UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

BRUNA LOURENÇO CRIPPA

O PAPEL DE *Staphylococcus* não-*aureus* (NAS) NA SAÚDE DO ÚBERE: UM ESTUDO POR MEIO DE ANÁLISE GENÔMICA E FENOTÍPICA

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

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

Orientador: Profa. Dra. Nathália Cristina Cirone Silva

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELA ALUNA BRUNA LOURENÇO CRIPPA, E ORIENTADA PELA PROF(A). DR(A) NATHÁLIA CRISTINA CIRONE SILVA.

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RESUMO

Staphylococcus não-*aureus* (NAS) são frequentemente isolados de casos de mastite e infecção intramamária, causando aumento de células somáticas no leite. Além de estarem abundantemente presentes no ambiente da fazenda, na pele e nas mucosas bovinas. Porém, a relação de NAS com a glândula mamária a nível espécie ainda não está clara. Sendo assim, o objetivo desse trabalho foi identificar espécies NAS isolados de leite de vacas saudáveis e com mastite provenientes de cinco estados do Brasil, sendo eles São Paulo, Santa Catarina, Pará, Paraíba e Goiás, analisar o perfil fenotípico e genotípico de resistência e virulência de todos os isolados, além de correlacionar a presença de NAS com as práticas de manejo adotadas pelas fazendas. Foram analisadas 1,468 amostras de leite de vacas com baixa contagem de células somáticas (CCS) e alta CCS, assim como amostras de leite de vaca com mastite clínica. As amostras foram semeadas em ágar sangue e, em seguida, as colônias características foram isoladas e foram realizados os testes de Gram, catalase e coagulase. Posteriormente, a identificação das espécies foi feita através do MALDI-TOF MS. Um questionário abordando informações sobre manejo adotado nas fazendas foi aplicado e uma análise estatística foi feita com o objetivo de correlacionar o isolamento de NAS com as variáveis relacionadas ao manejo. O perfil de susceptibilidade aos antimicrobianos através do método de disco difusão foi avaliado em todos os isolados. Foi avaliada a capacidade de formação de biofilme no ágar vermelho Congo e em superfície de aço inox. Através da técnica de PCR foram pesquisados genes responsáveis pela formação de biofilme (*bap*, *ica*A, *ica*D, *bbp*, *cna*, *ebps*, *eno*, *fib*, *fnb*A, *fnb*B, *clf*A and *clf*B), genes resistência à sanitizantes (*qac*AB e *qac*C), genes de resistência à meticilina (*mec*A e *mec*C) e genes de enterotoxinas estafilocócicas (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*). Por fim, isolados NAS foram selecionados e tiverem os genomas sequenciados através do sequenciamento completo do genoma (WGS). No total, 309 NAS foram isolados, sendo identificados 18 espécies. Foram identificados NAS resistentes a multiplas drogas (MDR) e NAS resistentes à meticilina (MRNAS). Os genes *eno* e *bap* foram os mais prevalentes entre os genes de biofilme e entre os genes de enterotoxinas, *seb* foi o mais prevalente. A capacidade de NAS de formar biofilme em aço inox foi confirmada. O WGS permitiu expandir os conhcecimentos sobre a patogenicidade de NAS, sendo constatado que NAS carregaram diversos genes de virulência e resistência além de apresentarem diversos elementos genéticos móveis (MGEs), que são responsáveis pela sua evolução e pela transferência de genes. Esses resultados evidenciam o potencial de NAS como causador de doenças não só em animais, mas também em humanos que forem expostos à esses isolados através dos alimentos.

Palavras chaves: Sequenciamento completo do genoma; Saúde Única; Resistência; Virulência; Biofilme;

ABSTRACT

Non-*aureus Staphylococcus* (NAS) are frequently isolated from cases of mastitis and intramammary infection, causing increased somatic cells in milk. In addition, they are abundantly present in the farm environment, on the skin, and in bovine mucous membranes. However, the relationship of NAS with the mammary gland at the species level is still unclear. Therefore, the objective of this study was to identify, at the species level, NAS isolated from the milk of healthy cows and cows with mastitis from five states in Brazil, namely São Paulo, Santa Catarina, Pará, Paraíba, and Goiás. To analyze the phenotypic and genotypic profile of resistance and virulence of all isolates, in addition to correlating the presence of NAS with the management practices adopted by the farms. A total of 1,468 milk samples from cows with low somatic cell count (SCC) and high SCC and milk samples from cows with clinical mastitis were analyzed. The samples were plated on blood agar, the characteristic colonies were isolated, and the Gram, catalase, and coagulase tests were performed. Subsequently, the identification at the species level was made through MALDI-TOF MS. A questionnaire addressing management information adopted on the farms was applied, and a statistical analysis was performed to correlate the isolation of NAS with the variables related to management. The antimicrobial susceptibility profile through the disk diffusion method was evaluated in all isolates. The biofilm formation capacity was evaluated on Congo red agar and stainless-steel surfaces. Genes responsible for biofilm formation (*bap*, *ica*A, *ica*D, *bbp*, *cna*, *ebps*, *eno*, *fib*, *fnb*A, *fnb*B, *clf*A, and *clf*B), sanitizer resistance genes (*qac*AB and *qac*C), methicillin resistance genes (*mec*A and *mec*C) and staphylococcal enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*) were investigated using the PCR technique. Finally, NAS isolates were selected, and genomes were sequenced using whole genome sequencing (WGS). In total, 309 NAS were isolated, and 18 species were identified. Multidrug-resistant (MDR) NAS and methicillin-resistant NAS (MRNAS) were identified. The *eno* and *bap* genes were the most prevalent among the biofilm genes, and *seb* was the most prevalent among the enterotoxin genes. The ability of NAS to form biofilm on stainless steel was confirmed. WGS allowed us to expand our knowledge about the pathogenicity of NAS, and it was found that NAS carried several virulence and resistance genes, in addition to presenting several mobile genetic elements (MGEs), which are responsible for their evolution and gene transfer. These results demonstrate the potential of NAS to cause disease not only in animals but also in humans who are exposed to these isolates through food.

Keywords: Whole genome sequencing; One Health; Resistance; Virulence; Biofilm.

SUMÁRIO

INTRODUÇÃO GERAL

O leite é considerado um alimento completo e necessário em todas as fases do desenvolvimento humano, desde o nascimento até a velhice. Sua qualidade é influenciada por uma série de fatores, dentre os quais os mais importantes são: o manejo dos animais, a alimentação e a sanidade do úbere (JAMAS et al., 2018). O leite é definido como produto obtido de ordenha completa, ininterrupta, em condições higiênicas, de vacas sadias, bem alimentadas e descansadas (BRASIL, 2017). A qualidade do leite é um termo amplo e abrange a segurança sanitária e o valor nutricional deste, sendo definida pela integridade, sabor, inocuidade e valor nutritivo. Existem duas formas de classificá-lo, sendo devido à sua integridade e sua composição. A integridade está relacionada a isenção da adição de substâncias, ou seja, ausência de fraude no produto, além de estar relacionada a deterioração física, química ou microbiológica (ausência de patógenos). Por outro lado, a composição está relacionada com o valor nutricional e industrial do produto (FAGAN, 2008).

Sendo um fluido rico e nutritivo, favorece o crescimento de micro-organismos, classificados como benéficos ou de uso industrial, patógenos ou deteriorantes. Visto isso, até o momento, estudos foram realizados com o propósito de analisar a microbiota do leite, sendo possível através do microbioma identificar os micro-organismos que componham um ecossistema complexo e interativo (QUIGLEY et al., 2013; DASH et al., 2022), relacionado ao leite e/ou à glândula mamária.

O Brasil é o terceiro maior produtor de leite do mundo, com mais de 34,6 bilhões de litros/ ano. No Agronegócio brasileiro, o leite está entre os seis primeiros produtos mais importantes, ficando à frente do milho, café beneficiado e arroz. Ele ainda representa um papel importante no suprimento de alimentos e de geração de emprego e renda para a

população. Para cada real aumentado na produção do sistema agroindustrial de leite, há um incremento, de cinco reais no Produto Interno Bruto do País, o qual coloca o agronegócio do leite à frente de setores como siderurgia e indústria têxtil (EMBRAPA, 2022; USDA, 2023; BRASIL, 2024).

A mastite nos bovinos de leite é uma das doenças mais importantes em todo o mundo e que gera prejuízos econômicos à cadeia produtiva do leite devido à redução da produção de leite, descarte de leite, custos com tratamento veterinário e uso de antimicrobianos. É uma reação inflamatória da glândula mamária em resposta à invasão por micro-organismos. A doença está associada à dor e à redução do bem-estar dos animais afetados (MCDOUGALL et al., 2009, HARAN et al., 2012; RUEGGE, 2017).

Embora o Brasil não possua números oficiais dos prejuízos causados pela doença, estima-se que o impacto econômico alcance até 10% do faturamento das fazendas. Nos Estados Unidos, estatísticas apontam perdas anuais da ordem de US$ 2 bilhões devido à redução na produção, ao descarte do leite e de animais e aos gastos com medicamentos e honorários veterinários (EMBRAPA, 2018). Entre os patógenos causadores de mastite estão os estafilococos, sendo que o maior causador dessas infecções é a espécie *Staphylococcus aureus*, seguido de espécies do grupo *Staphylococcus* não-*aureus* (NAS) (VIDLUND et al. 2022).

Embora considerados como patógenos com menor incidência clínica, NAS foram recentemente identificados como patógenos de mastite bovina relativamente comuns em muitos países (FREU et al., 2024). Na mastite subclínica, NAS são também o segundo achado mais frequente após *S. aureus* (PERSSON et al., 2011) em vacas leiteiras suecas. Em um estudo realizado no Brasil, um número maior de NAS foram encontrados em vacas com mastite comparado com os números de *S. aureus* encontrados no mesmo estudo (OLIVEIRA

et al., 2015). Em outros países (por exemplo, Finlândia, Holanda, Alemanha, e África do Sul), NAS são o achado mais prevalente na mastite (NYMAN et al., 2018). *Staphylococcus* não-*aureus* (NAS) formam um grupo heterogêneo de mais de 70 espécies, das quais aproximadamente 10 foram associados com infecções intramamárias em gado leiteiro (VANDERHAEGHEN et al., 2014; AGUIAR et al., 2022) e abundantemente presentes no leite a granel (DE VISSCHER et al., 2017). À medida que outras espécies de estafilococos se tornam mais comumente associadas com a doença, é de grande interesse aprender mais sobre a distribuição de NAS em fazendas leiteiras.

Staphylococcus não-*aureus* apresentam um alto número de fatores de virulência e o controle da mastite é dificultado pelo fato desse grupo conter muitas espécies diferentes (THORBERG et al., 2009). Um dos fatores de virulência de grande importância está relacionado à habilidade que estes micro-organismos têm em produzir biofilmes (AGUILAR et al., 2001), esse biofilme protege as bactérias da ação dos componentes do sistema imunológico, por dificultar a ação dos fagócitos (FOX et al., 2005), além de agir como uma barreira que dificulta a penetração de agentes antimicrobianos (STEWART, 1996). Além do biofilme, estafilococos são capazes de produzir toxinas, especialmente enterotoxinas que são secretadas nos alimentos e por serem termoestáveis, não são destruídas em altas temperaturas, o que os torna o principal responsável por casos de intoxicação alimentar (ARGUDÍN et al., 2010)

Além dos estafilococos apresentarem esses fatores de virulência, uma das principais preocupações em relação ao controle da mastite é a resistência dos agentes etiológicos aos antimicrobianos. O sucesso na terapia é dificultado pelo número crescente de isolados resistentes as drogas veterinárias usadas. NAS são mais resistentes a antimicrobianos em relação ao *S. aureus* e pode até apresentar característica de resistência a múltiplos fármacos

(FERGESTAD et al., 2021; TAPONEN et al., 2016; VALCKENIER et al., 2019). Essa resistência antimicrobiana vem aumentando nos últimos anos, dificultando o tratamento da mastite. NAS isolados de leite de vacas com mastite apresentaram genes de resistência antimicrobiano (FREY et al., 2013, KHAZANDI et al., 2018) que podem facilitar a infecção em vacas. Além disso, a multirresistência de NAS pode diminuir a taxa de cura da mastite causada por esses micro-organismos (KHAZANDI et al., 2018).

Os estudos genômicos, de fatores de virulência e resistência de espécies individuais ou intimamente relacionadas são necessários para entender o parentesco das espécies dentro deste grupo, bem como sua capacidade de causar doenças. Para se ter um controle e tratamento eficientes da mastite, os agentes causadores da infecção intramamária em rebanhos leiteiros precisam ser bem conhecidos e compreendidos (FREY et al., 2013, LEVISON et al., 2016).

Diversos estudos realizados no Brasil mostram *Staphylococcus* spp. isolados de leite de vaca com mastite em diferentes regiões (ALMEIDA et al., 2020). Porém, a grande maioria não contempla a identificação de NAS a nível de espécie. MÜLLER & REMPEL (2020), analisaram 15 artigos publicados no período de 2012 a 2020 sobre a qualidade do leite produzido no Brasil e três desses artigos, apresentaram o isolamento de *Staphylococcus* spp. nas amostras de leite analisadas, sendo esses estudos realizados nos estados de São Paulo, Minas Gerais e Espírito Santo. Em Pernambuco, MOTA et al., 2012 quantificaram a ocorrência de *Staphylococcus* isolados da mastite bovina e 39,3% das amostras apresentaram esse microrganismo. Dessas amostras, 51,1% foram classificados como NAS e 28,9% como *S. aureus*.

Oliveira et al., 2016 avaliaram a ocorrência de *Staphylococcus* spp. em amostras de leite de vacas com mastite clínica procedentes das Regiões Sul e Sudeste do Brasil. Entre os

microrganismos isolados, 9,31% eram *Staphylococcus* spp., sendo eles 77,77% classificados como estafilococos coagulase negativa (NAS) e 22,22% como estafilococos coagulase positiva. No Rio Grande do Sul, VESCO et al., 2017 avaliaram a prevalência de mastite subclínica entre os anos de 2010 á 2015 e das 2350 amostras coletadas, 44% foram identificadas como estafilococos coagulase negativa, 26% identificadas como estafilococos coagulase positiva e 10% como *Streptococcus* spp.

A principal aplicação do uso de sequenciamento completo do genoma (WGS) para vigilância de patógenos visa a busca por correspondências próximas entre os isolados clínicos e ambientais (MELLMANN et al., 2014; ASHTON et al., 2015; DALLMAN et al., 2016). Além de encontrar "correspondências" semelhantes nos bancos de dados, outra vantagem dos dados WGS como ferramenta de subtipagem para rastreabilidade de patógenos, é a capacidade de reconstruir a história evolutiva dentro de um cluster de isolados clonais, permitindo a identificação de um ancestral comum recente ou uma eventual fonte de contaminação (CHEN et al., 2017; HOFFMANN et al., 2016).

Apesar de terem diversos estudos sobre *Staphylococcus* spp. isolados de leite de vacas com ou sem mastite, a maioria se concentra principalmente em *S. aureus* ou não identifica as espécies NAS. Além disso, faltam estudos que contemplem vários estados brasileiros, considerando diversos parâmetros como: virulência, resistência, dados sobre o manejo e o sequenciamento completo do genoma.

Sendo assim, o objetivo desse trabalho foi identificar as espécies NAS isolados de leite de vacas saudáveis e com mastite provenientes das 5 regiões do Brasil. Analisar o perfil fenotípico e genotípico de resistência e virulência de todos os isolados, além de correlacionar a presença de NAS com as práticas de manejo adotadas pelas fazendas, sendo esses dados obtidos através de um questionário. Dessa forma, os objetivos específicos foram:

- Realizar uma revisão de literatura sobre o papel de NAS na mastite bovina e na Saúde Única;

- Realizar a aplicação de um questionário sobre manejo nas fazendas visitadas;

- Identificar as espécies NAS e avaliar o perfil de virulência e resistência através da busca de genes relacionados a formação de biofilme, produção de enterotoxinas, resistência a sanitizantes e resistência à meticilina pela técnica de PCR, além de caracterizar a resistência antimicrobiana através da realização de antibiograma;

- Correlacionar a presença de NAS com práticas de manejo adotadas nas fazendas onde as amostras de leite foram coletadas;

- Avaliar a capacidade da formação de biofilme de todos os NAS identificados, através do teste do Ágar Vermelho Congo (CRA) e verificar a capacidade de formação de biofilme na superfície de aço inoxidável de isolados NAS pré-selecionados de acordo com a presença de genes de interesse;

- Analisar o genoma de isolados meticilina resistentes através do sequenciamento completo do genoma (WGS).

CAPÍTULO 1

Revisão de literatura: Non-*aureus* staphylococci and mammaliicocci (NASM): their role in bovine mastitis and One Health

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Non-*aureus* staphylococci and mammaliicocci (NASM): their role in bovine mastitis and One Health

Running head: NASM: their role in One Health

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Abstract

Non-*aureus* staphylococci (NAS) are gaining importance in mastitis and public health, and some NAS have been reclassified as mammaliicocci (NASM). Bovine milk production has a major influence on the world economy, being an essential source of income for small, medium and large producers, and bovine mastitis caused by NASM can cause an economic impact. Mastitis generates financial losses due to reduced revenue, increased veterinary costs and expenses associated with animal slaughter. However, it is also a public health issue involving animal health and welfare, human health and the ecosystem. Furthermore, it is an increasingly common infection caused by NASM, including antimicrobial-resistant strains. Despite all these adverse effects that NASM can cause, some studies also point to its protective role against mastitis. Therefore, this review article addresses the negative and positive aspects that NASM can cause in bovine mastitis, the virulence of the disease and resistance factors that make it difficult to treat and, through the One Health approach, presents a holistic view of how mastitis caused by NASM can affect both animal and human health at one and the same time.

Keywords: non-*aureus* staphylococci and Mammaliicocci (NASM); milk; mastitis; public health; food safety; One Health.

Introduction

Milk and milk products are important global dietary products consumed by more than 6 billion people worldwide. In 2021, recorded milk consumption was 928 million tons, making the dairy industry a very profitable market (Codex Alimenentarius Commission, 2016; Food and Agriculture Organization of the United Nations, 2021). However, an infection of the mammary gland caused mainly by bacteria, mastitis, is a major problem affecting animal welfare, productivity and the economy, especially in dairy production, which can lead to losses for the dairy industry (Abera, 2020).

Among the main microorganisms involved in mastitis, we can mention *Staphylococcus* sp., of which *S. aureus* is the most significant mastitis pathogen, whilst the NAS (non-*aureus* staphylococci) are regarded as minor mastitis pathogens. Among all NAS found among cattle, *S. haemolyticus, S. chromogenes, S. epidermidis, S. warneri, S. cohnii, S. simulans, S. hominis, S. capitis, and S. xylosus* are the most prevalent species (Traversari *et al*., 2019). Besides NAS, it is important to highlight the new genus reclassified from *Staphylococcus*, the *Mammaliicoccus*. A study published by Madhaiyan *et al*. (2020) suggests the reassignment of five species of *Staphylococ*cus (*S. sciuri, S. fleurettii, S.lentus, S. stepanovicii* and *S. vitulinus*) to this novel genus, with *Mammaliicoccus sciuri* as the type species. By whole genome sequences, these mammaliicocci species are as distant from staphylococci (64.2 to 67.3% amino acid identity - AAI) as *Macroccus* is distant from *Staphylococcus* (61.1 to 64.3%). Non-*aureus* staphylococci and mammaliicocci (NASM) have become a concern among dairy producers, as they are the most common microorganisms isolated from aseptically collected quarter milk samples (Fergestad *et al*., 2021).

According to the World Health Organization (2022) 420,000 lives are lost due to food poisoning, and *Staphylococcus* spp. are characterized as important agents that can cause foodborne diseases. Poisoning occurs due to ingesting enterotoxins produced in food, including those produced by NASM, and symptoms include vomiting, diarrhea and cramps (da Silva Cândido *et al*., 2020). But there are other problems as well. Political agendas, legislation, development of therapies and educational initiatives are essential to mitigate the increasing rate of antibiotic resistance amoingst pathogenic organisms. In addition to bacteria having the ability to form biofilms, other genes are also involved in antibiotic resistance, and the process of becoming resistant can also occur through mutation at the genetic level (Zaman *et al*., 2017). In addition, it has been suggested that NASM might act as a potential reservoir for resistance genes, which can be transferred and integrated into the genome of *S. aureus* (Vitali *et al*., 2014). Despite being under debate, it is essential to highlight that there are species of *Mammaliicoccus* that are suggested to be the evolutionary origin of the *mec*A gene in *S. aureus* (Lakhundi & Zhang, 2018a).

For the most part, NASM canregarded as minor pathogens that do have the ability to casue mastitis. However, some studies have demonstrated that NAS can reduce infection by *S. aureus* and other pathogens Therefore, this review article aims to address the negative and positive aspects that NASM can have in bovine mastitis, in addition to addressing the importance of adopting the concept of One Health.

Non-*aureus Staphylococcus*

Differences among and within bovine NAS species have been reported regarding epidemiology and ecology (Souza *et al*., 2016), antimicrobial resistance (Fergestad *et al*.,

2021), virulence (Wuytack *et al*., 2020), potential protective traits (Toledo-Silva *et al*., 2021), host-interaction (Piccart *et al*., 2016) and impact on udder health and milk yield (Valckenier *et al*., 2019, 2020, 2021) as recently reviewed by De Buck *et al*. (2021). Non-*aureus* staphylococci can be present in a broad range of ecological habitats, which includes bovine teat canals (Traversari *et al*., 2019) and teat apices (Adkins *et al*., 2018) as well as the farm environment including bulk tank milk, bedding (Adkins *et al*., 2022;) and feces (Wuytack *et al*. 2020). *S. chromogenes* and *S. epidermidis* are predominantly isolated from milk. Therefore, they are considered host-adapted species, whereas *S. equorum* and *S. devriesei* are mostly found in the environment and are classified as environmental NAS species (Adkins *et al*., 2022). Other species, such as *S. haemolyticus,* originate from various (extra)mammary sites and so are labeled opportunistic (Adkins *et al*., 2022). Unfortutely, multiple studies contradict this current species-stratification (De Visscher et al., 2014; Supré et al., 2011; Thorberg et al., 2009; Vanderhaeghen et al., 2015), and these confounding observations could be attributed to strain differences within NAS species. Wuytack *et al*. (2019), for example, suggested in a recent study on strain distribution of NAS in multiple bovine-associated habitats that different NAS strains within a species appear to be habitat-specific or site-specific.

After several taxonomic reclassifications, introducing the coagulase test enabled differentiation between CoNS and CoPS (Fairbrother, 1940). Non-*aureus* staphylococci is a group of Gram-positive bacteria that were before known as coagulase-negative staphylococci (CoNS); they are usually found in the normal flora of humans and different animals, and some species are recognized as human and facultative animal pathogens (Belhout *et al*., 2022). For a long time, only *S. aureus* was considered to be harmful to its host, however, as a consequence of patient- and procedure-related changes, NAS now includes a large number

of nosocomial microorganisms, with *S. epidermidis* and *S. haemolyticus* being the most significant ones (Pereira-Ribeiro *et al*., 2019).

Some of the pathogenicity of NAS strains is probably due to their ability to adhere to surfaces and form biofilm on polymer surfaces of medical devices used during the hospitalization process in hospitals (Le *et al*., 2019;). In addition, NAS were also recognized to be capable of "hiding" from the immune system by being internalized by nonprofessional phagocytes, a mechanism that is mediated by the AtlE gene that helps the microorganism not only bind but also be phagocyted by human endothelial cells (Christner *et al*., 2010). Besides that, NAS showed antimicrobial resistance and carried multiple resistance genes, which can be shared with other species (Lee *et al*., 2020).

NAS brings issues not only to human health but also to animals. They are often found as the majority in bovine intramammary infection, especially in dairy heifers, and several studies in different areas of the globe have found NAS in different proportions in bovine udder, mainly in non-bred or first lactation animals (De Buck *et al*., 2021). This could have happened because the NAS group was considered to comprise minor pathogens and so were typically not treated. However, grouping these species contributed to a better understanding of their distribution and importance to the animal's health care (Condas *et al*., 2017).

Despite NAS being a large group, we can highlight some individual species. *S. epidermidis* is recognized as a pathogen and is the most important cause of nosocomial infection (Hugh & Ellis, 1968). This species can also adapt quickly to extreme conditions. For example, to survive in a place with a high salt concentration, the species has a different number of ion/proton exchangers and systems for osmo protectants (Gill *et al*., 2005), and carries many genes that allow it to survive in these severe situations (Otto, 2009). Also, it is known that *S. epidermidis* is capable of producing an extracellular polysaccharide adhesin,

termed polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine, by ica operon-encoded enzymes. This is a significant virulence factor of the specie, helping them to colonize medical devices and leading to device-related infections (Le *et al*., 2019). Besides this, they can also produce different toxins (Namvar *et al*., 2014). However, studies suggest that this bacterium has evolved to play an important role in maintaining the microbiomes of humans and other mammals (Otto, 2009).

Due to its potential to produce biofilm, *S. haemolyticus* is also a critical species for nosocomial infections in humans, especially from blood infections. However, there is not as much information for them as for *S. epidermidis* (Barros *et al*., 2015). When the resistance to β-lactam antibiotics is considered, *S. aureus* and *S. haemolyticus* present enough similarity to say that they have the exact same resistance mechanism (Czekaj *et al*., 2015).

In several countries, *S. chromogenes* has been identified as the most prevalent species in milk samples from healthy cows and those with subclinical mastitis and clinical mastitis (Valckenier *et al*. 2020). *S. chromogenes* could overcome the physical barrier of the udder and form biofilms. In addition, this species is more adapted to the cow's mammary gland and may become a microbial reservoir and possible source of infection (Isaac *et al*., 2017; Tomazi *et al*., 2015). Molecular studies have also shown that *S. chromogenes* has several antibiotic resistance genes, in addition to having this resistance tested and confirmed in phenotypic tests (Qu *et al*., 2019).

Mammaliicoccus

Mammaliicoccus are Gram-positive, non-motile, non-spore-forming, paired cocci cells with aerobic to facultative anaerobic growth. They are catalase-positive and possess variable oxidase activities. The G+C conent varies from 31.6 to 35.7% in genomes that range from

2.44 to 2.81 Mbp. Based on the analyses of 16S rRNA gene sequences, five species (*M. sciuri, M. fleurettii, M. lentus, M. stepanovicii*, and *M. vitulinus*), are now in this novel genus (Madhaiyan *et al*., 2020) and are as distant from *Staphyloccus* species as *Macrococcus* (another genus) is from *Staphylococcus*. Recognized to colonize humans and animals, they are also considered potentially opportunist pathogens capable of affecting the immune system (Beuckelaere *et al*., 2021). Like the NAS, this group may also cause mastitis in different species, and/or may serve to transmit resistance genes to other more pathogenic species, as suggested by the study of Lienen *et al*. (2022) where *M. sciuri, M. lentus, M. fleurettii* and *M. vitulinus* were isolated from milk and swab samples collected from German dairy cows.

M. sciuri (previously *Staphylococcus sciuri*) is usually found on the skin of wild and domestic animals. Based on their ribotype pattern, the species was divided into three different subspecies called *S. sciuri* subsp. *sciuri*, *S. sciuri* subsp. *carnaticus* and *S. sciuri* subsp. *rodentium* (Nemeghaire *et al*., 2014). In addition, some studies suggest this species as the origin of the *mec*A gene, which is located on a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec), and is the gene responsible for methycillin-resistance in *S. aureus* (MRSA) or more generally in *Staphylococcus* spp (MRSS: Couto *et al*., 1996; Fuda *et al*., 2007). Whilst being characterized as a typical colonizer of a wide range of animals, this species has also been associated with various health problems including skin disease in goats and pigs (Nemeghaire *et al*., 2014). Despite displaying various virulence factors (lipolytic, proteolytic and hemolytic), these bacteria are not related to infection in humans, except after previous infection due to a pathogen (Nemeghaire *et al*., 2014).

M. lentus (previously *S. lentus*) was first characterized as a subspecies of *S. sciuri*. However, after analysis of DNA-DNA hybridization, it was placed into a new species

(Schleifer *et al*., 1983). It was originally isolated from poultry and other animals and can produce enterotoxins, but only to a minoir extent (less than 100ng/ ml supernatant: Irlinger, 2008). Guerre et al. (1999) showed that the enterotoxigen strain of *M. lentus* inoculated in milk for the production of Camembert-type cheeses does not produce enterotoxins despite high levels of the microorganisms.

For *M. fleurettii* (previously *S. fleurettii*), it was also possible to identify enterotoxin production, and some studies suggest that this species, rather than *S. sciuri,* is the origin of the *mec*A gene in MRSA. However, the exact origin is still debated (Lakhundi & Zhang, 2018a). In this context, *M. stepanovicii* was also reported with the presence of the gene **(**Loncaric *et al*., 2013). Table 1 provides a complete list of NASM and the different samples from which they were isolated.

Table 1. NASM isolated from different samples (human, animal, environment, and food) and different countries.

Specie	Prevalence (%)	Sample collection	Number of samples	Presence of enterotoxin	Place	Reference
S. arlettae *S. xylosus* *M. sciuri*	12 12 12	Subclinical mastitis milk samples	209	*seb* (16,6%) No presence *sei* (8,3%)	China	(J. Xu *et al*., 2015)
S. chromogenes *S. simulans* *S. xylosus*	49 17 12	Intramammary infection	5,434	Not analyzed	Canada	(Condas *et al*., 2017)
S. equorum	7	Rectal feces	80	Not analyzed	Belgium	(Wuytack *et al*. 2020)
S. chromogenes *S. xylosus* *S. vitulinus*	29 9 9	Quarters from 82 heifers	324	Not analyzed		(Valckenier *et al*., 2020)
S. chromogenes *S. simulans* *S. warneri*	49 23 5	82 dairy cows	328	Not analyzed	Finland	(Taponen *et al*., 2008)
S. chromogenes *S. haemolyticus* *S. warner*	47 32 7	Composite milk samples	441	Not analyzed	Argentina	(Raspanti *et al*., 2016)
S. chromogenes *S. epidermidis* *S. capitis*	30 13 11	Quarter milk samples	4220	Not analyzed	Netherlands	(Sampimon *et al*., 2009)
S. warneri *S. chromogenes* *S. xylosus*	37 33 23	Udder quarters	46	Not analyzed	Poland	(Malinowski *et al*., 2006)
S. chromogenes *S. haemolyticus* *S. simulans*	48 18 7	Milk samples	604	Not analyzed	USA	(Jenkins *et al*., 2019)
S. epidermidis *S. hominis*	75 15	Human Nasal swab	176	Not analyzed	Germany	(Martínez-Meléndez *et al*., 2021)

S. warneri/ S. pasteuri	7,1					
S. epidermidis	57,9	Prosthetic joint infection	113	Not analyzed	USA	(Tande *et al*., 2014)
S. capitis/caprae	5,3					
S. lugdunensis	7,9					
S. epidermidis	83,3	Prosthetic joint infection	6	Not analyzed	Poland	(Bogut *et al*., 2014)
S. warneri	16,7					
S. epidermidis	56,7	Blood, pus, wound swab, drain fluid, tracheal aspirate, peritoneal fluid, and pleural Fluid	120	Not analyzed	India	(Bora *et al*., 2018)
S. haemolyticus	21,7					
S. lugdunensis	11,7					
S. epidermidis,	80,7	Human nasal swab among patients undergoing haemodialysis	71	Not analyzed	Poland	(Kozioł-Montewka *et al*., 2006)
S. lugdunensis	7,7					
S. haemolyticus	3,8					
S. warneri	3,8					
S. capitis	3,8					
M. sciuri	58,8	Environment	17	Not analyzed	German	(Schnitt *et al*., 2021)
S. lentus	17,6					
S. fleurettii	5,8					
S. epidermidis	5,8					
S. haemolyticus	5,8					
S. cohnii	5,8					
S. haemolyticus	1	Ready-to-eat raw fish	200	Not analyzed	Japan	(Hammad *et al*., 2012)
S. haemolyticus	1					
S. pasteuri	1					
S. intermedius	25	Meat	286	*sec* (12,5%)	Jordan	(Al-Tarazi *et al*., n.d.)
S. warneri	12,5			No presence		
S. lugdenesis	12,5			No presence		
S. epidermidis	6,3%			*sec* (66,6%)		

M. sciuri	3,1	Bicycles/ Environment	79	*sec* (100%)	China	(Z. Xu *et al*., 2019)
S. intermedius	3,1			No presence		
S. haemolyticus	29,0					
S. hominis	29,0			Not analyzed		
S. epidermidis	14,0					
S. saprophyticus	33,3	Cheese/ Raw milk/ Pasteurized milk/Food handlers /Environment/ Brine process, and Clot	229	*seb* (2,1%), *see* (2,1%) *seg* (3,2%) *seh* (1,1%), and *sei* (1,1%)	Brazil	(da Silva Cândido *et al*., 2020)
S. warneri	15,8			*sea* (1,1%), *see* (1,1%), *seg* (1,1%), and *seh* (3,2%)		
S. xylosus,	12,3			*sed* (1,1%), *see* (1,1%), *seg* (2,1%), *seh* (1,1%), and *sei* (1,1%)		
S. epidermidis	10,5			*see* (1,1%) and *seg* (1,1%)		
S. chromogenes	8,8			*see* (2,1%) and *seg* (1,1%)		
S. hyicus	5,3			No presence		
S. haemolyticus	2			*see* (2,1%) and *seh* (1,1%)		
S. agnetis, S. capitis, S. kloosii, S. pasteuri, M. sciuri and S. simulans	1,8			*seg* (1,1%), *seb* (1,1%), and *seh* (1,1%)		

NASM as a cause of mastitis

NAS forms a heterogeneous group of more than 50 species, of which approximately ten are associated with intramammary infections in dairy cattle and are abundantly present in bulk milk (Vanderhaeghen *et al*., 2015). As other species of Staphylococci become more commonly associated with the disease, it is of interest to learn more about the distribution of NAS on dairy farms. They have many virulence factors, and mastitis control is complicated because this group contains many species (Thorberg *et al*., 2009).

The main ways to diagnose subclinical mastitis from NAS are from the results of somatic cell count (SCC) and culture. Tomazi *et al*. (2015) observed that mammary quarters infected by NAS had elevated SCC (>306,000 vs <65,000 in contralateral uninfected quarters), without any affect on milk yield or contents of fat, crude protein, casein, lactose, total solids, and solids-not-fat. Although considered as pathogens with a lower clinical incidence, NAS infections have been identified as relatively common (Gao *et al*. 2017; Persson Waller *et al*. 2011; Heikkilä *et al*. 2018), clinical cases being due mainly to *S. chromogenes*, *S. simulans* and *S. haemolyticus* (Persson Waller *et al*., 2011). In subclinical mastitis cases, NAS are the second most commonly identified pathogen after *S. aureus* in Swedish dairy cows, *S. epidermidis* followed by *S. chromogenes, S. simulans,* and *S. haemolyticus* being the most common (Persson Waller *et al*. 2011). Another study isolated *S. chromogenes, S. fleurettii, S. haemolyticus, S. sciuri, S. simulans, S. succinus* and *S. xylosus* from mastitic bovine milk (Khazandi *et al*., 2018).

The main pathogenic factors of NASM

According to Vanderhaeghen *et al*. (2014), there are three virulence factor groups involved in the pathogenesis of staphylococcal infections: secreted proteins (e.g.,

superantigens, cytotoxins and tissue-degrading enzymes), cell surface-bound proteins (e.g. microbial surface components recognizing adhesive matrix molecules: MSCRAMM) and cell wall components, with the polysaccharide capsule and lipoteichoic being examples of those. Naushad and Naqvi (2019), using whole genome sequencing analyzed the distribution of 191 virulence factors in 441 genomes of 25 NAS species; the authors reported virulence factors in functional categories such as toxins (n = 93), iron metabolism (n = 29), adherence (n = 28), exoenzymes (n = 21) and immune evasion (n = 20). Another study by Wuytack *et al*. (2020) compared the presence of virulence genes (*agr*A, *bap*, and cap5H and the *mec*A methicillin resistance gene) of NAS isolated from the milk samples of healthy quarters and quarters with clinical mastitis. Based on this comparison, they suggested that NAS has the potential to cause mild clinical mastitis.

Enterotoxins

Staphylococcal poisoning is widespread and mainly transmitted by food products, as it can affect healthy people. NAS is also able to produce enterotoxins, which can be one of the causes of many food diseases (da Silva Cândido *et al*., 2020). Enterotoxins belong to a group of extracellular single-chain proteins that are water-soluble and resistant to proteolytic enzyme action in the digestive system. This means that they remain active after ingestion and can compromise human health and, consequently, the public health system. These proteins are also thermostable and resist thermal treatments such as pasteurization (Vanderhaeghen *et al*., 2014). There are more than 25 types of staphylococcal enterotoxins (SE) and enterotoxin-like (SEL) toxins; the former have an emetic effect in primates, whilst the latter do not. The most known of the enterotoxins are the classical serotypes (SEA through SEE: Bergdoll *et al*., 1971), followed by the non-classical SEG through SET as well as SEY and more recently SE02 (Munson *et al*., 1998; Omoe *et al*., 2013; Ono *et al*., 2008, 2015, 2019; Su & Lee Wong,

1995; Suzuki *et al*., 2020). The SELs identified until today are SELJ and then SELU through SELX, SELZ and SEL 01 (Hisatsune *et al*., 2017; Letertre *et al*., 2003; Spoor *et al*., 2015; Thomas *et al*., 2006; Wilson *et al*., n.d.; Zhang *et al*., 1998). NASM are capable of producing the majority of these (da Silva Cândido *et al*., 2020; Rodrigues *et al*., 2017). However, this has largely been ignored by authorities as NAS can establish a commensal relationship with humans and animals since they are also part of their normal microbiota (Podkowik *et al*., 2013).

When de Freitas Guimarães *et al*. (2013) studied 263 isolates of *Staphylococcus* spp. from mastitic bovine milk samples they were able to show that 49% were NAS, and of these 66% contained the gene responsible for enterotoxin production, with *sea, seb* and *sec* (producing the equivalent classical SEs) being the ones that appeared most. In addition, Ünal and Çinar (2012) found that 46% of isolated NAS tested positive for having one or more SE genes, with 63% out of the total presenting the *seh-sej* gene, which belongs to the non-classical SEs. Corroborating these results, Rodrigues *et al*. (2017) characterized NAS isolated from raw milk, Minas cheese, and Minas cheese production lines. They detected the genes *seb, seg, seh, sek* and *seu* in the NAS species identified.

Biofilm formation

Different species of bacteria can live in communities, adhere to an inert or living surface, and be protected by a polymeric matrix called a biofilm. This matrix is a self-product of its metabolism, consisting mainly of sugar and proteins (Shemesh & Ostrov, 2020). Biofilm formation in staphylococcal species is associated with a number of factors, and several studies in the literature show NAS as carriers of biofilm genes. Production of PIA is mediated by the genes *icaA, icaB, icaC* and *icaD*, representing an important contribution to

cell-cell adhesion (Srednik *et al*., 2017). One study observed biofilm formation in 50% of the NAS strains studied (Rumi *et al*. (2013). Naushad *et al*. (2019), in a study based on whole-genome sequencing data for 441 isolates from 25 species, identified that the *icaC* gene of the ica operon, believed to be involved in biofilm formation, was the second most frequent gene, and it was present in 17 isolates out of all species studied. Other genes of the ica operon, namely *icaA*, were found in eight species, *icaB* in seven species and *icaD* in eight.

Bap is another protein involved in biofilm formation, which is responsible for adhesion. In their research, Piessens et al. (2012) showed that 11.2% (41/366) of NAS were bap gene positive. Zuniga *et al*. (2015) isolated 67 strains of NAS from subclinical bovine mastitis and tested for the presence of the *bap* gene; they reported that 45 (67.2%) isolates tested positive and could thus produce biofilms. Rohde *et al*. (2007) found that nearly 90% of *S. epidermidis* strains produced a biofilm, and up to 27% of biofilm-positive *S. epidermidis* isolates produced PIA-independent biofilms, which involves the expression and accumulation of the associated protein. This indicates that, besides Bap, other proteins are involved in ica-idependent biofilm formation**.** Also, NAS produces MSCRAMM proteins, which mediate staphylococcal adherence to the host's extracellular matrix components. In addition to the operon *ica* that produces PIA adhesion, these components also form a biofilm (Christner *et al*., 2010).

Microbial surface components recognizing adhesive matrix molecules (MSCRAMM)

Adhesion to host proteins is usually mediated by bacterial cell-wall-associated proteins called MSCRAMMs (Walsh *et al*., 2008). A gene related to biofilm formation is *embp*, which is a MSCRAMM that plays a role during primary attachment to conditioned surfaces (Christner *et al*., 2010). According to Turchi *et al*. (2020), this was their study's most

frequently detected gene, followed by *eno* and *fbe*, then *altE*. Srednik *et al*. (2017) showed in their research that NAS isolated from cows with mastitis can produce biofilm in vitro, and they also showed that 90% of the isolates tested positive for one of the above genes.

Zuniga *et al*. (2015) also studied the components on microbial surfaces that recognize the adhesive matrix and found the presence of the *can* gene in 17% of isolates, and the *eno* gene at 82.1% was the most prevalent gene among all. The *ebpS* gene was positive in 17.9%, and there were 72.6% strains positive for the *fnbA* gene and 5.7% for the *fnaB* gene. The *fib* gene was among the most prevalent, present in 76/106 (71.6%), and the *bap* gene was in 60/106 (56%). Darwish and Asfour (2013) identified the presence of the *eno* gene in 92.6% of all 68 NAS isolates. Simojoki *et al*. (2012) also showed a prevalence of the *eno* gene, which was positive in 75% of the 84 studied strains, and the genes *bbp* (1.2%), *cflA* (4.8%), *cflB* (3.6%), *ebpS* (4.8%), *fib* (4.8%), *fnbA* (3.6%), and *fnbB* (2.4%) were also detected. The *cflA* gene is involved with adhesion and immune evasion whilst the *ebpS* gene is responsible for encoding elastin-balding proteins present in the extracellular matrix. Fibrinogen-binding proteins are encoded by the *fib* gene, and fibronectin-binding protein A and fibronectin-binding protein B are encoded by the *fnbA* and *fnbB* genes respectively (Zuniga *et al*., 2015).

Resistance to antibiotics

Biofilm formation is also connected to antibiotic resistance. According to Srednik *et al*, (2017), NAS isolates growing within biofilms are less susceptible to antimicrobials commonly used on farms. Antibiotics are manufactured at an estimated scale of about 100,000 tons annually worldwide, and their use profoundly impacts bacterial life on Earth. More pathogenic strains have become antibiotic-resistant, and some have become resistant to multiple antibiotics, known as multidrug resistance (Nikaido, 2009). El-Seedy *et al*. (2017)

tested how biofilm formation could affect antibiotic resistance, and a total of 95 NAS were identified and tested for biofilm formation, of which 95.8% were resistant to ampicillin, 77.9% were resistant to cefoxitin, 35.8% were resistant to cefuroxime and 31.6% were resistant to amoxycillin.

Besides biofilm formation, there are also genes involved in antibiotic resistance. Xu *et al*. (2015) studied the presence of some of these genes in 76 NAS isolates. They reported the following results: the most prevalent gene was *linA*, which gives resistance to lincosamides, followed by *tetK*, which is responsible for resistance to tetracycline. Conferring resistance to penicillin, the gene *blaZ* was found in 30.3% Also present were the genes *aacA – aphD* which provide resistance to aminoglycoside. Another gene studied was *msrA/B*, which was positive in 17.1% and 19.7% of isolates, respectively, conferring resistance to macrolide and erythromycin. The gene *mecA* is one of the most studied genes, and it is responsible for methicillin resistance and was positive in 17.1% of isolates; the genes *ermB/C* that confer resistance to erythromycin and clindamycin were present in 9.2% and 13.2% respectively whilst the genes *aac (6')/aph (2')*, responsible for resistance to streptomycin, were present in 10.5% of the strains (Kumar *et al*., 2010).

Another study identified 109 NAS, all of which were positive for the *blaZ* gene, and 73% of isolates had the *mecA* gene, both of which resist β-lactam groups. The genes that grant resistance to tetracycline *tetK*, *tetL* and *tetM* all showed a high presence, and other genes identified in a significant amount of isolates were the MLSb resistance genes, *ermB*, *inuA*, *msrA*, and *mphC*. In addition, *dfrG*, *aacA-aphD* and *aphA3* were present in 25, 24, and 43 NAS isolates (Qu *et al*., 2019). Ahmed *et al*. (2020) found NASM isolates exhibited a high prevalence of the resistance genes *mecA*, *blaZ* and *tetK*. Approximately half of the isolates harbored the *aac-aphD*, *erm*(C), and *erm*(B) genes.

Klibi *et al*. (2018) studied 83 isolates; sixty-eight of these strains were identified as NAS. In the study, 29.41% showed oxacillin and cefoxitin resistance whislt some strains were *mec*A positive. Eleven of those twenty methicillin resistant non-*aureus* staphylococci (MRNAS) showed erythromycin-resistance and were positive for *ermB, ermT, ermC, mphC* or *msrA.* Four strains were *tetK* positive, which were resistant to tetracycline. Silva *et al*. (2014) also studied MRNAS, and they reported 26 strains with oxacillin and cefoxitin resistance that were positive for the *mecA* gene. In the same study, the SCC*mec* gene, a mobile genetic element that carries the central determinant for broad-spectrum beta-lactam resistance encoded by the *mecA* gene, was analyzed; it was present in 9 out of 26 strains. MRNAS isolates were also found to display additional resistance to tetracycline, streptomycin, tobramycin and gentamicin, and harbored the genes: *tet*(K), *str*, *ant*(4')-I, and *aac*(6')*aph*(2'').

Multidrug resistance

Studies suggest that the frequent use of drugs in veterinary practices, such as penicillin and tetracycline, especially among cattle, has increased the resistance of microorganisms over the years. The Food and Drug Administration (FDA) and the European Medicine Agency (EMA) show that tetracycline, penicillin, and sulfonamides are the classes of antibiotics most sold in the USA and Europe (Dorneles *et al*., 2019). Several studies in the literature point to multidrug-resistant NAS and these are enumerated in Table 2. In addition, NAS are known to be more resistant to antibiotics when compared to *S. aureus,* and this resistance can be to different drugs and contribute to a low cure rate. The resistance can be due to NAS, typically on the cow's body, being more exposed to antimicrobials (Khazandi *et al*., 2018).

Table 2. Number of multidrug resistant Non-*aureus* Staphylococci; Antibiotic groups they have resistance to; Percentage of resistant strains for each drug or group.

Number of MDR-NAS	Antibiotic resistance	% of MDR	Reference
73/152	Penicillin + ampicillin + erythromycin	51%	(Phophi *et al*., 2019)
60/107	Enrofloxacin + oxacillin + penicillin + tetracycline	56%	(Cheng *et al*., 2019)
14/37	Oxacillin + chloramphenicol + novobiocin + tetracycline	38%	(Khazandi *et al*., 2018)
3/8	Ampicillin + clindamycin + oxacillin erythromycin + gentamycin + penicillin + sulfonamide + trimethoprim/sulfamethoxazole + tetracycline	37.5%	(Dorneles *et al*., 2019)
2/8	Ampicillin + penicillin + trimethoprim/sulfamethoxazole	25%	
1/8	Ampicillin + gentamycin + oxacillin + penicillin + sulfonamide + trimethoprim/sulfamethoxazole + tetracycline	12.5%	
2/8	Ampicillin + gentamycin + oxacillin + penicillin + trimethoprim/sulfamethoxazole + tetracycline	25%	
18/170	Same group of antibiotics (beta-lactam or MLS compounds)	10.6%	(Sampimon *et al*., 2011)
18/170	Different groups	10.6%	

NASM involvement in mastitis prevention

While studies show NASM as the cause of mastitis, some studies have demonstrated that NAS can reduce infection by *S. aureus* and other pathogens (Brouillette *et al*., 2022). In another study by Toledo-Silva *et al*. (2021), it was observed that NASM suppressed the biofilm dispersion of a strain of *S. aureus.* Some studies also point to a possible protective role of NASM, as described by Piepers *et al*. (2013), who in comparing infected heifers with uninfected heifers, observed that heifers infected with NASM had fewer cases of clinical mastitis throughout lactation.

Some gram-positive bacteria can produce bacteriocins, inhibiting the growth of other bacterial strains. Bacteriocins produced by NASM include epidermin, hyicin 3682, nukacin ISK-1, nukacin 3299 and nukacin KQU-131. *Staphylococcus epidermidis* produces Tu 3298, *Staphylococcus hyicus* 3682, *Staphylococcus warneri* ISK-1, *Staphylococcus simulans* 3299, and *Staphylococcus hominis* KQU-131 (Yong *et al*., 2019), so it can be seen that there is potential for NASM to negatively affect the growth of pathogenic bacteria. In this context, Ferronatto *et al*. (2019) used the cross-streaking method to test growth inhibition of mastitis-causing pathogens. Using this method, it is possible to determine if there was partial inhibition, total inhibition (no colony was observed), or no growth inhibition (same size and number of colonies as on the positive control plate). It was reported that all of the 19 NAS studied could inhibit growth partially, and in the case of *S. chromogenes* there was total inhibition. Another study showed the capacity of 38 strains of NAS to inhibit *Listeria innocua* growth, with the strain that most displayed this ability being *S. quorum* (Braem *et al*., 2014).

De Vliegher *et al*. (2004) also studied how *S. chromogenes* could inhibit the growth of some bacteria, such as *S. aureus*, *S. dysgalactiae* and *S. uberis*, and the result was positive. The NAS studied inhibited the growth of these strains but not that of *E. coli*, which is typically expected, as the inhibitory effect of bacteriocins is usually more intense against phylogenetically related bacterial species (Ferronatto *et al*., 2019). Furthermore, the same authors in a previous study suggested that colonization of the teat apex by *S. chromogenes* in pre-calving heifers protected the udder against increased SCC in the early postpartum period (De Vliegher *et al*., 2003).

According to Isaac *et al*. (2017), NASM is a group of bacteria classified as either minor mastitis pathogens or commensal microbiota. A study showed that *S. epidermidis*, *S. simulans*, *S. hominis*, *S. saprophyticus*, and *S. arlettae* isolated from milk were able to

produce antimicrobial compounds to inhibit the growth of some mastitis pathogens, including *S. aureus* (Nascimento *et al*., 2005). Beuckelaere *et al*. (2021) observed a probiotic effect of *S. chromogenes* when colonizing dry quarters with this microorganism. As a result, the authors observed a shift to the Th1 response in late pregnancy and early lactation, increasing IgG2 concentration. In a study with a murine mastitis model, the authors observed that *S. aureus* intramammary colonization is reduced by exoproducts from *S. chromogenes* and *S. simulans* (Brouillette *et al*., 2022). Some NAS species (*S. chromogenes*, *S. simulans* and *S. epidermidis*) inhibited the growth of *S. aureus* and downregulated the expression of rnaIII, important molecule of the QS system (Toledo-Silva *et al*., 2021). In addition, other studies point out that NASM protects the mammary quarters against IMI caused by primary pathogens (De Buck *et al*., 2021). oth characteristics of NASM, to cause or prevent mastitis, could be explained by differences among the species and intraspecies once the host determines the infection, the virulence of microorganisms and biofilm formation capacity (Brouillette *et al*., 2022). However, much still needs to be understood about these ratehr complex interactions within the microbiota.

One Health and public health

The One Health approach conceptualises that we cannot truly understand human, animal and environmental health by only addressing each in isolation. Because of this, we must understand the connections of these areas using holistic interpretations to face public health challenges. In other words, One Health focuses equally or more on the relationships between the factors in the system rather than on the individual-level factors themselves (Dalton *et al*., 2020).

We can observe in Table 1 and Table 2 NASM being isolated from food, clinical infections and environment, as well as their resistance to antibiotics. Several NASM strains from these studies (Hammad *et al*., 2012; Kozioł-Montewka *et al*., 2006; Schnitt *et al*., 2021; Z. Xu *et al*., 2019) hve been confirmed as MRNAS. Also, the topics about virulence and part of Table 1 showed the capacity of these species to produce toxins and form biofilm. All these components place NASM as a concern for public health.

Advances in molecular techniques allow us to relate strains from community and clinical infections, which helps us understand transmission among humans, animals and environments. The species most studied in *Staphylococcus* spp. is *S. aureus* and many studies have observed clones common in clinical infections, in animals or in the environment (Aires-de-Sousa, 2016; Silva *et al*., 2013, 2014; Zhou *et al*., 2018). But, we also observe the same route for NAS in studies that demonstrate the presence of identical clones in hospitals and communities (Martínez-Meléndez *et al*., 2016; Pinheiro-Hubinger *et al*., 2021; Xu *et al*., 2019). So, the prevalence of NASM in the dairy chain and the increasing prevalence of resistance genes raises further concern. The prevalence of antimicrobial-resistant pathogens has increased while the approval of new drugs has decreased, which is a public health challenge. However, the number of antimicrobials used in veterinary medicine is immense and should be discussed since they select resistant microorganisms. Animals are the reservoirs of these organisms and can transmit them through food (FDA, 2018; Gohl *et al*., 2016).

According to the FDA, each year in the United States at least 2.8 million antibiotic-resistant infections occur, and more than 35,000 people die as a result. Therefore, agencies around the world are trying to minimize the harm caused by the incorrect use of antibiotics, for example, using them for purposes for which they are not intended (to treat diseases caused

by viruses, fungi or yeasts), in addition to the frequent use of broad-spectrum antibiotics (Hildreth *et al*., 2009). The FDA and the European Food Safety Authority (EFSA) are two organizations that have been playing a crucial role in the dissemination of vital information to preserve the effectiveness of currently available antimicrobial drugs and promote the development of new medical products that can help reduce the emergence and spread of antimicrobial resistant bacteria (EFSA; FDA 2018).

Stevens *et al*. (2018) analyzed the consumption of antimicrobials in dairy herds and its association with the diameters of the antimicrobial inhibition zone of NAS isolated from subclinical mastitis. They reported a high incidence of antimicrobial treatment for fourth generation cephalosporins, a class of antibiotics important for human health. Strains of MRNAS were identified in milk samples with mastitis in a study in Korea, and one of the strains persisted on one of the analyzed farms for more than two years (Kim *et al*., 2019). The role of NAS as a cause of disease in humans has been described. Diseases such as endocarditis, tract infections, catheter-related sepsis, endophthalmitis, surgical site infections, peritonitis in patients with continuous ambulatory peritoneal dialysis and foreign body infections were due to infections caused by NAS (Piette & Verschraegen, 2009). Approximately 55-75% of nosocomial isolates are resistant to methicillin, and NASM is responsible for about 30% of all nosocomial bloodstream infections (Piette & Verschraegen, 2009).

In the context of One Health and mastitis, there is a solid recommendation for reducing antimicrobial use. Taking into acount that milk with antibiotic residues does not enter the supply chain, as it is withdrawn at source, this recommendation emphasises the need to periodically search for antimicrobial residues in raw milk on farm. Combined with microbiome analysis and research on antimicrobial residues, we have the somatic cell count,

which acts as an indicator of the health of the mammary gland, helping to reveal the situation of the dairy herd within the context of health (Gohl *et al*., 2016). When milk contains residues, this indicates that it comes from unhealthy animals, so it can cause foodborne pathogens and be a potential allergen for consumers. This concern is even more significant in developing countries, where there is no supervision on using antimicrobials in animals or the opportunity to test milk and meat for drug residues. The presence of adulterating antimicrobials in milk can have a profound impact on the health of babies and children in regions that already face high rates of diarrheal diseases and malnutrition. Several studies have shown a relationship between the intestinal microbiome, health status and disease. Therefore, ensuring that milk does not contain antimicrobial residues significantly impacts public health and producers' economic livelihood (Garcia *et al*., 2019; Gordon *et al*., 2012; Mohan *et al*., 2008). Since NASM is a large group that contains species with different levels of pathogenicity, virulence, resistance profile and host adaptation, analyses of the genetic profile of these species are necessary to understand the role of each NAS species in bovine mastitis (De Buck *et al*., 2021). Thus, the One Health approach is also required when we are specifically talking about NAS.

This article gathers evidence that NASM has the potential to cause mastitis, and that all of this can cause public health problems since there is a lack of specific studies and strategies to combat these pathogens. Even with this evidence, NASM is still overlooked as causing disease in both humans and animals. With all these points raised, we must apply a One Health approach to address this critical public health problem, including implementing good antimicrobial administration practices in human and veterinary health settings to delay the development of resistance and prolong the life of useful antimicrobials (FDA, 2018).

In conclusion, since it is an important food at all stages of life, milk consumption increases each year along with population growth. We know that NASM mastitis can harm the health and welfare of animals and, consequently, human health, either by food poisoning or by the recurrent use of antimicrobials to treat the disease, which can leave residues in the milk that will be consumed in different ways. Studies related to NASM mastitis are extremely important to learn more about the role of these microorganisms in the disease and how their virulence factors can make treatment difficult. Questions that need more concrete answers are why some NASM strains that cause mastitis appear to have a protective role against other microorganisms and why some strains can harm milk production and animals.

In addition, the increased resistance of these microorganisms to antimicrobials is worrying, requiring the implementation of residue monitoring in milk and studies of NASM antimicrobial resistance used to combat mastitis. Thus, a holistic vision that encompasses safer food and solutions to improve animal, human and ecosystem health seems to be the best alternative.

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CAPÍTULO 2

Analysis of the relationship between management practices and virulence and resistance factors of Non-*aureus* Staphylococci (NAS) isolated from cow's milk

Analysis of the relationship between management practices and virulence and resistance factors of Non-*aureus* Staphylococci and mammaliicocci (NASM) isolated from cow's milk

* Research Article to be Submitted

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ABSTRACT

Non-*aureus* staphylococci and mammaliicocci (NASM) are frequently isolated from cows' milk with mastitis or cases of intramammary infections. In addition, their isolation from the extramammary environment has also been reported in several studies. In most of these studies, NASM are not identified at the species level, which limits in-depth studies at this level. Therefore, this study aimed to identify the species of 309 NASM isolated from milk of cows with low and high somatic cell count (SCC), and milk of cows with clinical mastitis from the five producing regions of Brazil using the MALDI-ToF MS technique. All isolates were then tested for antimicrobial susceptibility, the presence of resistance genes (*mec*A, *mec*C, *qac*AB, and *qac*C), and the presence of virulence genes such as enterotoxin genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and *sei*). In addition, an analysis was performed to evaluate the association between NASM isolation and the different management practices adopted by the farms where the milk samples were collected. Eighteen different NASM species were identified in this study, the three most prevalent being *S. chromogenes* (58.6%), *S. simulans* (8.4%), *S. haemolitycus* (8.1%). Of the 309 NASM, 41 (13.3%) were classified as multidrug-resistant (MDR). Gene research showed 15 *mec*A-positive NASM, and the most prevalent enterotoxin was *seb*, with 47 (15.2%) NASM positive. NASM in general, was associated with good management and milking practices according to the responses obtained in the questionnaires, but the association of NASM with the use of gloves may also mean that the use of gloves served as a vehicle and dissemination of NASM in the herd. This study provided information on the virulence and resistance profile of NASM species isolated from cow's milk in Brazil, as well as providing data for future studies on the epidemiology of NASM.

Keywords: staphylococci; multidrug-resistant; mastitis; somatic cell count; milk; dairy cow.

INTRODUCTION

Non-*aureus* staphylococci and *mammaliicocci* (NASM) are frequently isolated from aseptically collected milk samples from dairy cows with subclinical mastitis and, to a lesser extent, from those with clinical mastitis (Naushad et al., 2016). Currently, the genus is composed over 70 species and subspecies, five of which were reclassified under the new genus *Mammaliicoccus*, formerly *S. sciuri*, *S. fleurettii*, *S. lentus*, *S. stepanovicii* and *S. vitulinus*, with *M. sciuri* as the type species (Madhaiyan et al., 2020).

Different species of NASM have been identified in various regions of the body of dairy cows and in their milk production environment, occupying diverse habitats. For instance, *Staphylococcus cohnii* is primarily found in the feces and environment of dairy cows, while *Staphylococcus chromogenes* is commonly isolated from milk samples (Piessens et al., 2011; Vanderhaeghen et al., 2014; De Visscher et al., 2014; Vanderhaeghen et al., 2015b; Wuytack et al., 2019; De Buck et al., 2021a). Furthermore, substantial differences between and within NASM species associated with dairy cows have been documented in several aspects, including epidemiology and ecology (Vanderhaeghen et al., 2015a; Souza et al., 2016), antimicrobial resistance (Fergestad et al., 2021), virulence (Vanderhaeghen et al., 2014;Wuytack et al., 2020; França et al., 2021) interaction with the host (Simojoki et al., 2012; Piccart et al., 2016; Souza et al., 2016, 2022) and impact on udder health and milk production (Piepers et al., 2010; Tomazi et al., 2015; Valckenier et al., 2019, 2020a, 2021; Crippa et al., 2024). Despite their frequent isolation in dairy herds, the role of NASM in udder health remains unclear, with further studies on NASM helping to close this knowledge gap (Addis et al., 2024; Freu et al., 2024).

Furthermore, a study showed that information related to herd management practices, such as herd size, milking frequency, frequency of disinfection before and after

milking, and the climate of each herd influenced the prevalence of the microorganisms identified. Therefore, studies that analyze the different factors related to herds are important to clarify further the size of this effect (Lan et al., 2017). Therefore, this study aimed to identify the NASM species isolated from cow's milk in dairy herds across all regions of Brazil and to determine their phenotypical antimicrobial susceptibility, as well as the presence of enterotoxins, and disinfectants and methicillin resistance genes. In addition, management data collected from the dairy farms through a questionnaire were correlated with the overall NASM and their respective species isolated from cows' milk samples.

MATERIAL AND METHODS

Milk sampling and SCC measurement

In the present study, 1,468 milk samples (convenience samples; 1270 composite milk samples representing the complete milking of each animal and 198 udder quarter milk samples from clinical cases of mastitis) were collected in each of the five regions of Brazil (South, Southeast, Central-West, Northeast and North) in the states of Santa Catarina (n=268), São Paulo (n=356), Goiás (n=258), Paraíba (n=285), and Pará (n=301), totaling collections in 17 herds.

Clinical mastitis cases were diagnosed based on macroscopic alteration in milk detected by strip cup test, as well as local and systemic clinical alterations. Complete milk samples from healthy dairy cows and those with subclinical mastitis were collected for automatic somatic cell count (SCC) analysis that was measured by flow cytometry (Fossomatic 5000basic®) according to (ISO 13366-2:2006). All samples were transported under refrigeration (1-10ºC) in isothermal boxes with recyclable ice via air or land transport.

Dairy herd management practices

At the time of milk sampling, a questionnaire was administered to the person responsible for each dairy farm to obtain information related to dairy herd management. This questionnaire was used for statistical correspondence analysis carried out in this study. This questionnaire addressed several questions related to milking management and mastitis control including the type of milking, execution of the milking line, use of disposable gloves, washing teats before milking, elimination of the first three jets of milk [strip cup test], pre-dipping, drying the teats, post-dipping, carrying out the California Mastitis test (CMT), immediate treatment of clinical mastitis, choice of antibiotic use,

routine culture of milk samples; antibiogram of mastitis pathogens, feeding during milking, providing food soon after milking, treatment with antibiotics at drying-off, use of teat sealant, mastitis vaccines, mastitis for culling animals due to mastitis, use of hot water to clean milk machine equipment, use of alkaline detergent, acid detergent, and sanitizer to clean the milk machine equipment). Furthermore, the questionnaire gathered information related to dairy farms such as daily milk production volume, number of lactating cows, breeding system, technical assistance, and maintenance of milking equipment. The questionnaire (Appendix 1) was submitted to the Research Ethics Committee (CEP) and the Animal Use Ethics Committee (AUEC) at the University of Campinas and was approved under protocol Number 3.556.852.

Isolation and identification of NASM

NASM was isolated from milk samples collected using methodologies according to the National Mastitis Council (NMC; Hogan et al., 1999) guidelines. 0.01 μL of each sample was plated on sheep blood agar (5%) (Oxoid) and incubated at 37°C under aerobic conditions. Plates were read after 24, 48, and 72 hours of incubation (Oliver et al., 2004). After incubation, up to three characteristic colonies were isolated based on their morphology and coloration. Biochemical tests, such as Gram staining, catalase, and coagulase, were then performed to confirm the gender. The species were identified using the Matrix Associated Laser Desorption-Ionization – Time of Flight (MALDI-ToF MS) technique. Then, a batch of isolated bacterial colonies was diluted and homogenized in a solution of Milli-Q water and HPLC-grade ethanol. The supernatant was removed by centrifugation (13,000 × g for 2 minutes), and the pellets were air-dried at room temperature for up to 10 minutes. Next, formic acid (70%) was added to the pellets until completely dissolved. After 10 minutes, an equal volume of 100% acetonitrile was added. The sample was centrifuged once more, and 1.0 μL of each bacterial extract was placed

onto a steel plate and allowed to air-dry for 15 minutes. Once dry, the samples were overlaid with 1.0 μL of matrix solution consisting of α-cyano-4-hydroxycinnamic acid (HCCA) diluted in 50% acetonitrile and 2.5% trifluoroacetic acid and allowed to dry for approximately 7 minutes before MALDI-ToF MS analysis. The spectral data were processed using FlexControl v.3.4 Software and the MBT Biotyper Compass v.4.1.100 library for microorganism identification (Barcelos et al., 2019; Almeida et al., 2024).

Antimicrobial resistance

The phenotypical antimicrobial resistance in NASM isolates was tested using the agar diffusion method and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2024). The disk diffusion sensitivity test was performed with disks impregnated with gentamicin (10 μg), oxacillin (1 μg), cefoxitin (30 μg), tetracycline (30 μg), erythromycin (15 μg), clindamycin (2 μg), tobramycin (10 μg), chloramphenicol (30 μg), penicillin (10 μg).

DNA extraction and detection of genes for enterotoxin production, resistance to disinfectants, and methicillin resistance

DNA extraction of NASM isolates was performed using the InstaGene™ Matrix kit (Bio-Rad Laboratories, Hercules, California, USA), following all manufacturer's instructions. Then, the DNA of bacterial samples were quantified using a NanoDrop Lite (Thermo Fischer Scientific). After the DNA extraction and quantification step, oligonucleotides were used to amplify the genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *sei*, *sej*, *seh*, *qac*AB, *qac*C, *mec*A and *mec*C. The PCR conditions to determine the presence of the analyzed genes can be seen in Table 2.

Table 2. Oligonucleotides and their properties used in the detection of genes involved in the production of enterotoxins, resistance to methicillin and disinfectants in NASM isolates.

Gene	Primer	Sequence	Product size (bp)	Annealing temperature	References
Sea	SEA-1	TTGGAAACGGTTAAAACGAA	120	50°C	(Johnson et al., 1991)
	SEA-2	GAACCTTCCCATCAAAAACA			
Seb	SEB-1	TCGCATCAAACTGACAAACG	478	50°C	(Johnson et al., 1991)
	SEB-2	GCAGGTACTCTATAAGTGCC			
Sec	SEC-1	GACATAAAAGCTAGGAATTT	257	50°C	(Johnson et al., 1991)
	SEC-2	AAATCGGATTAACATTATCC			
Sed	SED-1	CTAGTTTGGTAATATCTCCT	317	50°C	(Johnson et al., 1991)
	SED-2	TAATGCTATATCTTATAGGG			
See	SEE-1	AGGTTTTTTCACAGGTCATCC	209	57°C	(Mehrotra et al., 2000)
	SEE-2	CTTTTTTTTCTTCGGTCAATC			
Seg	SEG-1	AAGTAGACATTTTTGGCGTTCC	287	55°C	(Omoe et al., 2013)
	SEG-2	AGAACCATCAAACTCGTATAGC			
Seh	SEH-1	GTCTATATGGAGGTACAACACT	213	55°C	(Omoe et al., 2013)
	SEH-2	GACCTTTACTTATTTCGCTGTC			
Sei	SEI-1	GGTGATATTGGTGTAGGTAAC	454	55°C	(Omoe et al., 2013)
	SEI-2	ATCCATATTCTTTGCCTTTACCAG			
*qac*AB	qacAB	TCCTTTTAATGCTGGCTTATACC	220	56 ºC	(Zmantar et al., 2011)
		AGCCKTACCTGCTCCAACTA			
*qac*C	qacC	GGCTTTTCAAAATTTATACCATCCT	249	56 ºC	(Zmantar et al., 2011)
		ATGCGATGTTCCGAAAATGT			
*mec*A	mecA-1	GGGATCATAGCGTCATTATTC	527	57°C	(Gómez-Sanz et al., 2010)
	mecA-2	AACGATTGTGACACGATAGCC			
*mec*C	mecC-1	GCTCCTAATGCTAATGCA	304	51°C	(Cuny et al., 2011)
	mecC-2	TAAGCAATAATGACTACC			

For PCR, a GoTaq Hot Start Polymerase kit (Promega Corporation, Madison, USA) was used, this kit consisting of Buffer PCR 1X, 200µM of each deoxynucleotide triphosphate (dNTP), MgCl2, 1 U of Taq Polymerase. 1µl of DNA and 10 pmol of each primer were used, in addition to Milli-Q ultrapure water with the ultimate goal of reaching a volume of 25µL. PCR was performed using a Bio-Rad C-1000 Thermocycler (BioRad, Hercules, USA) and the parameters used are described in Table 2. An agarose gel composed of 0.5X TBE (45 mM Tris-borate) and SYBR (Safe DNA Gel Stain) and then, by electrophoresis, the DNA fragments were analyzed. After this step, the DNA

fragments were visualized using a photodocumentator from BioRad Laboratories (BioRad, Hercules, USA).

Statistical analysis

A statistical analysis of correspondence between the management data collected through the questionnaire, the isolation of NASM and the clinical status of the animals from which the milk samples were collected was performed according to Asensio (1989), using the Infostat 2020 (Di Rienzo et al., 2020). Some questions in the questionnaire were not used in the analysis due to a lack of answers or, in the case of 100% identical answers (without statistical variation). The questions used in the statistical analysis can be seen in Table 3.

A chi-square test was also performed between the 309 NASM and the presence of the virulence genes and resistance genes analyzed, as well as between the results of the antimicrobial susceptibility test. Only the variables that presented significance between $P \leq 0.05$ and $P \leq 0.20$ were considered. Next, the *odds ratio* values were determined, with values above 1.0 indicating an association between the NASM presence and the variable studied, and this association corresponds to a risk factor, and values below 1.0 representing protective factors. In the final through the chi-square test, the ideal fit of the model was considered one in which the test did not present significance $P \geq 0.05$. Finally, the correspondence analysis graph was produced using the Microsoft® Office Excel® 2021 program.

Table 3. Variables selected for correspondence analysis of the questionnaire applied on farms

Question	Response
How many times a day are animals milked?	1; 2; 3
What is the type of milking?	Manual; Mechanics (bucket milking); Mechanical (piped)
Do you maintain milking equipment?	Periodic; Occasional (when there is a problem); Never
Do you do a milking line, that is, do you milk the cows with mastitis last or separately?	Always; Sometimes; Never
Does the milker(s) use disposable gloves to perform milking?	Yes; No
Do you wash the teats with water?	Always; When very dirty; Never
Do you do pre-dipping?	Yes; No
Do you dry the teats?	Yes/paper towel; Yes/Cloth; No
Do you provide food for the cows during milking?	Yes; No
Do you feed the cows immediately after milking?	Yes; No
Does the CMT test (California Mastitis Test) take place?	Yes/regularly; Yes/occasionally/ No
Do individual somatic cell count (SCC) of lactating cows?	Yes/regularly; Yes/occasionally/ No
Do you treat subclinical mastitis during lactation?	Yes; No
How do you choose antibiotics to treat mastitis?	Seller recommendation; Veterinarian's recommendation;
Do you do bacteriological culture and antibiogram of milk?	Yes/regularly; Yes/occasionally/ No
Do you treat the cows with antibiotics when drying them (dry cow antibiotics)?	Yes, of all dry cows; Yes, from some dry cows; No
Do you use internal teat sealant on dry cows?	Yes, in all dry cows; Yes, in some dry cows; No
Do you use the vaccine for mastitis?	Yes; No
Do you cull animals due to mastitis?	Yes; No
Do you sanitize the milking machine with chlorine solution before milking?	Yes; No
Who do you sell the milk to?	Dairy under Federal Inspection Seal (SIF); Dairy under State Inspection Seal (SIE); Cheese factory
Is payment for milk based on quality?	Yes; No
Lactating cow housing system	Free stall; Tie stall; Compost barn; Pickets

RESULTS

In total, 309 NASM were isolated and identified, being *S. chromogenes* (n=181), *S. simulans* (n=26), *S. haemolyticus* (n=25), *S. epidermidis* (n=14), *S. xylosus* (n=14), *S. warneri* (n=9), *S. equorum* (n=9), *S. cohnii* (n=7), *S. saprophyticus* (n=7), *S. hyicus/agnetis* (n=5), *M. sciuri* (n=4), *S. pasteuri* (n=2), *S. succinus* (n=1), *S. auricularis* (n=1), *S. caprae* (n=1), *S. capitis* (n=1), *S. kloosii* (n=1), *S. arlettae* (n=1). Of these 309, 167 (54%) originated from milk from cows with SCC >200,000 cells/mL, 109 (35.3%) originated from cows with SCC <200,000 cells/mL, and 33 (10.7%) were from cows with clinical mastitis. Furthermore, 107 (34.6%) NASM were isolated from milk samples from the state of São Paulo, 92 (29.8%) from the state of Santa Catarina, 49 (15.8%) from the

state of Pará, 39 (12.6%) from the state of Paraíba and 22 (7.12%) from the state of Goiás. The distribution of NASM species isolated among the states can be seen in Figure 1.

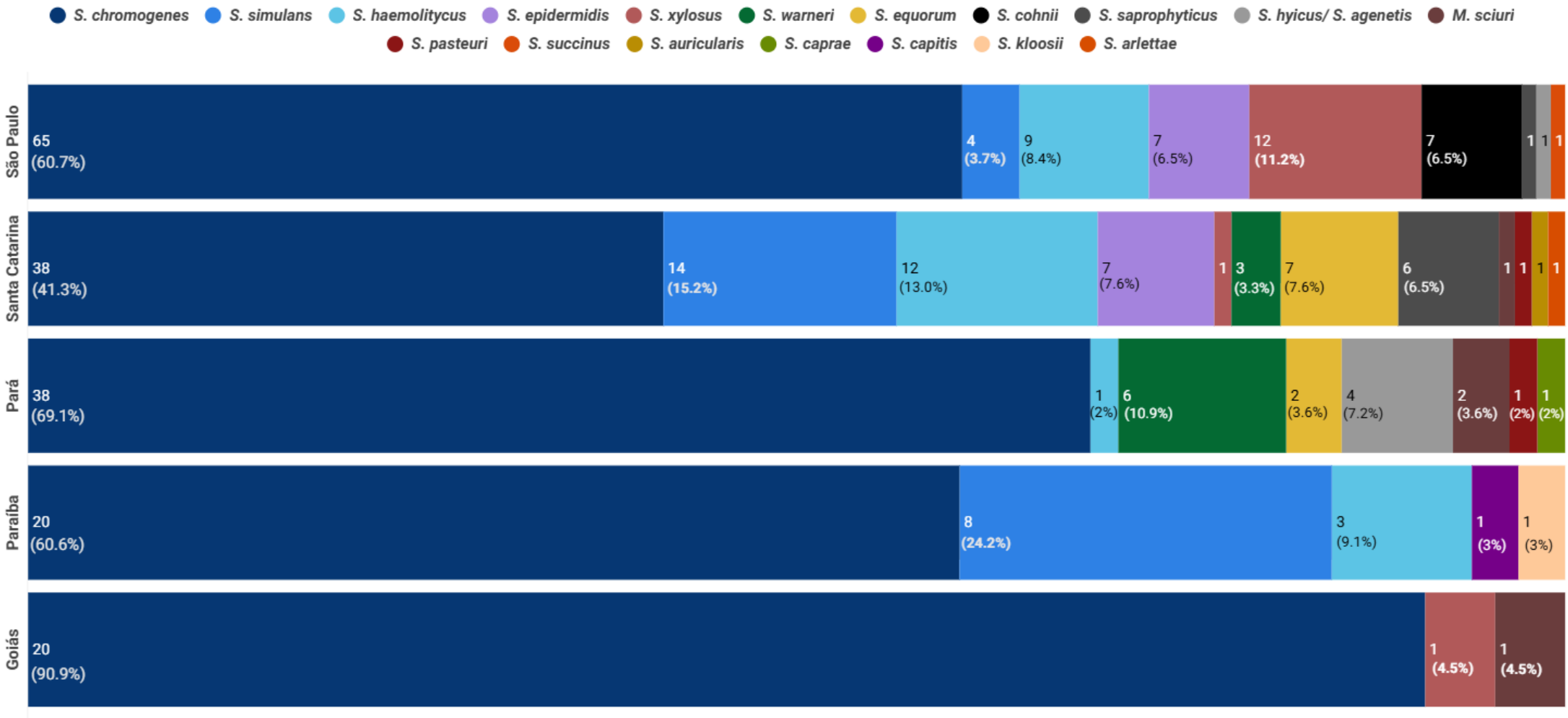

Figure 1. The distribution of the 309 NASM isolated in each state

Management data and the association with NASM isolation

To demonstrate the associations between the variables of the questionnaire on the management applied on the farms and the isolation of NASM, a correspondence analysis was performed, and the results are presented in Figure 2 and Table 4. The interpretation is made by evaluating the proximity of the variables (questionnaire responses) with the response variable, the presence of NASM. In short, the closer the analyzed variables are to NASM, the greater the association between them. The cumulative chi-square value for this analysis was 63.79%, which means that the selected variables efficiently represent the phenomenon studied, explaining more than 60% of this variation.

Therefore, it can be observed that the variables that presented a stronger association with the isolation of NASM were: “SCC <200,000 cells/mL”; that is, NASM were associated with cow's milk samples with SCC <200,000 cells/mL. NASM were associated with the milking line and the practice of milking cows with mastitis last or separately. “Pre-dipping-Yes”, NASM are associated with the practice of pre-dipping. “Mechanical (piped)’’ NASM are associated with piped milking. “Gloves-Yes”, NASM are associated with using gloves during milking. “Post-milkfeeding-Yes”, NASM are associated with providing food to cows immediately after milking. “CCSlactation-Regularly”, NASM are associated with regularly performing individual somatic cell counts on lactating cows. “Veterinarian's recommendation”, NASM are associated with the choice of antibiotic to treat mastitis on the veterinarian's recommendation. “AntibioticDryCows-Of all cows”, NASM are associated with antibiotic treatment when drying off cows (dry cow antibiotic). “Qualitypayment-Yes”, NASM are associated with the payment for milk according to quality.

Therefore, it is possible to observe that the variables most strongly associated with NASM isolation are related to good management practices, which is reflected in animals with low SCC and clinically classified as healthy.

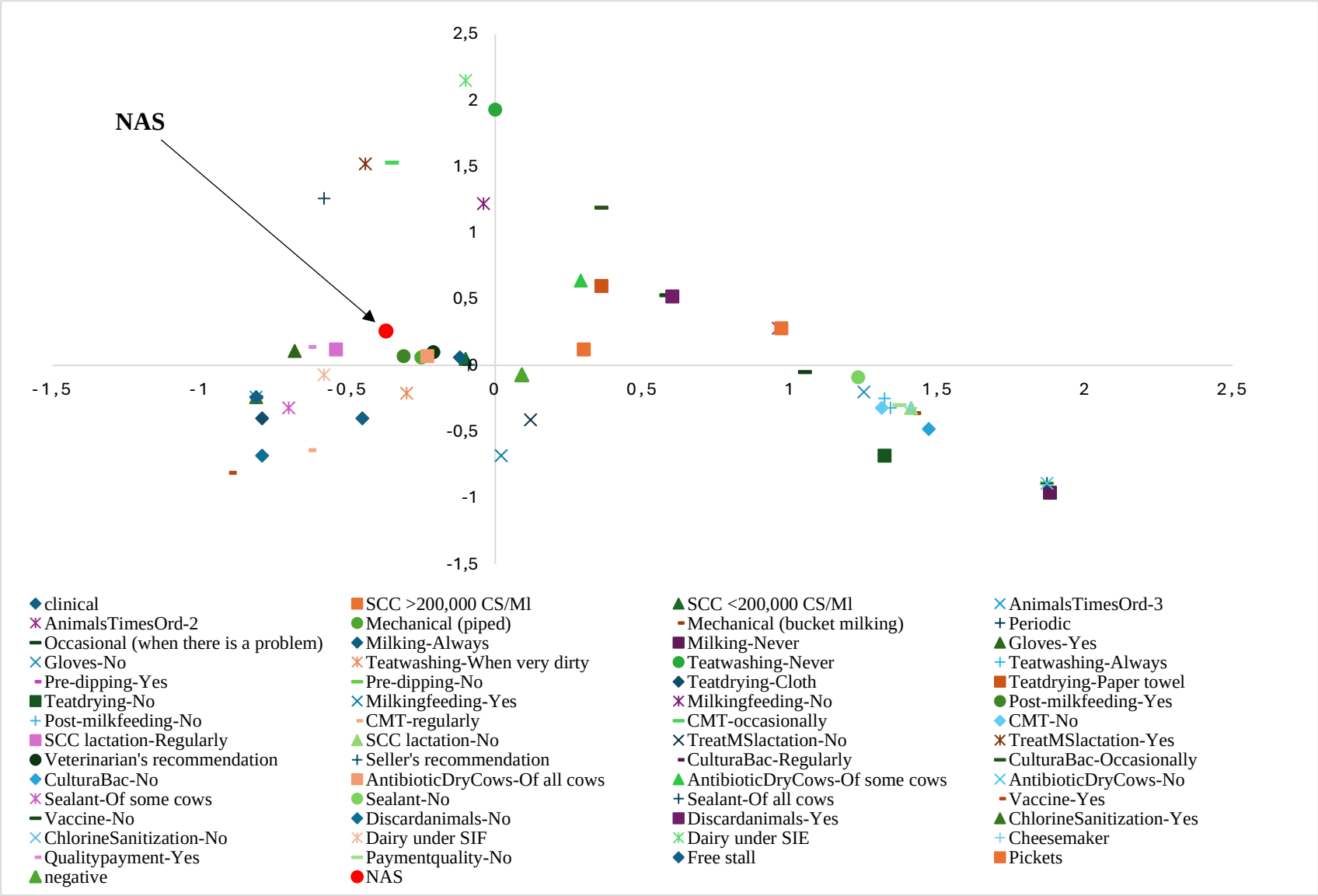

Figure 2. Graph of correspondence analysis of the questionnaire applied on the farms where the milk samples were collected and NASM isolates.

Table 4. Legend of the correspondence analysis graph of the applied Questionnaire

Question	Graph Response Legend
How many times a day are animals milked?	TimesAnimalsOrd-2
	TimesAnimalsOrd-3
What is the type of milking?	Mechanics (bucket milking)
	Mechanical (piped)
Do you maintain milking equipment?	Occasional (when there is a problem)
	Periodic
Do you do a milking line, that is, do you milk the cows with mastitis last or separately?	Milking-Always
	Milking-Never
Does the milker(s) use disposable gloves to perform milking?	Gloves-Yes
	Gloves-No
Do you wash teats with water?	Washing teats-Never
	Washing teats-Always
	Washing teats-When very dirty
Do you do pre-dipping?	pre-dipping-Yes
	pre-dipping-No
Do you dry the teats?	Dryingteats-No
	Dryingteats-Cloth
	Dryingteats-Paper towel
Do you provide food for the cows during milking?	Milkingfeeding-Yes
	Milkingfeeding-No
Do you feed the cows immediately after milking?	Post-milkfeeding-Yes
	Post-milkfeeding-No
Does the CMT test (California Mastitis Test) take place?	CMT-occasionally
	CMT-regularly
	CMT-No
Do individual somatic cell count (SCC) of lactating cows?	SCC lactation-No
	SCC lactation-Regularly
Do you treat subclinical mastitis during lactation?	TreatMSlactation-Yes
	TreatMSlactation-No
How do you choose antibiotics to treat mastitis?	Veterinarian's recommendationrinary
	Seller's recommendation
Do you do bacteriological culture and antibiogram of milk?	CulturaBac-No
	CulturaBac-Occasionally
	CulturaBac-Regularly
Do you treat the cows with antibiotics when drying them (dry cow antibiotics)?	AntibioticDry Cows-Of all cows
	AntibioticDry Cows-From some cows
	AntibioticDry Cows-No
Do you use internal teat sealant on dry cows?	Sealant-From all cows
	Sealant-From some cows
	Sealant-No
Do you use the vaccine for mastitis?	Vaccine-Yes
	Vaccine-No
Do you dispose of animals due to mastitis?	Discard animals-Yes
	Discard animals-No
Do you sanitize the milking machine with chlorine solution before milking?	SanitizationChlorine-Yes
	SanitizationChlorine-No
Who do you sell the milk to?	SIF Dairy
	SIE Dairy
	Cheese factory
Is payment for milk based on quality?	Paymentquality-Yes
	Paymentquality-No
Lactating cow housing system	Free stall
	Pickets
Samples where NASM were isolated	NASM
Samples where NASM were not isolated	negative

Antimicrobial susceptibility of NASM isolates

When analyzing the results of antimicrobial susceptibility (Figure 3), can be observed that the antibiotics that presented a greater number of resistant NASM were oxacillin with 83 (19.62%) resistant NASM isolates, penicillin with 77 (18.20%), clindamycin with 55 (13.00 %), tobramycin with 47 (11.11%), chloramphenicol with 40 (9.47%), tetracycline with 38 (8.98%), erythromycin with 34 (8.04%), cefoxitin with 27 (6.38%) and gentamicin with 22 (5.20%). All species showed resistance to at least one antibiotic, except the species *S. succinus*, which was sensitive to all antibiotics tested.

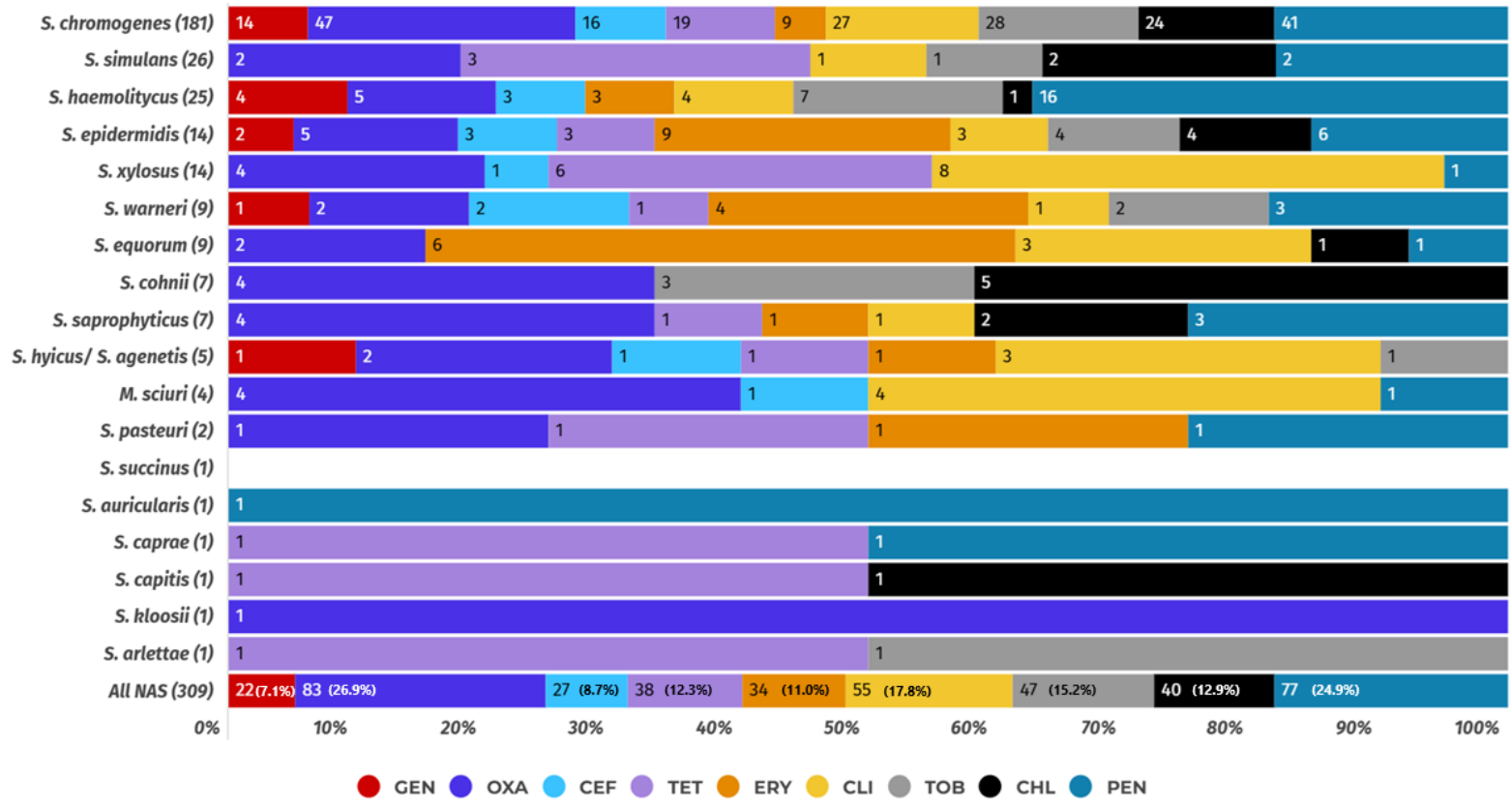

Figure 3. Results of the antimicrobial susceptibility test of the 309 NASM against the antibiotics tested. Total number of resistant NASM and the result of resistance by species. Antibiotics: GEN: Gentamicin (10 µg), OXA: Oxacillin (1 µg), CEF: Cefoxitin (30 µg), TET: Tetracycline (30 µg), ERY: Erythromycin (15 µg), CLI: Clindamycin (2 µg), TOB: Tobramycin (10 µg), CHL: Chloramphenicol (30 µg) and PEN: Penicillin (10 µg).

A statistical analysis (chi-square test) was performed to evaluate the association between resistance to the antibiotics tested and the 309 NASM analyzed. Separating the species into two groups was necessary for this analysis to be possible. The group "*S. chromogenes*" where the 181 NASM identified as *S. chromogenes* were inserted, and the group "Non-*chromogenes*'' where the remaining 128 species were inserted. Of all the antibiotics tested, erythromycin was the only one that showed a statistical difference between the two groups analyzed. The chances of "Non-*chromogenes*" species being resistant to this antibiotic are almost 5 times (4.99) greater (Table 5).

Table 5. Result of the chi-square test between the antibiotic susceptibility test and the 309 NASM.

Antibiotic	Species group	Odds ratio	Confidence Limits (95%)	Chi-Square	P value
Erytromicin	Non-*chromogenes*	4.99	2.20 - 11.30	19.59	<0.0001
	S. chromogenes	0.20	0.09 - 0.45		

The antimicrobial susceptibility results also show us that among the 309 NASM isolates, 25.0% showed resistance to at least one of the tested antibiotics, 17.8% showed resistance to up to 2 different antibiotics, 7.1% showed resistance to 3 different antibiotics, 2.9% showed resistance to 4 different antibiotics, 1.9% showed resistance to 5 different antibiotics, 1.0% showed resistance to 6 different antibiotics, 1.6% showed resistance to 7 different antibiotics, 1.0% showed resistance to 8 different antibiotics and 1.0% showed resistance to 9 different antibiotics (Figure 4).

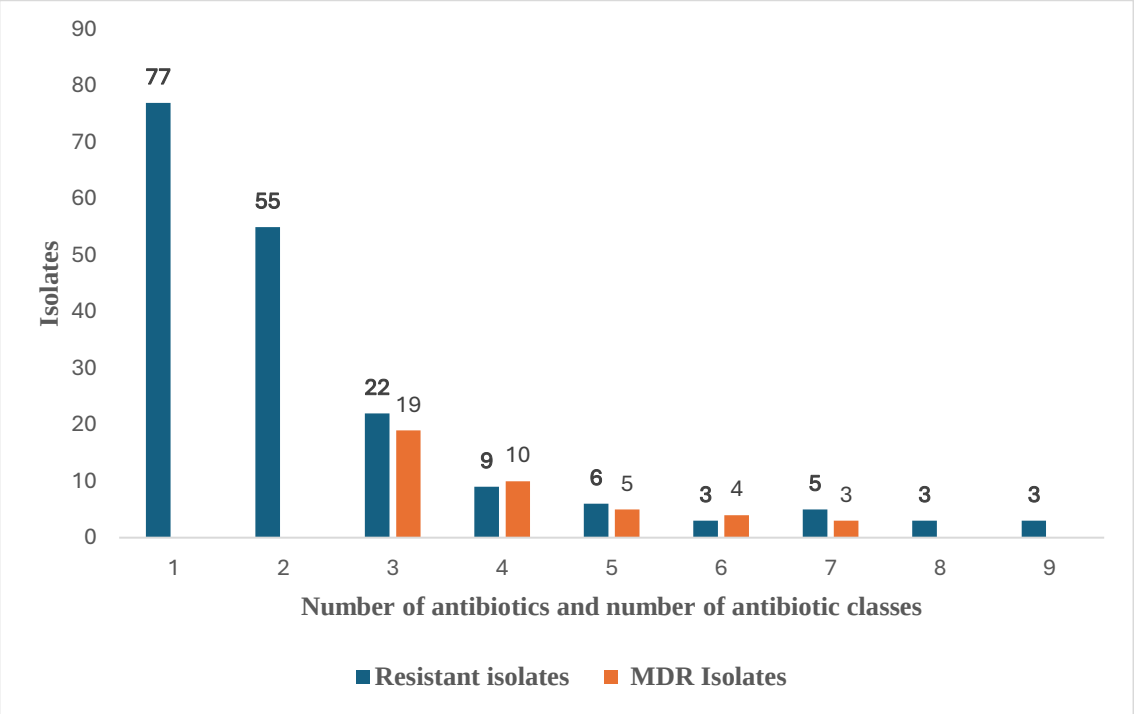

Figure 4. Number of NASM isolates resistant to one or more different antibiotics and number of Multidrug-resistant (MDR) NASM.

Multidrug-resistant (MDR) strains are those that are resistant to 3 or more different classes of antibiotics. This work classified 41 (13.3%) NASM isolates as MDR (Figure 4). Being 25 *S. chromogenes* (61%), 4 *S. haemolyticus* (9.7%), 4 *S. epidermidis* (9.7%), 2 *S. warneri* (4.9%), 2 *S. xylosus* (4.9%), 1 *S. cohnii* (2.4%), 1 *S. hyicus/agnetis* (2.4%), 1 *S. pasteuri* (2.4%), 1 *M. sciuri* (2.4%).

Deepening the analysis of the MDR isolates regarding the clinical status of the cows from which the milk samples were collected, we have the following result:17 *S. chromogenes* isolated from milk of cows with SCC >200,000 cells/mL, 5 *S. chromogenes* isolated from milk of healthy cows and 3 *S. chromogenes* isolated from milk of cows with clinical mastitis. Three *S. haemolyticus* isolates from milk from cows with SCC >200,000 cells/mL and 1 isolate from healthy cow's milk. Three *S. epidermidis* isolates from milk from cows with clinical mastitis and 1 isolate from milk from healthy cows. Two *S. warneri* isolates from milk from cows with SCC >200,000 cells/mL. One *S. xylosus* isolated from healthy cow's milk and 1 isolated from cow's milk with SCC >200,000

cells/mL. One *S. cohnii* isolated from cow's milk with SCC >200,000 cells/mL. One *S. hyicus/agnetis* isolate from milk from healthy cows. One *S. pasteuri* isolate from milk from healthy cows. One *M. sciuri* isolated from cow's milk with SCC >200,000 cells/mL. Totalizing 25 (61%) MDR NASM isolated from milk from cows with SCC >200,000 cells/mL, 10 (24.4%) MDR NASM isolated from milk from healthy cows and 6 (14.6%) MDR NASM isolated from milk from cows with clinical mastitis.

Resistance and virulence genes

The search for methicillin resistance genes in the 309 NASM using the PCR technique showed the presence of 15 (4.8%) isolates carrying the gene *mec*A, 4 isolates from cow's milk with clinical mastitis, 5 isolates from milk with SCC <200,000 cells/mL and 6 isolates from cow's milk with SCC >200,000 cells/mL, and 0 (0%) the gene *mec*C. Sanitizer resistance genes *qac*AB e *qac*C were present in 4 (1.3%) NASM and 12 (3.8%) NASM, respectively. Of the 4 isolates positive for the *qac*AB gene, 2 come from cow's milk with clinical mastitis, 1 isolate from cow's milk with SCC <200,000 cells/mL, and 1 isolate from cow's milk with SCC >200,000 cells/mL. Among the 12 isolates positive for the *qac*C gene, 2 come from cows with clinical mastitis, 7 from cow's milk with SCC <200,000 cells/mL, and 3 from milk with SCC >200,000 cells/mL.

Regarding the presence of genes responsible for enterotoxin production in NASM isolates, the *sea* gene was present in 20 (6.5%) NASM, being 2 isolates from cow's milk with clinical mastitis, 8 isolates from cow's milk with SCC <200,000 cells/mL and 10 isolates from cow's milk with SCC >200,000 cells/mL; *seb* was present in 47 (15.2%) NASM, being 2 isolates from cow's milk with clinical mastitis, 23 isolates from milk with SCC <200,000 cells/mL and 22 isolates from cow's milk with SCC >200,000 cells/mL; *sec* was present in 2 (0.64%) NASM, being 1 isolate from cow's milk with clinical mastitis and 1 isolate from milk with SCC >200,000 cells/mL; *sed* was not present in any (0%) NASM; *see* was present in 15 (4.8%) NASM, being 4 isolates from cow's milk with

clinical mastitis, 5 isolates from cow's milk with SCC <200,000 cells/mL and 6 isolates from cow's milk with SCC >200,000 cells/mL; *seg* was present in 14 (4.5%) NASM, being 3 isolates from cow's milk with clinical mastitis, 6 isolates from cow's milk with SCC <200,000 cells/mL and 5 isolates from cow's milk with SCC >200,000 cells/mL; *seh* was present in 5 (1.61%) NASM, being 1 isolate from cow's milk with clinical mastitis, 1 isolate from cow's milk with SCC <200,000 cells/mL and 3 isolates from cow's milk with SCC >200,000 cells/mL; and *sei* was present in 5 (1.61%) NASM, being 3 isolates from cow's milk with SCC <200,000 cells/mL and 2 isolates from cow's milk with SCC >200,000 cells/mL. Totaling 75 (24.3%) NASM that presented at least one enterotoxin gene.

The research results on the resistance and virulence genes of NASM isolates can be seen in Figure 5.

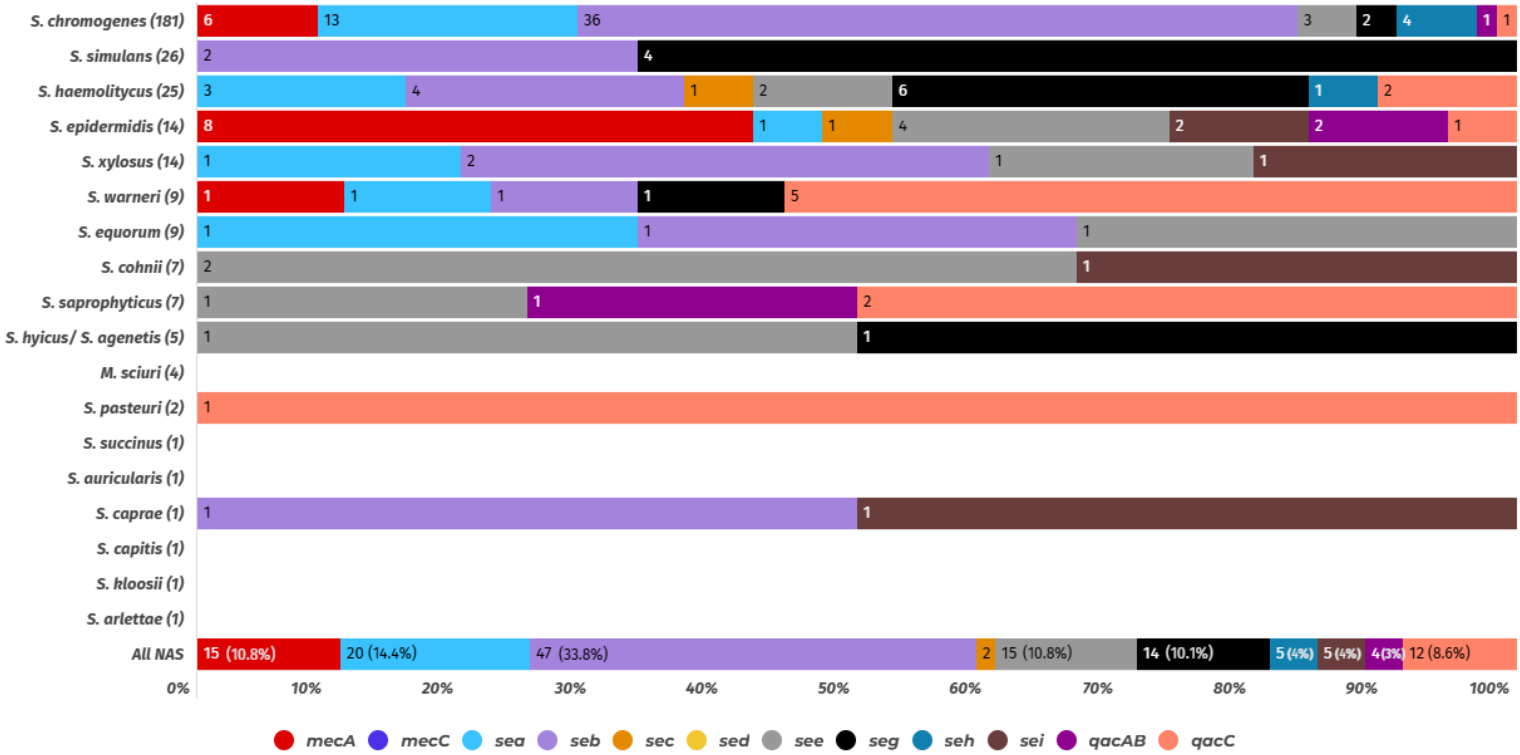

Figure 5. Prevalence of resistance and virulence genes among the 309 NASM analyzed.

A chi-square test was also performed to assess the association between resistance and virulence genes and the 309 NASM. The genes that showed statistically significant differences were *mec*A, *seb*, *see*, *seg*, and *qac*C (Table 6). The chances of the *mec*A gene being present in *S. chromogenes* species were 4 times greater when compared to Non-*chromogenes*. The same was observed for the presence of the *see*, *seg*, and *qac*C genes, where the chances of these genes being present in the *S. chromogenes* species were 6.14, 9.26, and 16.92 higher than in Non-*chromogenes* species, respectively. The opposite occurred with the seb gene since the chances of this gene being present in Non-*chromogenes* species were 2.64 higher than in *S. chromogenes* species.

Table 6. Result of the chi-square test between the genes analyzed and their presence in the 309 NASM.

Genes	Species group	Odds ratio	Confidence Limits (95%)	Chi-Square	P value
*mec*A	Non-*chromogenes*	0.24	0.08 - 0.73	6.62	0.0101
	S. chromogenes	4.16	1.36 - 12.68		
Seb	Non-*chromogenes*	2.64	1.30 - 5.35	7.42	0.0065
	S. chromogenes	0.38	0.19 - 0.77		
See	Non-*chromogenes*	0.16	0.05 - 0.55	9.67	0.0019
	S. chromogenes	6.14	1.83 - 20.54		
Seg	Non-*chromogenes*	0.11	0.03 - 0.43	11.86	0.0006
	S. chromogenes	9.26	2.33 - 36.72		
*qac*C	Non-*chromogenes*	0.06	0.01 - 0.33	12.99	0.0003
	S. chromogenes	16.92	3.04 - 94.25		

DISCUSSION

As already mentioned in the introduction, several studies in the literature indicate the isolation of NASM in samples of milk from healthy cows and those with mastitis in Brazil and worldwide (Persson Waller et al., 2011; Sampimon et al., 2011; Mota et al., 2012; Oliveira et al., 2016; Gao et al., 2017; Stevens et al., 2018; Müller and Rempel, 2020). However, most of these studies do not include the identification and differentiation of species, and this group, which is so heterogeneous, is treated as something unique. This study aims to contribute to this gap, mainly because the NASM isolates come from

Brazil, the world's third-largest milk producer (USDA, 2024), providing information on the prevalence, virulence, and resistance profile of individual NASM species.

As shown in the results, among the 1,468 milk samples analyzed from the 17 herds, 309 NASM were isolated and identified, with 18 different species identified (Table 1). In this study, the most prevalent species isolated and identified were *S. chromogenes, S. simulans, S. haemolitycus, S. epidermidis, S. xylosus, S. warneri,* and *S. equorum*. In a review article, De Buck et al., (2021) surveyed the three most frequently isolated NASM species from cows with clinical mastitis and subclinical mastitis in different countries. In Belgium, in one of the studies, the three most prevalent NASM species were *S. equorum* (34%), *S. haemolyticus* (13%) and *S. epidermidis* (9%). In another study carried out in Belgium, the three species were *S. chromogenes* (41%), *S. sciuri* (13%) and *S. cohnii* (11%). In a study carried out on herds in the United States of America, the three most prevalent species were *S. chromogenes* (48%), *S. haemolyticus* (18%) and *S. simulans* (7%). In China, they were *S. arlettae* (12%), *S. sciuri* (12%), and *S. xylosus* (12%), and in Argentina, the three most prevalent NASM species were *S. chromogenes* (47%), *S. haemolyticus* (32%) and *S. warneri* (7%). The authors do not mention any studies involving NASM isolated in Brazil. In this study, the three most frequently isolated species were *S. chromogenes (*58.6%), *S. simulans* (8.4%), *S. haemolitycus* (8.1%), which corroborates the findings in the literature.

In Brazil, de Freitas Guimarães et al., (2013) analyzed 1,318 milk samples from cows with mastitis from 10 farms in the state of São Paulo and isolated 128 NASM. Of these 128 NASM, the three most prevalent species were *S. warneri* (31.3%), *S. epidermidis* (14.8%), and *S. hyicus* (12.5%), and even though this is a study also carried out in Brazil, these findings differ from the results found in this study. If we consider only the 107 NASM isolated from the state of São Paulo (Figure 1) in this study, the difference between the results found by De Freitas Guimarães and collaborators (2013) remains. In

another study carried out in Brazil, but with samples also coming only from the state of São Paulo, Tomazi et al., (2015) analyzed 1,440 samples of milk from mammary quarters and isolated 141 NASM, of which 108 could be identified, with three species being more prevalent *S. chromogenes* (74%), *S. saprophyticus* (5.5%) e *S. haemolyticus* (4.6%), results that most closely resemble the findings in this present study. It's important to highlight the species *S. chromogenes* which represents 58.6% of the NASM species identified in this study and which corroborates the findings of other studies where this species was also the most prevalent in the dairy herds analyzed (Taponen et al., 2007; Persson Waller et al., 2011; Condas et al., 2017; Valckenier et al., 2020b) in addition to the studies already mentioned above. This can be explained by the fact that this species is more adapted to the cow's udder when compared to other NASM species and can use it as a reservoir and possible source of infection. (Tomazi et al., 2015).

Regarding the SCC of the mammary quarter samples from which NASM were isolated, 54% came from samples with high SCC; 35.3% were composed of milk samples with low SCC, and 10.7% of the samples came from cows with clinical mastitis. In general, NASM are much more associated with cases of subclinical mastitis (SCC >200,000 cells/mL) and, to a lesser extent, also with cases of clinical mastitis. In addition, they are frequently reported in cases of intramammary infections (IMI) in several studies (Naushad et al., 2016; Valckenier et al., 2020b). Furthermore, IMI-causing NASM causes increased SCC in bulk milk tanks (De Buck et al., 2021). The increase or not of SCC can also vary between NASM species, as is the case of *S. chromogenes*, which in a study in Canada, was the species most associated with high SCC compared to the others analyzed in the study. (De Buck et al., 2021). In our study, the species *S. chromogenes* was present in 62% of the milk samples with high SCC, which corroborates the information mentioned above. Studies indicate that differences in the prevalence of NASM in herds are related not only to geographical issues but also to management practices (De Buck et

al., 2021). In our study, the presence of NASM in herds was mainly associated with SCC <200,000 cells/mL, the practice of forming a milking line, performing pre-dipping, mechanical milking (channeled), using gloves, and feeding the animal immediately after milking, i.e., in general, associated with good practices before, during and after milking. Both *S. aureus* and NASM are commonly isolated from milk samples from clinically healthy cows or low SCC (Jones et al., 1984; Rall et al., 2014; Valckenier et al., 2020b). Additionally, some NASM species such as *S. cohnii* and *S. sciuri* causing IMI, do not present an increase in SCC in milk compared to *S. chromogenes* and *S. simulans*, for example (Condas et al., 2017). De Visscher et al., (2014) analyzed the distribution of NASM in extramammary niches that were linked to milking and compared them with NASM that caused IMI in these same herds; some species of NASM, such as *S. auricularis*, *S. cohnii*, *S. equorum*, *S. haemolyticus* and *S. hominis*, were associated with the use of gloves, with the use of gloves being indicated as a vehicle for transmission of these species between cows.

On the other hand, in this same study, *S. chromegenes* was not associated with the use of gloves, which suggests that this species is more related to the cow's udder, in addition to several studies associating *S. chromogenes* with milk, the teat apex and the teat canal (Vanderhaeghen et al., 2015b). It is also important to highlight that NASM can persist in the udder for months, even during the lactation period, in addition to being widely present in the bovine skin and mucosa as well as in the farm environment (De Visscher et al., 2014; Naushad et al., 2016).

Discussing the antimicrobial resistance profile of NASM, studies suggest that the frequent use of drugs in veterinary practice, such as penicillin and tetracycline, especially among cattle, has increased the resistance of microorganisms. The FDA and the European Medicine Agency (EMA) show that Penicillins, Tetracyclines, and Sulfonamides are the most widely sold classes of antibiotics in the US and Europe (FDA, 2022; Dorneles et al.,

2019; EMA, 2023) for use in animals. In our study, 24.9% of NASM isolates showed resistance to penicillin, 26.9% showed resistance to oxacillin, and 8.7% showed resistance to cefoxitin. Klibi et al., (2018) studied 68 NASM isolates from milk of cows with mastitis, and of these 68, 20 (29.4%) showed resistance to oxacillin and/or cefoxitin. All NASM species identified in this study, except *S. succinus*, showed resistance to at least one antibiotic tested. However, when analyzing by species and especially regarding resistance to multiple drugs of different classes, among the 41 MDR NAS (13.3%) found in this study, we have *S. chromogenes* as the species classified as the most MDR, followed by *S. haemolyticus* and *S. epidermidis*. In the study carried out by Nobrega et al., (2018), 8.8% (n=150) of the 1,702 NASM analyzed were MDR; the authors also hypothesize that the increase in antimicrobial-resistant NASM is due to exposure to prolonged doses and subtherapeutic antimicrobial concentrations in the udder, especially after treatments not indicated for IMI and in other tissues. Furthermore, according to the authors, these microorganisms, when eliminated in the environment, may cause IMI. Several other studies in the literature present MDR NASM (Sampimon et al., 2011; Cheng et al., 2019; Dorneles et al., 2019; Phophi et al., 2019).

All the above not only raises concerns about methicillin-resistant *Staphylococcus aureus* (MRSA) but also concerns about the increasingly frequent emergence of methicillin-resistant *Staphylococcus* non-*aureus* (MRNAS), such as those found in this study. MRSA is associated with several infections and high mortality. It was previously restricted to the hospital medical environment, but today we know that MRSA is also present in the community (food, animals, industrial equipment, and food handlers) (da Silva Abreu et al., 2021; Medeiros et al., 2024; Roy et al., 2024). The concern now extends to MRNAS, which also becomes resistant to β-lactam antibiotics due to the presence of the *mec*A gene, which in turn encodes the penicillin-binding protein (PBP2a or PBP2), reducing the ability of β-lactam antibiotics to act against these microorganisms

(Roy et al., 2024). In this study, NAS species carrying the *mec*A gene were *S. epidermidis* (n=9), *S. chromogenes* (n=5) and *S. xylosus* (n=1). In Brazil, Silva et al., (2014) evaluated the presence of the *mec*A gene in 128 NAS isolated from milk of cows with subclinical mastitis from 10 dairy herds in the state of São Paulo, and 26 (20.3%) harbored the *mec*A gene. Among the species carrying this gene were *S. epidermidis* (7), *S. chromogenes* (7), *S. warneri* (6), *S. hyicus* e (5) and *S. simulans* (1). When compared with the results obtained in this study, there is a higher prevalence of identified MRNAS and a greater diversity of *mec*A-positive species. In addition to widespread resistance to several antibiotics, in a study carried out in Belgium, MRNAS were considered an important reservoir of virulence genes and resistance genes to other antibiotics (De Buck et al., 2021), which raises further concern about MRNAS as these genes can be transferred to other species (Smith and Andam, 2021).

Another concern is the resistance of microorganisms to the sanitizers used, which contributes to their survival and increased resistance. Disinfectants such as peracetic acid, sodium hypochlorite, and quaternary ammonium compound (QAC) are commonly used for disinfection in the food industry and milking equipment. In this study, some NASM presented the resistance genes *qac*AB and mainly *qac*C in the analysis performed by PCR; these genes encode membrane protein efflux pumps and promote tolerance to QAC disinfectants. (Czarnecka et al., 2025).

Regarding the presence of genes involved in the production of enterotoxins in NASM isolates, except the *sed* gene, all the others investigated were found in this study. Staphylococcal enterotoxins (SEs) are extracellular, heat-stable proteins capable of remaining present even where no longer viable bacteria exist. (Bellio et al., 2019). The classic SEs, the most defined currently, were classified as SEA, SEB, SEC, SED, and SEE, responsible for 95% of food poisoning by *S. aureus* (Cai et al., 2021). NASM are also capable of producing enterotoxins, which can be one of the causes of foodborne

illness (da Silva Cândido et al., 2020). Currently, 6 SEs are known (SEA, SEB, SEC, SED, SEE, and SEG), 21 SE-like toxins (H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, 27 and 28) (Chieffi et al., 2020).

Xavier et al., (2017) characterized NASM isolated from raw milk, Minas cheese, and Minas cheese production lines and detected the *seb*, *seg*, *seh* genes in the identified NASM species. In Brazil, Rall et al., (2014) evaluated the prevalence of staphylococcal enterotoxins in 128 NASM isolated from milk of healthy cows and cows with mastitis, and 69 (53.9%) presented the *sea* gene, which was the most prevalent. This differs from the results found in this study, where *seb* was the most prevalent gene.

In Brazil, RDC 724/2022 and IN No. 313/2024 provide for the control of staphylococcal enterotoxins in milk and dairy products, in addition to the control of SEs in ready-to-consume products. The limit should be the absence of SEs ng/g in all units tested (N=5) (Brazil, 2019). In the European Union, Commission Regulation (EC) 1441/2007 provides for the enumeration of coagulase-positive staphylococci (CPS) and the detection of staphylococcal enterotoxins (SEs) in milk and dairy products and that SE detection should be performed when the CPS count exceeds 10^5 colony forming units per gram (CFU/g) (European Commission [EC] 2007; Bellio et al., 2019). In other words, this EU regulation does not cover the production of enterotoxins by most NASM species, and SEs are only tested when the CPS count is higher than 10^5 (CFU/g). However, despite foods having low counts of this microorganism or after the vegetative cells have been inactivated, the enterotoxins may be viable since they are heat-stable, thus underestimating the identification of these enterotoxins in foods (Chieffi et al., 2020).

It is important to emphasize that most of the milk samples where these NASM isolates presented sanitizer resistance genes, methicillin resistance genes, and enterotoxin genes are from cows with low SCC and high SCC. Therefore, these samples will go to

the tanks that will be sold, and these pathogenic isolates may be disseminated in the dairy processing chain, thus representing a risk to the consumer's health.

CONCLUSION

The results of this study allowed us to expand our knowledge about the widespread NASM species in different Brazilian herds. Despite the diversity of species, the prevalence of *S. chromogenes* was shown to be much higher. There was a higher prevalence of NASM isolates in some states, and geography and farm characteristics, such as management practices, may have influenced this isolation.

Although the numbers regarding resistance and virulence may not be considered high, they are still worrying since the samples from which most NASM were isolated were considered suitable for commercialization. In addition, NASM can transfer resistance and virulence genes to other microorganisms. Therefore, control and identification of NASM are necessary to better understand the level of pathogenicity among these species and the risk they pose, and subsequently, to design more effective control strategies.

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Appendix 1

PRODUCER RESEARCH QUESTIONNAIRE

Producer Name:

_____

Property Name: _____

Municipality:

_____

Contact telephone:_____

Contact e-mail:_____

Date:

1 MILKING AND MASTITIS CONTROL

1.1 How many times a day are animals milked?
( ) 1
( ) 2
( ) 3

1.2 What is the number of milkers?
( ) 1
( ) 2
( ) 3
( ) Other:_____

1.3 What is the type of milking?
( ) Manual
( ) Mechanics (bucket milking)
( ) Mechanics (piped)

1.4 How many milking sets do you have?_____

1.5 What is the material of the liners?
( ) Rubber
( ) Silicone

1.6 Does it have automatic cup extraction?
( ) Yes
( ) No

1.7 Do you maintain the milking equipment?
( ) Periodic
( ) Occasional (when there is a problem)
( ) Never

1.8 Do you do a milking line, that is, do you milk cows with mastitis last or separately?
( ) Always
( ) Sometimes
( ) Never

1.9 Does the milker(s) use disposable gloves to perform milking?
( ) Yes
( ) No

1.10 Do you wash teats with water?
( ) Always
( ) When very dirty
( ) Never

1.11 Do you do the mug test (elimination of the first three jets of milk)?
( ) Yes
( ) No

1.12 Do you pre-*dip*?
( ) Yes
( ) No

1.13 Do you dry the teats?
( ) Yes. ( ) Paper towel ( ) Cloth
( ) No

1.14 Do you do post-*dipping*?
( ) Yes
( ) No

1.15 Do you provide food for the cows during milking?
( ) Yes
( ) No

1.16 Do you provide food to the cows immediately after milking?
( ) Yes
( ) No

1.17 Do you take the CMT test (California Mastitis Test)?
( ) Yes, regularly
( ) Yes, occasionally
( ) No

1.18 Do individual somatic cell count (SCC) of lactating cows?
( ) Yes, regularly
( ) Yes, occasionally
( ) No

1.19 Do you treat clinical mastitis immediately?
( ) Yes.
What criteria(s) should be treated?
_____
( ) No Which product(s)?_____

1.20 Are you treated for subclinical mastitis during lactation?
( ) Yes.
What criteria(s) should be treated?
_____
( ) No Which product(s)?_____

1.21 How do you choose antibiotics to treat mastitis?
( ) Seller recommendation
( ) Veterinarian's recommendation
( ) Rotation
( ) Culture and antibiogram results
( ) Other: _____

1.22 Do you perform bacteriological culture and antibiogram of milk?
( ) Yes, regularly.
( ) Yes, occasionally
( ) No

1.23 Do you treat the cows with antibiotics when drying them (dry cow antibiotic)?
( ) Yes, of all dry cows. Which product(s)?_____
( ) Yes, from some dry cows. What criteria(s)?
_____
( ) No

1.24 Do you use internal teat sealant on dry cows?
( ) Yes, in all dry cows. Which product(s)?_____
( ) Yes, in some dry cows. What criteria(s)?_____
( ) No

1.25 Do you use the vaccine for mastitis?
( ) Yes. Which one?
_____
( ) No

1.26 Do you discard animals due to mastitis?
( ) Yes. What criteria(s)?
_____
( ) No

1.27 Do you use warm water to rinse the milking machine after milking?
( ) Yes
( ) No

1.28 Do you use alkaline detergent to clean the milking machine?
( ) Yes, with hot water
( ) Yes, with water at room temperature
( ) No

1.29 Do you use acid detergent to clean the milking machine?
( ) Yes
( ) No

1.30 Do you sanitize the milking machine with chlorine solution before milking?
( ) Yes
( ) No

2 FARM DATA

2.1 Daily milk production (liters/day):_____

2.2 Number of lactating cows in the herd:_____

2.3 Somatic cell count (SCC) of tank milk:_____

2.4 Total bacterial count (CBT) of tank milk:_____

2.5 For those who sell the milk:_____
( ) Dairy under
Federal Inspection
Seal (SIF)
( ) Dairy under State
Inspection Seal (SIE)
( ) Cooperative
( ) Cheesemaker

2.6 Is payment for milk according to quality?
( ) Yes
( ) No

2.7 Housing system for lactating cows
( ) Free stall
( ) Tie stall
( ) Compost barn
( ) Pickets
( ) Other: _____

2.8 Technical assistance
( ) Yes
( ) Dairy
( ) Private
( ) Other: _____
( ) No

CAPÍTULO 3

Presence of genes responsible for biofilm formation and biofilm formation by *Staphylococcus* non-*aureus* (NAS) isolated from cow's milk

Artigo submetido no periódico "Biofouling"

Presence of genes responsible for biofilm formation and biofilm formation by *Staphylococcus* non-*aureus* (NAS) isolated from cow's milk

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Highlights

- *Staphylococcus* non-*aureus* (NAS) isolated from cow's milk carries biofilm genes
- NAS formed biofilm on Congo red agar (CRA)
- NAS isolated from milk could form biofilm on stainless steel

Graphical abstract

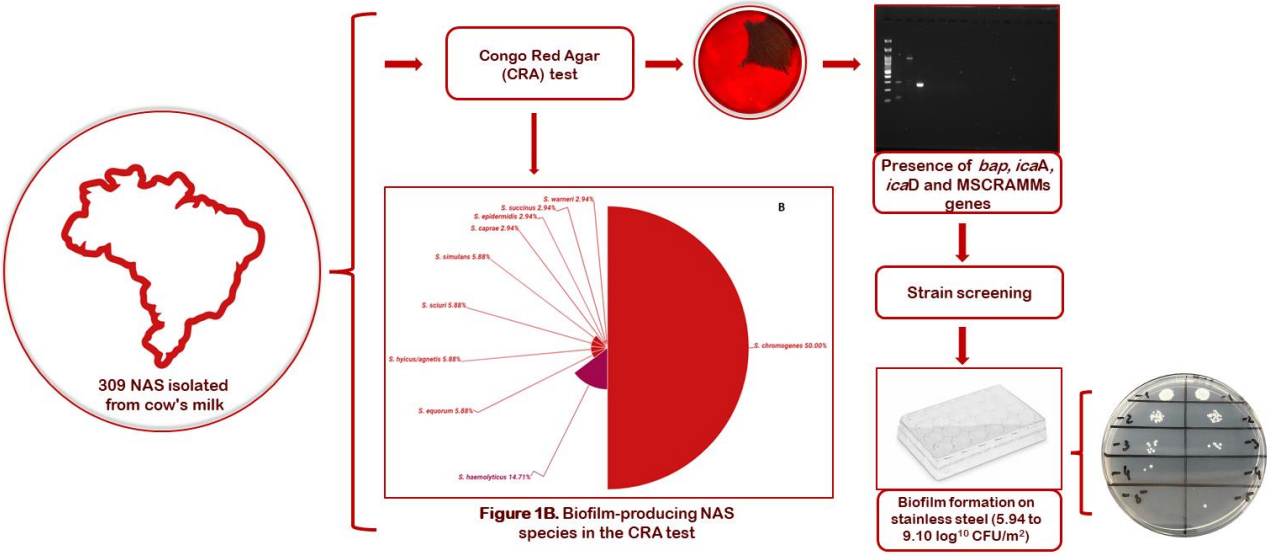

Figure 1B. Biofilm-producing NAS species in the CRA test

Abstract

Non-*aureus Staphylococcus* (NAS) isolated from the milk of healthy cows and milk from cows with mastitis has been frequently reported. However, there are few in-depth studies regarding their virulence profile and the ability of these microorganisms to form biofilms. Therefore, this research aimed to evaluate the biofilm formation capacity of NAS isolates from Brazilian milk. In this work, 309 NAS isolates were subjected to the Congo Red Agar (CRA) phenotypic test. Next, genotypic characterization was carried out by screening the *bap*, *ica*A, *ica*D, and MSCRAMMs genes: *bbp*, *cna*, *ebps*, *eno*, *fib*, *fnb*A, *fnb*B, *clf*A and *clf*B. Finally, ten isolates that presented the highest frequency of the genes analyzed were selected to evaluate the ability to form biofilm on the contact surface of the stainless-steel discs through microbiological counting (log10 CFU/cm^2). This was measured in three periods (24h, 48h, and 72h) at a temperature of 25ºC. The results showed that 35 NAS (11.32%) produced biofilm in the CRA test. The genotypic analysis showed the *eno* (38.5%) and *bap* (27.5%) genes as the most prevalent. In the analysis of biofilm formation on stainless steel, the factor 'growth time' had no significant difference in the microbiological count. All selected isolates formed biofilm on stainless steel, and microbial counts were observed in the 5.94 to 9.10 log10 CFU/m^2 range. Therefore, these results provide evidence that NAS isolated from milk may represent a risk to human and animal health since they carry several virulence genes and demonstrate the ability to form biofilm.

Keywords: biofilm; stainless steel; CRA test; virulence genes.

Introduction

Mastitis is a common and costly disease of dairy cows, affecting milk quality and quantity, animal welfare, and profitability. Among the main causative agents of mastitis is *Staphylococcus aureus*, and recent studies have shown that Non-*aureus* staphylococci (NAS), previously considered minor or opportunistic mastitis pathogens, are increasingly associated with subclinical and clinical mastitis cases worldwide (Piepers *et al.* 2007, Traversari *et al.* 2019).

NAS is a diverse group of *Staphylococcus* genius, being the most prevalent in mastitis *Staphylococcus chromogenes, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus simulans,* and others (Hosseinzadeh and Dastmalchi Saei 2014, Traversari *et al.* 2019).

Different species of bacteria can live in communities, adhere to an inert or living surface, and be protected by a polymeric matrix called a biofilm. This matrix is a self-product of its metabolism, consisting mainly of sugar and proteins. Bacteria generally produce a biofilm to protect themselves from fluctuations in environmental conditions. The quality, quantity, and safety of food products are affected by the persistence of some foodborne pathogens on food contact surfaces and biofilms, and this problem has been reported more frequently (Satpathy *et al.* 2016, Shemesh and Ostrov 2020).

Biofilm formation in staphylococcal species is associated with several factors, and several studies in the literature show NAS is a carrier of biofilm genes. The production of the polysaccharide intercellular adhesin (PIA), which consists of linear b-1, 6-linked glucosaminylglycan, whose synthesis is mediated by the genes *ica*A, *ica*B, *ica*C, and *ica*D, represent an important contribution to cell-cell adhesion (Arciola *et al.* 2012, Srednik *et al.* 2017). Also, Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are important during the development of biofilms (Foster 2019). Examples of MSCRAMMs include the genes *clf*A and *clf*B (which encode

clumping factors A and B), *fib* (which encodes fibrinogen-binding protein), *fnb*A and *fnb*B (which encode fibronectin-binding proteins A and B), *cna* (which encodes collagen-binding protein), *eno* (which encodes laminin-binding protein), *ebps* (which encodes elastin-binding protein), and *bbp* (which encodes sialoprotein-binding protein) (Khoramian *et al.* 2015).

Biofilm formation is also connected to antibiotic resistance, according to Srednik et al., (Srednik *et al.* 2017), NAS isolates growing within biofilms are less susceptible to antimicrobials commonly used on farms. Researchers and organizations are highlighting the increasing rate of antibiotic resistance. This is because, in addition to bacteria having the ability to form biofilms, other genes are also involved in antibiotic resistance; the process of becoming resistant can also occur through mutation at the genetic level (Frieri *et al.* 2017, Zaman *et al.* 2017). In addition, it has been suggested that NAS might act as a potential reservoir for resistance genes, which can be transferred and integrated into the genome of *Staphylococcus aureus* (Holmes and Zadoks 2011, Otto 2013, Vitali *et al.* 2014).

Few studies have evaluated the presence of genes responsible for biofilm formation and MSCRAMMs in NAS isolates at the species level, mainly food-source isolates. Most studies have focused on *S. aureus* and clinical isolates from hospitals. Considering milk, an important food used as an ingredient for several other dairy products, and considering that these NAS isolates come from milk samples from Brazil, the third largest milk producer in the world, it is necessary to study these species further (USDA 2024, Garcia *et al.* 2019). Therefore, this study aimed to evaluate the presence of genes responsible for biofilm formation and adhesion and the biofilm formation capacity of NAS isolated from dairy farms in Brazil.

Material and methods

Origin and identification of isolates

This study used 309 NAS isolates from healthy cows' milk and cows with mastitis from a previous study (unpublished data) in Brazil. These isolates belong to the Food Microbiology Laboratory II, Faculty of Food Engineering (FEA), State University of Campinas (UNICAMP). The species used were *S. chromogenes* (N=181), *S. simulans* (N=26), *S. haemolyticus* (N=25), *S. epidermidis* (N=14), *S. xylosus* (N=14), *S. warneri* (N=9), *S. equorum* (N=9), *S. cohnii* (N=7), *S. saprophyticus* (N=7), *S. hyicus/agnetis* (N=5), *M. sciuri* (N=4), *S. pasteuri* (N=2), *S. succinus* (N=1), *S. auricularis* (N=1), *S. caprae* (N=1), *S. capitis* (N=1), *S. kloosii* (N=1), *S. arlettae* (N=1). Furthermore, of these 309 NAS, 15 (4.8%) were positive for the *mec*A gene (unpublished data) in a previous PCR analysis.

Genotypic tests

Cultures were grown in BHI broth at 37ºC for 24 hours and centrifuged once at 12,000rpm/30s at room temperature. The supernatant was discarded, and the obtained pellet was used for DNA extraction. The latter step was carried out according to the instructions of the manufacturer of the InstaGene™ Matrix kit (Bio-Rad Laboratories, Hercules, California, USA).

A spectrophotometer was used to quantify and determine the purity of the collected samples in nanograms per microliter (ng/μL). These parameters were calculated using the following ratios: A260/A280 nm and A230/A260 nm.

After extraction and quantification of the 309 strains, oligonucleotides were used for the amplification of *bap*, *ica*A, *ica*D, *bbp*, *cna*, *ebp*S, *eno*, *fib*, *fnb*A, *fnb*B, *clf*A, and *clf*B genes. Table 1 shows details about this analysis and the parameters used.

Table 1. Oligonucleotide sequence of each gene that was used for genotypic identification, and parameters applicable to the Thermal Cycler for the PCR reaction for biofilm genes.

Gene	Oligonucleotides sequence (5´-3')	Amplicon size (bp)	Conditions	Controls	Reference
bap	5'-CCCTATATCGAAGGTGTAGAATTG-3' 5'-GCTGTTGAAGTTAATACTGTACCTGC-3'	971	94ºC/4 min; 94ºC/1 min; (40 cycles); 59ºC/1 min.; 72ºC/ 1 min; 72ºC/ 10 min.	(5008E)	(Cucarella *et al.* 2001)
*ica*A	5'-CCTAACTAACGAAGGTAG-3' 5'-AAGATATAGCGATAAGTGC-3'	1315	94ºC/2 min.; 94ºC/1min; (32 cycles); 49ºC/1 min; 72ºC/ 1 min; 72ºC/ 5 min.	(ATCC6538)	(Vasudevan *et al.* 2003)
*ica*D	5'-AAACGTAAGAGAGGTGG-3' 5'-GGCAATATGATCAAGATAC-3'	381		(N315)	
bbp	5'-AACTACATCTAGTACTCAACAACAG-3' 5'-ATGTGCTTGAATAACACCATCATCT-3'	575	95ºC/5 min; 94ºC/1 min; (25 cycles); 52ºC/1 min; 72ºC/1 min; 72ºC/10 min.	(WB49, N315)	(Tristan *et al.* 2003)
*clf*A	5'-ATTGGCGTGGCTTCAGTGCT-3' 5'-CGTTTCTTCCGTAGTTGCATTTG-3'	292	95ºC/5 min. 94ºC/1 min (25 cycles) 56ºC/1 min 72ºC/1 min 72ºC/10 min.	(WB49, USA300)	(Tristan *et al.* 2003)
*clf*B	5'-ACATCAGTAATAGTAGGGGGCAAC-3' 5'-TTCGCACTGTTTGTGTTTGCAC-3'	205		(6538, N315)	(Tristan *et al.* 2003)
cna	5'-GTCAAGCAGTTATTAACACCAGAC-3' 5'-GTCAAGCAGTTATTAACACCAGAC-3'	423		(N315, USA300)	(Tristan *et al.* 2003)
*ebp*S	5'-AGAATGCTTTTGCAATGGAT-3' 5'-AATATCGCTAATGCACCGAT-3'	652		(6538, 25923)	(Vancraeynest *et al.* 2004)
eno	5'-ACGTGCAGCAGCTGACT-3' 5'-CAACAGCATTCTTCAGTACCTTC-3'	302		(WB49, USA300)	(Tristan *et al.* 2003)
fib	5'-CTACAACTACAATTGCCGTCAACAG-3' 5'-GCTCTTGTAAGACCATTTTCTTCAC-3'	404		(6538, USA300)	(Jarraud *et al.* 2001)
*fnb*A	5'-CATAAATTGGGAGCAGCATCA-3' 5'-ATCAGCAGCTGAATTCCCATT-3'	127		(6538, WB49)	(Vancraeynest *et al.* 2004)
*fnb*B	5'-GTAACAGCTAATGGTCGAATTGATACT-3' CAAGTTCGATAGGAGTACTATGTTC-3'	524		(WB49, USA300)	(Tristan *et al.* 2003)

The amplification process was carried out using a kit that contained a Reaction Buffer PCR 1X, 200µM of each deoxynucleotide triphosphate (dNTP), $MgCl_2$, 1 U of Taq Polymerase (GoTaq Hot Start Polymerase, Promega Corporation, Madison, USA), 1µl of DNA, 10 pmol of each primer, and ultrapure water Milli-Q to achieve a final volume of 25µL. All treatments were performed in the Thermocycler Bio-Rad C-1000 (BioRad, Hercules, USA) following the parameters shown in Table 2. After amplification, the DNA fragments were analyzed through electrophoresis (80V, 80mA, 120 minutes) in a 1% (m/v) agarose gel buffer with 0.5X TBE (45 mM Tris-borate) and

SYBR Safe DNA Gel Stain. Photodocumentation of the gel was done using Image LabTM Software with the Molecular Imager Gel DocTM XR from BioRad Laboratories.

Biofilm production on Congo Red Agar (CRA)

The NAS isolates were reactivated in BHI broth at 37°C in aerobic conditions for 24 hours. BHI agar, 0.08% Congo red, and 5% sucrose (Cruzado-Bravo *et al.* 2018) were mixed to prepare Congo red agar. The samples were then manually inoculated onto Petri dishes containing CRA and incubated at 37°C for 24 hours. Biofilm formation was visible as black or gray, while non-biofilm formation appeared burgundy or red.

Biofilm formation in stainless steel

Ten samples (8, 14, 263, 267, 283, 885, 891, 895, 897, and 942) showed the highest prevalence of adherence and biofilm genes out of the 309 samples. The positive control, ATCC 6538, a highly biofilm-producing strain, was selected, and the negative control consisted of the absence of bacteria.

For the biofilm formation, stainless steel discs, each with a diameter of 12 mm, were meticulously prepared. They were first washed with a neutral detergent and distilled water, then immersed in 70% ethyl alcohol, washed again with distilled water, and finally sterilized at 121°C (1 atm) for 15 minutes (Cruzado-Bravo *et al.* 2018). The cultures were grown in BHI broth at 37°C for 24 hours and standardized using the McFarland scale (0.5; 10^8 CFU/ml). A 1mL suspension was then placed on a plate containing 24 wells containing a sterilized disk. Three discs were analyzed per sample. The microplates were then incubated at 25°C for 72 hours. Bacterial counts were taken at 24, 48, and 72 hours of incubation.

Additionally, discs were removed from the microplate sterilely and washed thrice with phosphate-buffered saline (PBS) to remove non-adherent bacteria. They were then transferred to tubes containing 9 ml of 0.1% (w/v) peptone water and sonicated in an ultrasonic bath at 40 kHz for 1 minute, followed by a vortex mixing for 1 minute. Serial

dilutions were performed on the suspension, and 0.01mL was plated on BHI agar using the microdrop method. The plates were then incubated at 37°C for 24 hours, and the number of colonies was counted. For bacterial counting, equation (1) was used (Guilherme *et al.* 2016):

(1) $$CFU/cm^2 = \frac{CFU/mL^1 \text{ x diluent volum (mL)}}{\text{area (cm)}}$$

Statistical Analysis

The data collected in this study are presented in two ways; quantitative data as means ± standard deviation and qualitative data as frequencies (%). The results were considered significant at p-values less than 0.05. All analysis was conducted using RStudio version 2023.03.0-daily+82.pro2 2020 and GraphPad. Statistical analysis of genes and CRA test for biofilm formation was done with Cochran's Q test (Cochran 1950), resorting to the RStudio. Assumptions of independence and binarity were met. Due to the binary nature of the variables, the presence of the gene was considered as one (1) and the absence of the gene as zero (0). As for CRA the same system was adopted: one (1) for positive strains and zero (0) for negative strains.

GraphPad Software, Inc. was used to perform normality tests Shapiro-Wilk for microbiologic counting and linear regression to analyze and compare the relationship between genes and biofilm formation

Results and discussion

CRA test

As seen in Figure 1 A, in Congo Red Agar, it is observed that strains that do not produce biofilm appear with color tones ranging from bright red to burgundy red, while for strains positive for biofilm production, black and dark tones are noted, color characteristics that are acquired when using the dye Congo Red, which is a representative of the category of secondary diazo compounds, and which, in addition to being used for

dyeing media, presents excellent stability characteristics in various areas of application in food processing (Sharma *et al.* 2021, Ruiz-Romero and Vargas-Bello-Pérez 2023).

According to Figure 1 B, among 309 strains analyzed, 34 (11.0%) were positive for biofilm-producing bacteria in CRA. Among those positive for *Staphylococcus* spp. the species with the highest occurrence were *S. chromogenes* (50%), *S. haemolyticus* (14.71%), and *S. equorum* (5.88%). Similar results for identified microorganisms were observed by Turchi *et al.* (2020) when investigating the species diversity of *Staphylococcus* spp. causes of subclinical mastitis using the PCR-RFLP (Restriction fragment length polymorphism) technique, concluding that *S. epidermidis* was the predominant species in thirty-nine isolates (53.4%) of its analyzed samples. However, unlike the present study, none of the isolates were biofilm producers on Congo Red Agar. Previous studies show that several factors can modify/favor the formation and quantification of bacterial biofilms, such as the properties and physicochemical profile of bacteria during the supply of nutrients in biofilm formation, use of different bacterial cultivation surfaces with different roughness and hydrophobicity profiles, and use of varied methods for quantifying biofilm adhesion (Bhagwat *et al.* 2021, Zheng *et al.* 2021, Simões *et al.* 2022).

In a study carried out by Salina *et al.* (2020), from a sample of 100 NAS strains collected from two dairy farms in the state of São Paulo, Brazil, from cows with subclinical and clinical mastitis, 8% were positive for CRA, results similar to those of this study. Another study, conducted by Azara *et al.* (2022), examined 125 isolates and found that only 4 (3.2%) were classified as strong biofilm producers due to the black color of their colonies; the remaining 121 (96.8%) were considered non-biofilm producers, presenting a red or burgundy color, indicating a lower biofilm production than that observed here. On the other hand, some studies show a higher proportion of biofilm

formation than ours. For example, Donadu *et al.* (2022) reported that 44.9% of NAS isolates were biofilm positive.

The sensitivity and accuracy of the different methods for evaluating adherence and formation of bacterial biofilms are pretty varied, with the tube adhesion assessment method (TM) being characteristic for having greater sensitivity compared to the Congo Red (CR) method, even though the specificity of both methods is similar (Thilakavathy *et al.* 2015, Shrestha *et al.* 2018). The CR technique is still widely used as it presents lower equipment requirements and is quicker to obtain results in the laboratory. However, compared to methods, it is the least advisable due to the high incidence of false negatives (García *et al.* 2004, Ruiz-Romero and Vargas-Bello-Pérez 2023)

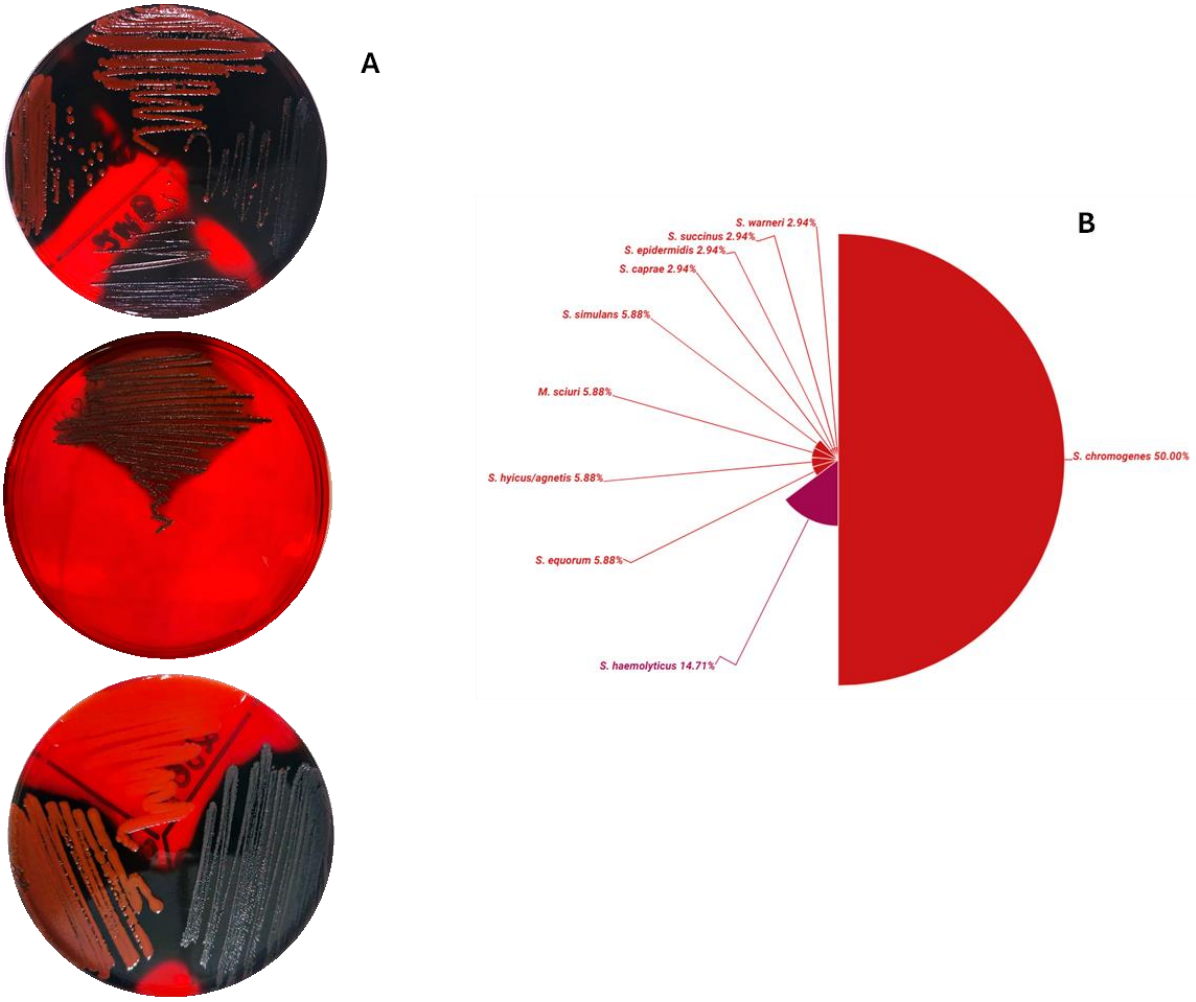

Figure 1. (A) Plates with red and black staining to identify biofilm formation on Congo Red Agar. **(B)** Frequency (f%) of species positive for biofilm formation in the CR.

Detection of genes encoding biofilm formation and adhesion factors

All 309 isolates were used to assess the relationship between the presence of *bap*, *ica*A, *ica*D, and MSCRAMMs genes and biofilm formation. Figure 2 shows the distribution of gene prevalence among all species. The most present gene was *eno*, appearing in 38.5% of isolates, followed by *bap* (27.5%). The prevalence of other genes in all isolates analyzed was: *ica*D (6.8%), *ica*A (5.2%), *fib* (4.9%), *clf*A (3.9%), *fnb*A (3.6%), *ebpS* (2.3%), *clf*B (1.9%), *cna* (1.6%), *fnb*B (1.0%) and *bbp* (1.0%).

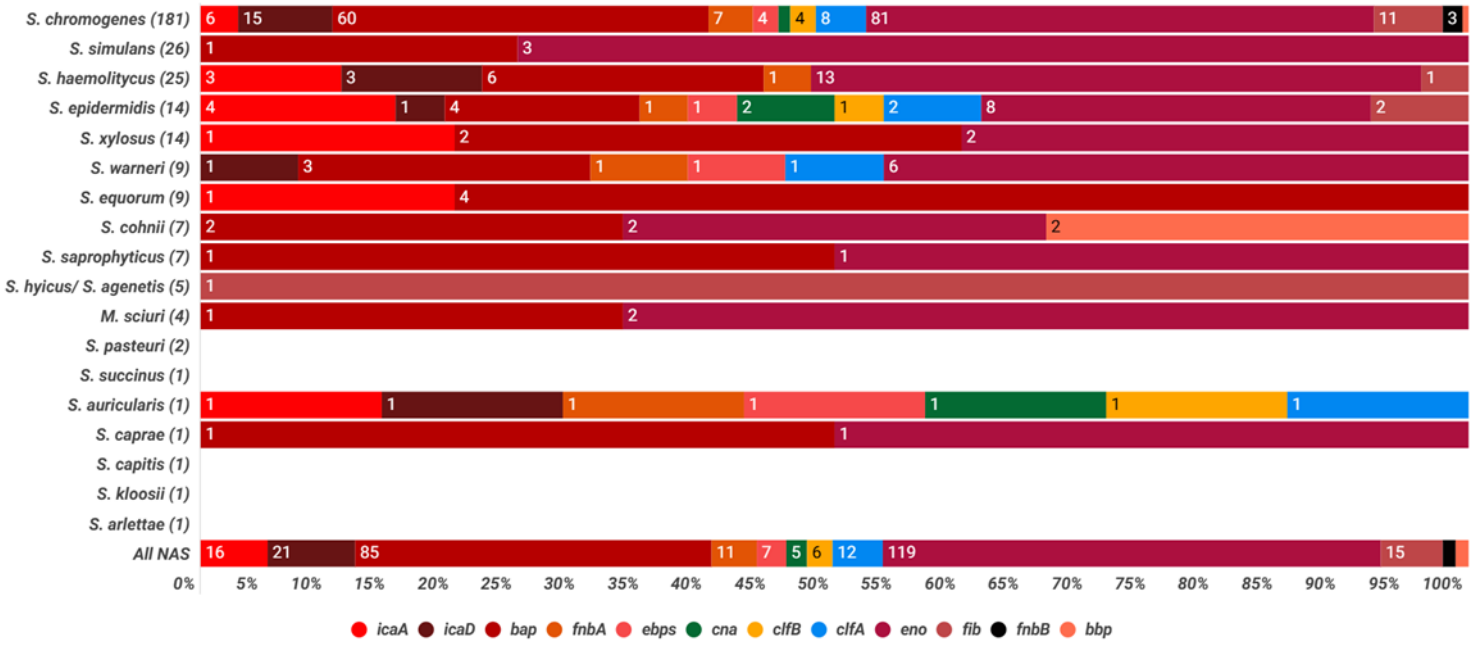

Figure 2. Prevalence of the *bap*, *ica*A, *ica*D and MSCRAMMs genes in the 309 NAS isolates presented overall and by species.

This analysis of frequency and distribution of biofilm genes passed the Shapiro-Wilk normality test, that is, it was classified as a normal distribution, with values W = 0.979 and p = 0.979, p > 0.05.

Q is a measure of variation or discrepancy in proportions between compared groups (genes), indicating substantial variability between them (Hoaglin 2016). This means that the presence of genes is not uniform in all samples, suggesting that at least one of the samples has a different proportion of gene presence compared to the others. In

summary, the very small p-value leads us to conclude that the genes do not have the same prevalence in all samples, indicating a significant difference in the distribution of gene presence.

The Cochran Q test was also performed to compare the presence of each gene with the result "biofilm formation in CRA". The binary system of presence/absence of genes was compared with the presence/absence of biofilm formation. The results can be seen in detail in Table 2.

Table 2. Cochran's Q test compares the prevalence of genes with the outcome of "biofilm formation in CRA".

	Cochran's Q test			
Genes	**Q**	**p-value**		**Significance**
*ica*A	55.48	9.44E-14	p<0.001	*
*ica*D	63.93	1.28E-15	p<0.001	*
bap	182.58	1.32E-41	p<0.001	*
fnbA	58.30	2.25E-14	p<0.001	*
ebps	57.78	2.93E-14	p<0.001	*
cna	59.56	1.18E-14	p<0.001	*
clfB	58.60	1.93E-14	p<0.001	*
clfA	60.71	6.60E-15	p<0.001	*
eno	274.23	1.36E-61	p<0.001	*
fib	62.95	2.12E-15	p<0.001	*
fnbB	61.97	3.48E-15	p<0.001	*
bbp	61.97	3.48E-15	p<0.001	*

This statistic for Cochran's Q test reflects the degree of variability between the observed proportions of the presence and absence of genes concerning biofilm formation. Higher values indicate more variability. The p-value indicates the probability that the observed variability could have occurred by chance. A smaller p-value indicates more robust evidