geração (NGS) do gene 16S rRNA e em paralelo caracterizar a resistência e capacidade de persistência (formação de biofilme) de isolados de *Staphylcocccus* spp., presentes nas mesmas amostras que foram sequenciadas. Tais isolados de *Staphylococcus* spp. já tinham sido previamente isolados e identificados em um estudo anterior do nosso grupo de pesquisa (CÂNDIDO et al., 2020).

Para o estudo da diversidade bacteriana foram comparadas 48 amostras de laticínios orgânicos com 48 amostras de laticínios convencionais, dentre essas foram avaliadas amostras de queijos, leite cru, leite pasteurizado e swabs de manipuladores, equipamentos, utensílios e superfícies da indústria, essas amostras foram sequenciadas e sua diversidade bacteriana identificada em famílias e gêneros bacterianos.

No total foram identificadas 51 famílias e 96 gêneros, sendo possível observar que a sua prevalência no geral consistiu nas famílias Enterobacteriaceae (45,4%), Planococcaceae (14,6%), Moraxellaceae (12,6%), Pseudomonadaceae (10,9%), Streptococcaceae (3,4%), Bacillaceae (3,1%), Aeromonadaceae (2.1%), Enterococcaceae (1,3%), Pseudoalteromonadaceae (1.1%), Staphylococcaceae (1,1%), Xanthomonadaceae (1,0%) outras famílias (< 1,0%), e nos gêneros Não identificados (38,3%), *Kurthia* (14,2%), *Pseudomonas* (10,9%), *Acinetobacter* (9,7%), *Escherichia-Shigella* (4,0%), *Bacillus* (3,1%), *Hafnia-Obesumbacterium* (3,0%), *Psychrobacter* (2,9%), *Lactococcus* (2,2%), *Aeromonas* (2,1%), *Enterococcus* (1,3%), *Streptococcus* (1,2%), *Pseudoalteromonas* (1,1%), *Stenotrophomonas* (1,1%), *Staphylococcus* (1,0%) e outros gêneros (< 1%).

Foram aplicados testes estatísticos com intuito de comparar as amostras Orgânicas *Versus* Convencional; a diferença de bactérias encontradas entre amostras de leite pasteurizado *versus* leite cru, queijos *versus* leite pasteurizado, queijos *versus* swabs, sendo essas 3 ultimas comparações aplicadas com objetivo de identificar diferença dos microbiomas nas etapas de produção. Dentre essas condições descritas apenas as famílias

Pseudoalteromonadaceae; Bacillaceae; Pseudomonadaceae e Aeromonadaceae; Vibrionaceae, Weeksellaceae, Pseudoalteromonadaceae, Dermacoccaceae e Aeromonadaceae e gêneros *Pseudoalteromonas*; *Bacillus*; *Pantoea*, *Pseudomonas* e *Aeromonas*; *Macrococcus*, *Staphylococcus*, *Sphingobacterium*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Morganella, Propioniciclava, Enhydrobacter, Solibacillus, Vibrio, Chryseobacterium, Elizabethkingia* e *Dermacoccus* apresentaram diferença estatística significativa, respectivamente a cada condição avaliada.

Por mais que sejam o mesmo tipo de queijo, a diferença encontrada em algumas famílias e gêneros, podem estar relacionadas com as matérias-primas e as condições de fabricação do queijo que podem desempenhar um papel critico na composição microbiológica das amostras. A alta diversidade bacteriana encontradas nas amostras pode sugerir que ela pode ter sido preparada com matérias-primas de alta diversidade bacteriana ou influenciada pela ecologia do ambiente de processamento (LUSK et al., 2012).

Por exemplo na condição avaliada entre produtos orgânicos e convencionais, apenas o gênero *Pseudoalteromonas* e sua família correspondente Pseudoalteromonadaceae, apresentou alguma diferença estatística significativa, apresentando uma maior prevalência em lacticínios de produção convencional. O gênero *Pseudoalteromonas* contém 57 espécies (PARTE et al., 2020). É uma bactéria com capacidade de sobreviver em ambientes extremos, como no fundo do mar e sobrevivem a temperaturas extremante baixas (PARRILLI et al., 2021). Segundo Paulo et al. (2021), que também realizou estudo de microbioma de queijo Minas Frescal, a contaminação, por *Pseudoalteromonas* pode estar relacionado com o sal ou salmoura utilizada no processo (PAULA et al., 2021). Felizmente nenhuma espécie de *Pseudoalteromonas* é descrita como um patógeno humano (Alikunhi et al., 2017; Sánchez-Díaz et al., 2019).

O único gênero que apresentou diferença estatística significativa entre leite pasteurizado e cru, foi o *Bacillus*, e sua respectiva família Bacillaceae. Que teve a capacidade de apresentar uma maior quantidade de *Amplicon Sequence Variants* (ASVs) no leite pasteurizado do que no leite cru. Segundo Kmiha et al. (2017), o gênero *Bacillus* é um importante contaminante do leite devido aos esporos resistentes ao calor, sendo capazes de sobreviver ao tratamento térmico do leite e deteriorar o produto final, sendo possível que eles germinem e se multipliquem após a pasteurização.

O NGS do gene 16S rRNA é uma técnica conhecida por ser capaz de identificar microrganismos que muitas vezes são considerados não cultiváveis por meios de análises microbiológicas convencionais, ou que se encontra com uma baixa carga microbiana (IANNI et al., 2020; QUIGLEY et al., 2012).

Já foi observado por outros autores que é comum encontrar em queijos e outros laticínios microrganismos que poderiam vir até mesmo ser considerados incomuns nesse tipo de alimento (QUIGLEY et al., 2012).

Como é demonstrado na nossa pesquisa uma alta diversidade, isso evidencia a importância que o NGS pode vir a ter na identificação de microrganismos não cultiváveis por métodos tradicionais, que muitas vezes podem estar sendo negligenciados nesse tipo de alimento, mas que podem vir a se tornar microrganismos emergentes.

Em relação à pesquisa de resistência a antibióticos os genes mais prevalentes encontrados foram *bla*Z (25,3%), *mec*A (13,7%), *lsa*(B) (6,3%). Os fenótipos de resistência mais frequente foram observados para os antibióticos penicilina (25,3%), oxacilina (21,1%) e clindamicina (11,6%), fenotípico do qual corrobora com a maior prevalência de seus respectivos genes envolvidos (*bla*Z, *mec*A e *lsa*(B)). Uma maior diversidade e quantidade de genes foi observada em laticínios orgânicos do que no convencional, mas não houve diferença estatística significativa por meio do teste Cochran's Q. Ao contrário dos resultados genotípicos, no fenótipo foi observada uma maior quantidade e diversidade de resistência no sistema de produção convencional, por mais que também não tenha sido estatisticamente significativo. Também foi possível observar que no sistema orgânico tem mais cepas com genes presentes e que não apresentaram o fenótipo correspondente do que as cepas isoladas no sistema convencional, podendo ser um indicativo de que o fentopico não é apresentado devido a baixo contato com antibióticos sintéticos.

O veloz surgimento de genes de resistência a antibióticos tem se tornado um grande problema global (WANG et al., 2021) e um desafio complexo à saúde pública. Planos recentes sobre resistência antimicrobiana destaca a importância de adotar abordagens de *One Health* que podem ultrapassar as fronteiras disciplinares tradicionais (CARS et al., 2016). A estrutura da *One Health*, proclamada por muitas agências globais de saúde, conhece que a resistência antimicrobiana é uma dificuldade inter-domínio no qual a saúde humana, a agricultura animal e o meio ambiente são os componentes

principais e inter-relacionados (TIEDJE et al., 2019). Em todo o mundo, quase 700.000 mortes por ano estão relacionadas a infecções bacterianas de cepas resistentes a antibióticos, número do qual estima-se que irá aumentar para 10 milhões de mortes por ano até 2050, sendo assim o controle da resistência aos antibióticos exigirá amplos esforços globais e multidisciplinares (CABRERA-PARDO et al., 2019).

A resistência a antibióticos de *Staphylococcus* em alimentos tem se tornado cada vez mais comum no mundo todo, principalmente em produtos lácteos, entre eles os queijos ALNAKIP et al., 2019; ARAGÃO et al., 2019; CAI et al., 2021; RODRIGUES et al., 2017; ZAYDA et al., 2020). Cada vez mais se tornando comum a prevalência da resistência a penicilina (CAI et al., 2021; RODRIGUES et al., 2017), eritromicina, clindamicina, (ALNAKIP et al., 2019; CAI et al., 2021; ZAYDA et al., 2020), cefoxitina e oxacilina (ALNAKIP et al., 2019; ZAYDA et al., 2020), tetraciclina, gentamicina (ALNAKIP et al., 2019) em queijos.

O uso generalizado e impróprio de antibióticos na pecuária por muito tempo foi considerado responsável pelo desenvolvimento gradual de resistência em *Staphylococcus* (CAI et al., 2021). Tal resistência dificulta tratamentos de doenças como a mastite nos animais (ZAYDA et al., 2020). A disseminação da resistência a antibióticos na cadeia de alimentos, pode vir a trazer bactérias resistentes até os consumidores (CAI et al., 2021), sendo assim seria de extrema importância o desenvolvimento de programas de prevenção para proteger os consumidores e evitar a disseminação de bactérias resistentes e potencialmente virulentas (RODRIGUES et al., 2017).

A incidência de resistência antimicrobiana tem aumentado em *Staphylococcus*, isso pode ser atribuído a fatores como administração frequente de antibióticos no gado ou o uso indevido e demasiado de antimicrobianos. Portanto, a cadeia alimentar é uma possível via de transferência de resistência, ambos os *Staphylococcus* coagulase positivos e negativos de origem alimentar exibem uma ampla gama de elementos genéticos de resistência antimicrobiana. Dentre os *Staphylococcus* resistentes, o *Staphylococcus aureus* resistente à meticilina (MRSA) é conhecido por ser resistente a vários antimicrobianos sendo o responsável por infecções de difícil tratamento. Portanto, constitui um sério problema de saúde pública, causando milhares de mortes por ano em todo o mundo e constituindo um grande fardo econômico global (OUOBA et al., 2019).

A resistência a diferentes antimicrobianos em *Staphylococcus* está relacionado com o transporte de elementos móveis, como o cassete estafilocócico cromossômico *mec* (SCC*mec*), conferindo resistência à meticilina e reduzindo suscetibilidade a múltiplos agentes antimicrobianos (WANG et al., 2019). Foi possível apenas identificar os SCC*mec* em *S. aureus*, sendo um classificado como tipo I em lacticínio orgânico, e um tipo IVc no lacticínio convencional. Infelizmente nenhum dos *Staphylococcus* coagulase negativa (SCN) submetido a tipagem de SCC*mec*, foi identificado, mas não é algo tão incomum, sendo que a outros trabalhos na literatura que normalmente tem um dificuldade de identificar os SCC*mec* em SCN (CUNY; WITTE, 2005; TANIGUCHI et al., 2019; VANDERHAEGHEN et al., 2012). Existem SCC*mec* que não são tipificáveis, principalmente em SCN isso pode ser devido aos complexos *mec* e *ccr* passam por processos complexos de recombinação e reordenação nos genomas, por meio deles que ocorre a geração de novos tipos SCC*mec*. Muito provavelmente, apenas uma pequena fração da diversidade SCC *mec* está presente em *S. aureus*. Não sendo possível detectá-los com a metodologia estabelecida para *S. aureus* (MARTÍNEZ-MELÉNDEZ et al., 2015), pois ainda a grande maioria dos trabalhos envolvendo a tipificação de SCC*mec*, estão envolvidos com coagulase positivo, em especial o *S. aureus*.

E não se tratando apenas em estudo do SCC*mec* em SCN, mas de forma geral por muito tempo os estudos de resistência e virulência de *Staphylococcus* foram voltados para *S. aureus*, os SCN antes eram considerados constituintes apatogênicos da pele humana saudável e da microbiota da mucosa. Durante as últimas décadas, entretanto, o SCN emergiu como causa comum de infecções nosocomiais (HEILMANN; ZIEBUHR; BECKER, 2019), e sua prevalência, virulência e resistência em alimentos tem sido cada vez mais explorada (CÂNDIDO et al., 2020; CRUZADO-BRAVO et al., 2019; KÜREKCI, 2016; RODRIGUES et al., 2017; SILVA; RODRIGUES; SILVA, 2020). Tanto o *S. aureus* e CNS podem produzir enterotoxinas capazes de provocar intoxicação alimentar (CÂNDIDO et al., 2020; GUIMARÃES et al., 2013; PODKOWIK et al., 2013). O impacto da produção de enterotoxinas por ambos os tipos de *Staphylcoccus* é tanta, que em 2019, foi publicada Instrução Normativa n° 60, de 23 de dezembro de 2019 (BRASIL, 2019), que prevê os padrões microbiológicos de alimentos, e finalmente colocando para categoria de alguns alimentos, em especial os lácteos, a pesquisa de enterotoxinas estafilocócicas (ng/g) e não mais apenas a pesquisa de contagem de estafilococo

coagulase positiva, como era disposto na antiga RDC nº 12, de 2 de janeiro de 2001 (ANVISA, 2001).

Em relação a pesquisa de genes de formação de biofilme e resistência a sanitizanes, houve uma maior prevalência dos genes *ica*D (41,1%), *ica*A (29,5%) (biofilme) e *qac*C (6,3%) (saniziantes QAC).

Os genes *ica*A, *ica*D, são genes frequentemente associados a produção de biofilmes de *Staphylococcus*, encontrados em laticínios, tais genes podem contribuir para persistência dos *Staphylococcus* nos animais facilitando problemas relacionados a infecções como mastite, ou persistência em equipamentos e utensílios na indústria, dificultando sua remoção com chances de contaminação dos produtos finais, e uma posterior intoxicação alimentar (CRUZADO-BRAVO et al., 2019; MARTIN et al., 2016).

Genes *qac*A/B e *qac*C estão relacionados com a classe de sanitizantes QACs, os mecanismos de resistência dos *Staphylococcus* se deve a capacidade que essas cepas tem um sistema de bombas de efluxo, baseados em proteínas transmembrana capazes de transportar uma única classe ou vários compostos, proteínas que transportam dois ou mais antimicrobianos ou biocidas são chamadas de bombas de efluxo multirresistentes. Entre os biocidas, os QAC são amplamente utilizados na indústria alimentícia (MARCHI et al., 2015). Os genes que conferem capacidade de bombas de efluxo em *Staphylcoccus* já tem sido relatados em lacticínios no Brasil (KRONING et al., 2020). Existem estudo que sugerem a correlação de cepas de *Staphylococcus* enterotoxigênicas com resistência a sanitizantes, podendo aumentar as chances de intoxicação alimentar em consumidores. Sendo assim é recomendado a rotação do uso de sanitizantes (HO; BOOST; O'DONOGHUE, 2015).

Após a caracterização dos genes de resistência e formação de biofilme, 14 cepas de *Staphylcooccus* com genes de interesse foram selecionadas para serem avaliadas em relação a sua capacidade fenotípica de resistência a sanitizantes, e formação de biofilmes na superfície do aço inox. Os sanitizantes BAC, NaClO e ácido peracético, foram submetidos ao teste de eficácia de sanitizantes da AOAC, e ao ensaio de Concentração Inibitória Mínima (CIM). Na avaliação do teste de eficácia do sanitizantes o ácido peracético se demonstrou o menos eficaz no geral, BAC e NaClO não apresentou

diferença significativa. Ao avaliar os resultados da CIM sanitizantes BAC se demonstrou o menos eficaz, sendo NaClO não apresentando diferença com o ácido peracético.

A CIM foi realizada com intuito de encontrar a concentração mínima necessária para eliminar os *Staphylococcus*, e avaliar essas concentrações na capacidade de remover os biofilmes. Os biofilmes foram formados em tratamentos de incubação de 24 horas e 48 horas, não apresentando diferença significativa. Todas as cepas avaliadas apresentaram formação de biofilmes, e que as concentrações aplicadas células planctônicas (livres em meio aquoso) não são aplicáveis para a remoção de células sésseis (aderidas a superfícies sólidas de aço inoxidável). Também foi possível perceber que mesmo cepas que não continham os principais genes formadores de biofilme, mas continham múltiplos genes de resistência a antibióticos, foram capazes de formar biofilmes e apresentar alguma persistência ao tentarem ser removidas com sanitizantes. Enfatizando a importância das cepas com genes de resistência a antibióticos, elas podem se tornar um adjuvante adicional para dificultar a eliminação desse microrganismo.

Os *Staphylococcus* é um dos principais patógenos envolvidos em intoxicação alimentar, em partes isso é devido a sua habilidade de formar biofilmes em superfícies de contato com alimentos. As células que podem se desprender de biofilmes maduros são uma fonte de contaminação cruzada na indústria de alimentos (ZHANG et al., 2021). A eliminação do biofilme microbiano é de extrema importância para a garantia da segurança alimentar, a remoção do biofilme maduro é particularmente importante, devido ao seu maior potencial de contaminação cruzada e maior resistência à remoção (SHAO et al., 2020). A presença de biofilme contribui para a disseminação de resistência a antimicrobianos, pois os biofilmes dificultam a penetração dos antimicrobianos, fazendo com que os *Staphylococcus* não sejam inibidos, o biofilme também pode promover a mutação espontânea de *Staphylococcus*, acelerando o surgimento de resistência aos antimicrobianos hereditários, aumenta a capacidade de obter e espalhar os genes responsáveis por tal resistência, sendo assim a formação de biofilmes pode ser um fator importante para aquisição de resistência dos *Staphylococcus* (OU et al., 2020).

A presente pesquisa por mais que dividida em 3 trabalhos, todas corroboram entre si, o NGS utilizado para caracterizar todas as bactérias em lacticínios orgânicos e convencionais, tendo apenas 1 família (Pseudoalteromonadaceae) e 1 gênero (*Pseudoalteromonas*) com diferença estatística, os resultados da pesquisa de genes e antibiogramas sem diferença estatística, os resultados de resistência a sanitizantes e de

formação de biofilmes similares entre os dois tipos de produção de queijo Minas Frescal, juntos são indicadores de que apenas o fato isolado de um lacticínio ser orgânico ou convencional não foi o suficiente para estimar se ele vai ter mais ou menos bactérias, e se tais bactérias serão mais ou menos resistentes.

As pesquisas voltadas em comparação de produtos lácteos orgânicos *Versus* convencional, são focadas em análises químicas, físico-químicas, ou nutricionais, diferenças de componentes químicos ou nutrientes (NØRSKOV et al., 2019), como ácidos graxos (LIU et al., 2020a; SAMARRA et al., 2021), iodo (STEVENSON; DRAKE; GIVENS, 2018; VAN DE KAMP; SARIDAKIS; VERKAIK-KLOOSTERMAN, 2019; WALTHER et al., 2018), nitrogênio (BRITO; SILVA, 2020), até mesmo avaliação de impactos ambientais (KNUDSEN et al., 2019). Raramente é abordada a diferença microbiológica desses produtos, muito menos o uso de métodos moleculares para tal comparação. Estudos que comparam a microbiota de produtos orgânicos com os convencionais, observam que as características microbiológicas entre os sistemas são similares (ABREU et al., 2021; CÂNDIDO et al., 2020; MULLEN et al., 2013), sendo muito difícil atribuir a contaminação microbiológica ao produto ser orgânico ou não, devido a inúmeros fatores que podem estar envolvidos na contaminação dos lacticínios, como por exemplo as contaminações provenientes do úbere (associada à mastite), contaminações cruzadas provenientes de patógenos de outros animais na fazenda, contaminação ambiental, má higiene das superfícies do úbere e da teta e de equipamentos de ordenha não higienizados, manipulação incorreta de ordenadores (KEBA et al., 2020; NDAHETUYE et al., 2020b), resfriamento inadequado durante o transporte, nos lacticínios as contaminações podem vir dos equipamentos, utensílios, manipuladores (NDAHETUYE et al., 2020b), armazenamento ineficiente (VERRAES et al., 2014). Levando em conta que o leite e seus derivados são alimentos ricos em nutrientes, com alta atividade de agua, pH ideal para o desenvolvimento microbiano, tornado um excelente meio de cultura para diversos microrganismos (NDAHETUYE et al., 2020b). A falta de Boas Práticas de Fabricação (BPF), programas de monitoramento e vigilância pode contribuir para a contaminação dos lacticínios (KEBA et al., 2020).

CONCLUSÃO

O NGS se mostrou uma ferramenta da biologia molecular extremante eficaz no quesito de identificação bacteriana, apresentando número de famílias e gêneros identificados em apenas uma corrida, mostrando a praticidade do uso dessa metodologia. Ao comparar com métodos convencionais de análise microbiológicas, existem inúmeros microrganismos não cultiváveis e que muitas vezes são negligenciados na pesquisa e na indústria, sendo que muitas vezes tais microrganismos podem vir a se tornar patógenos ou deteriorantes emergentes na área de laticínios.

As pesquisas realizadas a respeito de *Staphylococcus* spp. resistentes e produtores de biofilmes vem para nos mostrar que tantos produtos orgânicos e convencionais podem ter a presença desse patógeno com capacidade de resistência e persistência, e em paralelo chamar atenção para capacidade de virulência de *Staphylococcus* cogulase negativa, pois na literatura ainda se encontram muitos trabalhos focados apenas em *Staphylococcus* coagulase positiva, principalmente o *S. aureus*. No geral não obteve muitas diferenças estatísticas significativas entre os laticínios orgânicos e convencionais, mas demonstrou quais seriam os antibióticos mais eficazes contra esses patógenos, como por exemplo a alta capacidade do cloranfenicol na inibição dos *Staphylcooccu*s ou qual teve sua menor eficiência como a penicilina.

O estudo do biofilme veio para reforçar que as concentrações utilizadas dos sanitizantes em células de bactérias livres não são tão eficazes para remoção do biofilme, mostrando a importância da correta higienização e de controle nos processos de higienização da indústria de laticínios a fim de prevenir sua formação. E os perigos que associação da capacidade de formação de biofilmes podem vir a ter com os outros genes de virulência.

Por fim, tanto a análise por NGS e de caracterização de *Staphylococcus* spp., corroboram entre si, no sentido de que apenas o fato do produto ser orgânico ou convencional é muito difícil de predizer as diferenças nas características microbiológicas do mesmo, pois inúmeros são os fatores que contribuem para contaminação e proliferação dos microrganismos, como a falta de boas práticas de fabricação na indústria, boas práticas na higiene na ordenha, armazenamento inadequado, contaminação cruzada, entre outros, tais fatores podem afetar tanto os produtos orgânicos ou convencionais, sendo assim muito difícil atribuir a contaminação apenas ao tipo de sistema de produção.

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ASPECTOS ÉTICOS

Este projeto foi submetido ao Comitê de Ética em Pesquisa, uma vez que se tratou de pesquisa com seres humanos. Todavia, foram garantidos os critérios da resolução nº 466/2012 (BRASIL, 2012). O projeto foi aprovado pelo comitê de ética em pesquisa da Unicamp com o número do parecer: 1.383.294. O documento consta no Anexo 1.

ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA DA UNICAMP - CAMPUS CAMPINAS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Diversidade microbiana, caracterização molecular e expressão gênica de Staphylococcus aureus em plantas de processamento de queijo tipo Minas frescal orgânico e convencional

Pesquisador: Nathalia Cristina Cirone Silva

Área Temática:

Versão: 1

CAAE: 51326415.5.0000.5404

Instituição Proponente: Faculdade de Engenharia de Alimentos

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.383.294

Apresentação do Projeto:

A agricultura orgânica possui tratamento dos animais e pastagem diferente da agricultura convencional. Acredita-se que pelo uso de diferente forma de alimentação, prevenção e tratamento de doenças, a microbiota presente na matéria prima orgânica seja diferente da microbiota no produto convencional. Além da diversidade microbiana é possível que haja diferença nos clones de uma mesma espécie, bem como genes de resistência e virulência observados em isolados. Produtos farmacêuticos industrializados sofrem restrições na agricultura orgânica, exceto vacinas, como a de febre aftosa e brucelose em bovinos, cuja aplicação é obrigatória por lei (GADE, 2002; BRASIL, 2011). A alimentação de animais com resíduos de produtos agrícolas pode potencializar a possibilidade de introduzir nos micro-organismos novas propriedades de resistência, deterioração ou risco para a saúde (COOREVITS et al., 2008).Há preocupação por parte dos governos e da Organização Mundial de Sáude quanto ao uso de antibióticos na criação de animais (WHO, 2014, FDA, 2015). O governo dos Estados Unidos, desde 2014, vem desenvolvendo estratégias para limitar a resistência resultante da utilização de antibióticos em animais produtores de alimentos, buscando assegurar que haja antibióticos seguros e eficazes para utilização em animais e seres humanos (FDA, 2015).O gênero Staphylococcus é reconhecido mundialmente como causa de intoxicações alimentares, infeções em humanos e animais como bacteremia,

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infeções de feridas e mastite (HAYAKAWA et al., 2001; OTE et al., 2011, PODKOWIK et al., 2013). Os surtos envolvendo alimentos e o gênero Staphylococcus são comuns tanto no Brasil (LIMA et al., 2013) como no mundo (SATO'O et al., 2014). No Brasil, entre 2000 e 2014 19,50% dos surtos notificados teve o agente etiológico Staphylococcus aureus como causador de surtos de doenças transmitidas por alimentos (DTA) (SECRETARIA DE VIGILÂNCIA EM SAÚDE, 2014).Muitos surtos alimentares aconteceram devido à pouca higiene durante o processamento, preparação ou distribuição dos produtos alimentares somados à falha no resfriamento dos produtos depois de prontos, permitindo o crescimento de S. aureus e a produção de toxinas. Como colonizador frequente da pele e membranas mucosas, S. aureus pode entrar na cadeia de alimentos, principalmente, via manipuladores colonizados (HENNEKINNE et al., 2012).S. aureus é naturalmente suscetível aos antibióticos que já foram desenvolvidos. A resistência é muitas vezes adquirida por transferência horizontal de genes, por mutação cromossômica e seleção aos antibióticos. A susceptibilidade de S. aureus levou à descoberta da penicilina por Alexander Fleming, porém em meados da década de 1940, a resistência à penicilina foi observada em hospitais e em uma década tornou-se um problema significativo na comunidade. A partir desse período até hoje, foi possível notar que S. aureus tem boa capacidade para adquirir resistência a maioria dos antibióticos (CHAMBERS e DE LEO, 2009). A má higienização da planta de processamento pode facilitar a adesão dos micro-organismos às superfícies, consequentemente eles interagem com as mesmas e iniciam a multiplicação celular. Quando a massa bacteriana começa a agregar resíduos e outras bactérias, o biofilme está estabelecido, se tornando uma das principais fontes de contaminação na indústria de alimentos (ZOTTOLA & SASAHARA, 1994).A intoxicação alimentar típica ocorre devido ao consumo de alimentos nos quais houve a produção de uma ou mais enterotoxina de S. aureus durante o crescimento a temperaturas de abuso. A intoxicação do consumidor apresenta um início rápido e costuma variar entre 3 e 5 horas após a ingestão de alimentos contaminados (LOIR et al., 2003). A pasteurização do leite reduz a níveis seguros as células de S. aureus, entretanto, se as enterotoxinas foram sintetizadas no leite cru, o tempo e temperatura desse processo térmico não são suficientes para sua inativação (CARMO, 1997). Sendo comum relatos de surtos ocasionados por enterotoxinas estafilocócicas em leite e derivados lácteos (VERAS et al., 2008, SCHMID et al., 2009, JOHLER et al., 2015).São poucos trabalhos que relacionam micro-organismos em produtos lácteos orgânicos e convencionais (COOREVITS et al., 2008) e também são poucos os que realizaram estudos com Staphylococcus aureus para comparar a diversidade de padrão de resistência e clones desse micro-organismo em derivados lácteos orgânicos e convencionais (SATO et al., 2004, TIKOFSKY et al., 2003). Para a

tipagem e caracterização de S. aureus vários métodos foram desenvolvidos, entre eles, a tipagem por agr, spatyping, SCCmec type e PFGE (Campo pulsado em gel de eletroforese).A identificação de micro-organismos pela microbiologia tradicional pode demorar dias além de não ser possível detectar micro-organismos não cultiváveis ou que não se desenvolvam nas condições utilizadas (OIKONOMOU et al., 2012). O sequenciamento e análise de regiões variáveis dentro do gene 16S rRNA pode ser um método relativamente rápido e de baixo custo para avaliar diversidade bacteriana, podendo ser útil para descoberta e identificação de agentes patogênicos e deteriorantes em alimentos e outras matrizes (KOLBERT e PERSING, 1999).Além da genômica, transcriptoma e bioinformática são ferramentas que podem ser utilizadas a fim de obter uma visão abrangente sobre a fisiologia e virulência de Staphylococcus sp. Que são de grande importância em alimentos e para a saúde pública. A transição de um crescimento a um estado de não-crescimento da bactéria em decorrência de estresse é acompanhado por uma reprogramação do perfil de expressão gênica. Proteínas necessárias apenas em células em crescimento não são mais necessários em altas concentrações em células não-crescimento e a sua síntese diminuiu. Em contraste, expressão de genes que codificam proteínas com específica ou inespecífica funções adaptativas são fortemente induzidos em resposta aos estímulos que restringem o crescimento. O estudo dessas variações podem ser realizados por métodos com abordagem de transcriptomica (BECKER et al., 2007).

Objetivo da Pesquisa:

Objetivo Primário: Observar a diversidade microbiana, e isolar, identificar e caracterizar molecularmente e fenotipicamente isolados de Staphylococcus aureus, provenientes de planta de processamento queijo tipo Minas frescal de produção orgânica e convencional, além de avaliar a expressão de genes utilizando estratégias de transcriptomica.

Objetivo Secundário: - Identificar a diversidade microbiana através do sequenciamento do gene 16S rRNA.- Isolar e identificar cepas de Staphylococcus aureus em planta de processamento de queijo tipo Minas frescal com produção orgânica e convencional.- Realizar o perfil fenotípico e genotípico de resistência dos isolados quanto aos antibióticos: oxacilina, cefoxitina, cloranfenicol, clindamicina, eritromicina, penicilina, tobramicina, tetraciclina e gentamicina.- Identificar genes de resistência a sanitizantes (qacA, qacB e qacC)- Identificar genes associados à formação de biofilme (icaA, icaD, bap, bbp, cna, ebps, eno, fib, fnbA, fnbB, clfA e clfB) e à produção de enterotoxinas (sea-see, seg-selx).- Verificar a diversidade clonal das cepas através das técnicas de spa type e agr type de cepas de S. aureus.- Identificar o tipo do SCCmec dos isolados resistentes a meticilina.- Realizar PFGE nas cepas selecionadas através da observação dos spatyping e perfil genotípico de

enterotoxinas das cepas. - Avaliar a expressão gênica por PCR em tempo real e fenotípica por RPLA (Aglutinação Reversa Passiva com Látex) dos genes sea, seb, sec, sed sob diferentes temperaturas em uma cepa isolada de laticínio orgânico e uma de laticínio convencional que possua cada gene citado.- Avaliar a expressão gênica por PCR em tempo real e fenotípica pelo método de microplaca dos genes icaA, icaD e bap em aço inox e plástico sob diferentes temperaturas em uma cepa isolada de laticínio orgânico e uma de laticínio convencional que possua o gene bap e os genes icaA e icaD concomitantemente. - Caracterizar transcritos por RNA-seq em quatro cepas (produtoras de toxina e biofilme) sob diferentes temperaturas.

Avaliação dos Riscos e Benefícios:

Riscos:

Não há risco previsível ou desconforto para os voluntários participantes da pesquisa.

Benefícios:

No caso de detecção de micro-organismos indicadores, haverá orientação para melhor higienização das mãos.Além disso, com a permissão dos responsáveis pela creche, será apresentada uma pequena palestra sobre boas práticas de manipulação e armazenagem de alimentos para os funcionários responsáveis pela alimentação das crianças.

Comentários e Considerações sobre a Pesquisa:

Trata-se de uma pesquisa da Faculdade de Engenharia de Alimentos do Departamento de Ciências, da Profa Dra Nathalia Cristina Cirone Silva cujo objetivo primário é o de observar a diversidade microbiana, e isolar, identificar e caracterizar molecularmente e fenotipicamente isolados de Staphylococcus aureus, provenientes de planta de processamento queijo tipo Minas frescal de produção orgânica e convencional, que consta com um custeio no valor de R$20.000,00.

Considerações sobre os Termos de apresentação obrigatória:

*A folha de rosto confere com o título do projeto de pesquisa e apresenta a assinatura da pesquisadora responsável e do responsável pela instituição conforme a resolução 466/12 do CNS/MS.

*O cronograma está adequado, conforme compromisso do pesquisador com a resolução 466/12 do CNS/MS, o pesquisador pode executar sua revisão de literatura e outros itens relativos ao planejamento da pesquisa antes da emissão do parecer do CEP, como a seleção dos sujeitos e coleta dos dados através de prontuários, porém a análise dos dados só deverá ser realizada após a aprovação do

CEP. Neste caso a data de início desta fase da pesquisa é recomendável que seja posterior a aprovação do projeto pelo CEP e neste caso está de acordo.

Conclusões ou Pendências e Lista de Inadequações:
*Não há pendências ou inadequações

Considerações Finais a critério do CEP:
- O sujeito de pesquisa deve receber uma via do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado.

- O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado.

- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado. Se o pesquisador considerar a descontinuação do estudo, esta deve ser justificada e somente ser realizada após análise das razões da descontinuidade pelo CEP que o aprovou. O pesquisador deve aguardar o parecer do CEP quanto à descontinuação, exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de uma estratégia diagnóstica ou terapêutica oferecida a um dos grupos da pesquisa, isto é, somente em caso de necessidade de ação imediata com intuito de proteger os participantes.

- O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo. É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

- Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projetos do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma, junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial.

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- Relatórios parciais e final devem ser apresentados ao CEP, inicialmente seis meses após a data deste parecer de aprovação e ao término do estudo.

-Lembramos que segundo a Resolução 466/2012 , item XI.2 letra e, "cabe ao pesquisador apresentar dados solicitados pelo CEP ou pela CONEP a qualquer momento".

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_606272.pdf	12/11/2015 09:05:39		Aceito
Outros	Autorizacao.pdf	12/11/2015 09:04:42	Nathalia Cristina Cirone Silva	Aceito
Folha de Rosto	Folha_de_rosto.pdf	12/11/2015 09:02:35	Nathalia Cristina Cirone Silva	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	09/11/2015 14:17:29	Nathalia Cristina Cirone Silva	Aceito
Projeto Detalhado / Brochura Investigador	projeto_de_pesquisa.pdf	09/11/2015 14:16:50	Nathalia Cristina Cirone Silva	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 04 de Janeiro de 2016

Assinado por:
Renata Maria dos Santos Celeghini
(Coordenador)

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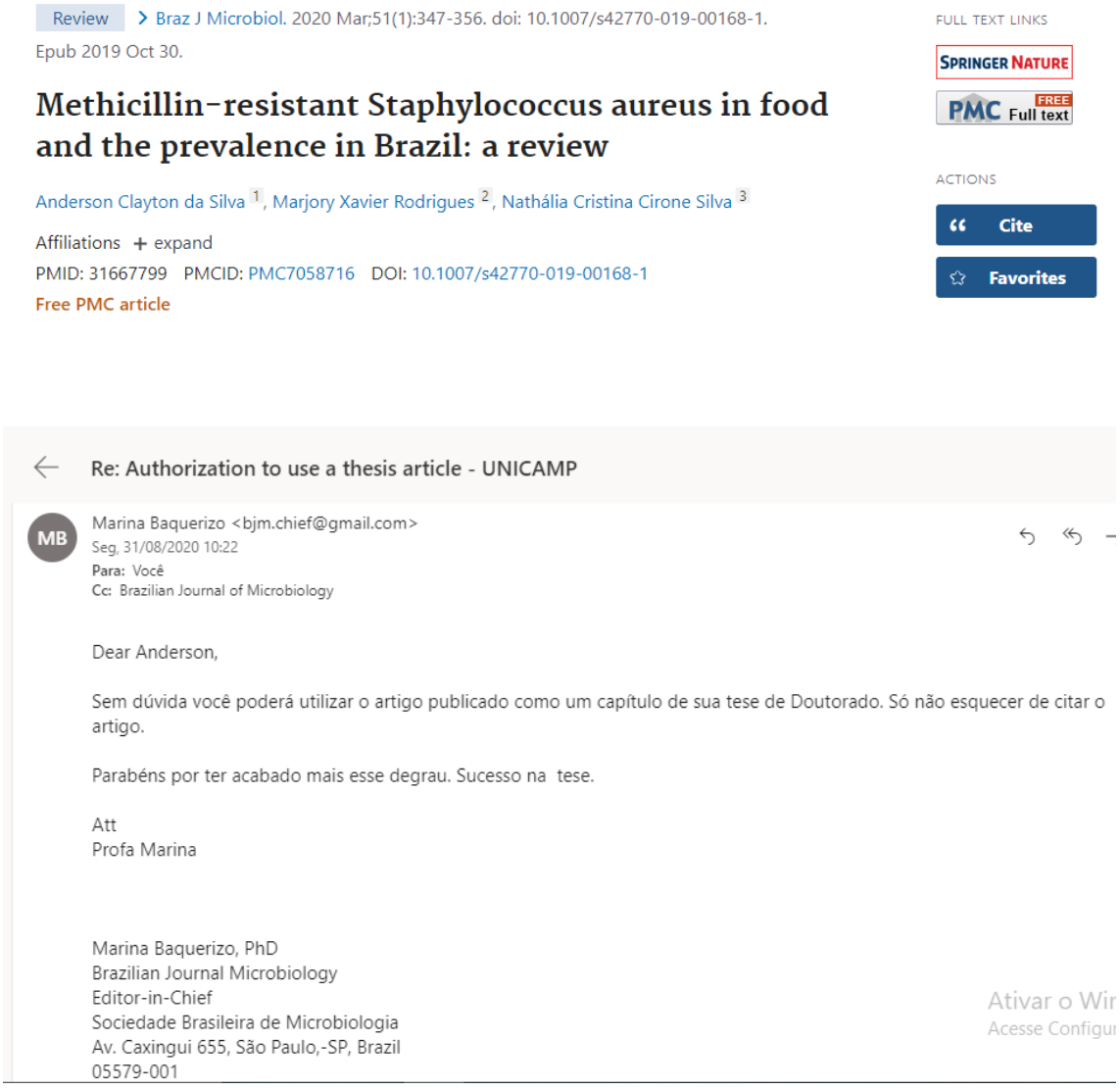
ANEXO 2

DECLARAÇÃO DE AUTORIZAÇÃO DE USO DE CONTEÚDO



Review > Braz J Microbiol. 2020 Mar;51(1):347-356. doi: 10.1007/s42770-019-00168-1. Epub 2019 Oct 30.

Methicillin-resistant Staphylococcus aureus in food and the prevalence in Brazil: a review

Anderson Clayton da Silva [1], Marjory Xavier Rodrigues [2], Nathália Cristina Cirone Silva [3]

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ANEXO 3

DECLARAÇÃO DE AUTORIZAÇÃO DE USO DE CONTEÚDO

ELSEVIER

International Dairy Journal

Available online 30 June 2021, 105139

In Press, Journal Pre-proof

Bacterial diversity in organic and conventional Minas Frescal cheese production using targeted 16S rRNA sequencing

Anderson Clayton da Silva Abreu [a], Marcelo Falsarella Carazzolle [b], Bruna Lourenço Crippa [a], Giovana Rueda Barboza [a], Vera Lúcia Mores Rall [c], Liliana de Oliveira Rocha [a], Nathália Cristina Cirone Silva [a]

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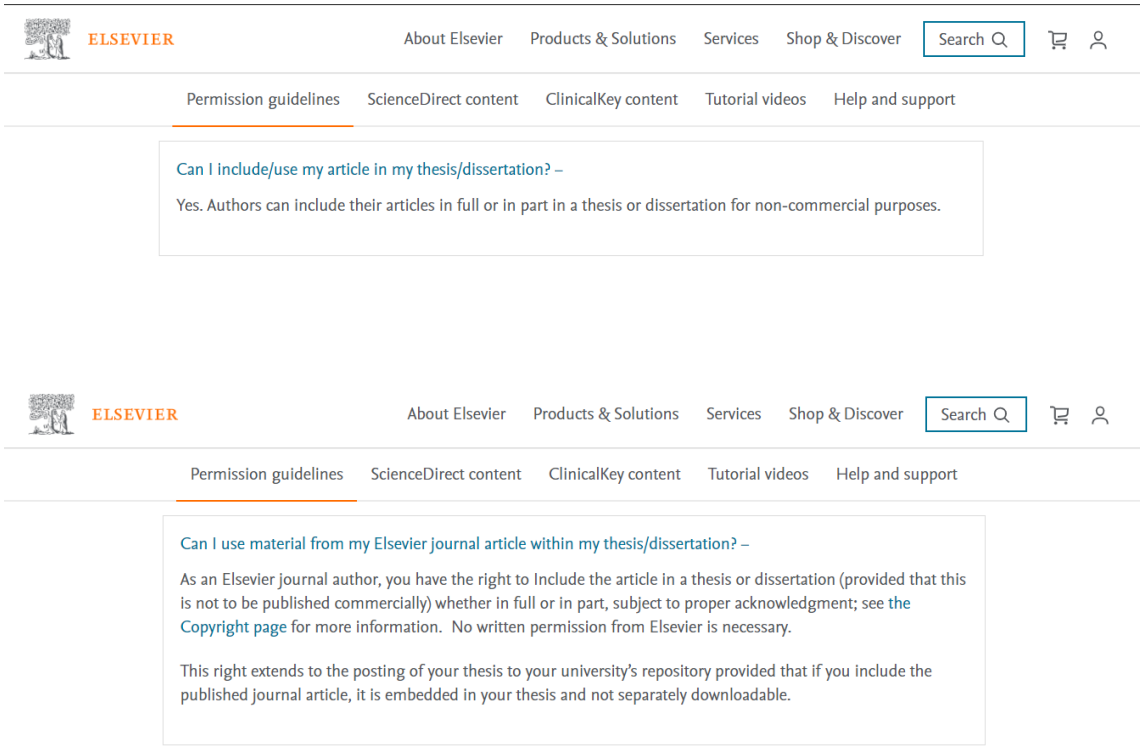

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ANEXO 4

DECLARAÇÃO DE AUTORIZAÇÃO DE USO DE CONTEÚDO

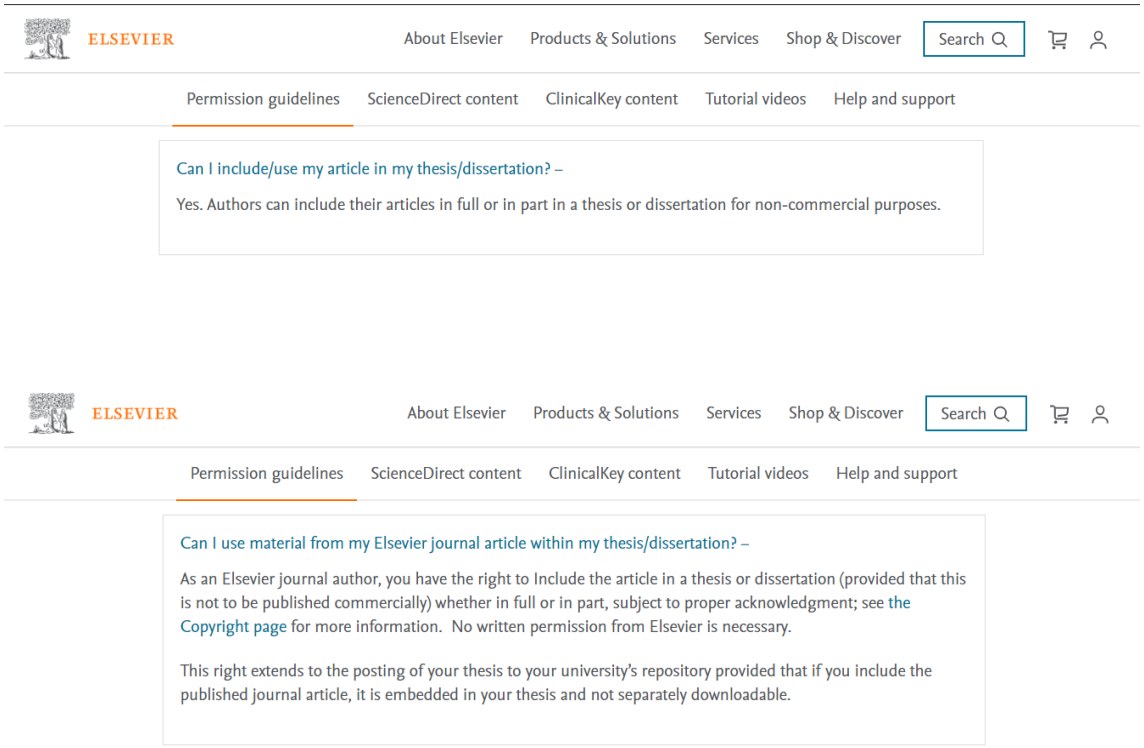
Journal of Dairy Science
Volume 104, Issue 4, April 2021, Pages 4012-4022

Research

Antimicrobial resistance of *Staphylococcus* spp. isolated from organic and conventional Minas Frescal cheese producers in São Paulo, Brazil

Anderson Clayton da Silva Abreu, Luiz Gustavo Matos, Talita Junia da Silva Cândido, Giovana Rueda Barboza, Victória Vilaça Martins Alencar de Souza, Karen Vanessa Munive Nuñez, Nathália Cristina Cirone Silva

Department of Food Science, School of Food Engineering, University of Campinas, Campinas, São Paulo, 13083-862, Brazil



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ANEXO 5

DECLARAÇÃO DE AUTORIZAÇÃO DE USO DE CONTEÚDO

International Dairy Journal
Available online 4 August 2021, 105171
In Press, Journal Pre-proof

Assessment of sanitiser efficacy against *Staphylococcus* spp. isolated from Minas Frescal cheese producers in São Paulo, Brazil

Anderson Clayton da Silva Abreu [a], Bruna Lourenço Crippa [a], Victória Vilaça Martins Alencar de Souza [a], Karen Vanessa Munive Nuñez [a], Jaqueline Milagres de Almeida [a], Marjory Xavier Rodrigues [b], Nathália Cristina Cirone Silva [a]

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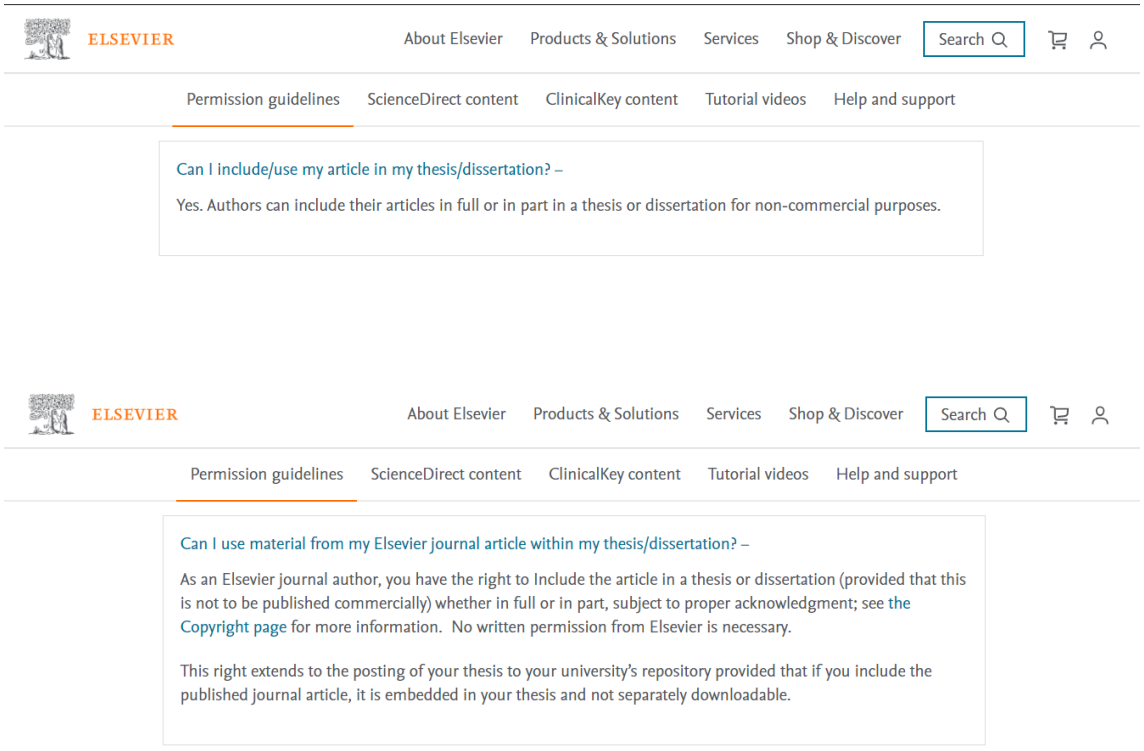

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ANEXO 6

DECLARAÇÃO CADASTRO SISGEN

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CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AC7D05D

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AC7D05D**
Usuário:	**UNICAMP**
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Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Staphylococcus aureus

Título da Atividade:	**Caracterização molecular de Staphylococcus spp. isolado da produção orgânica e convencional de queijo Minas Frescal no estado de São Paulo - Brasil.**

Equipe

Anderson Clayton da Silva Abreu	**UNICAMP**
Nathália Cristina Cirone Silva	**UNICAMP**

Data do Cadastro:	**19/07/2020 14:29:31**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **18:57** de **01/09/2020**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN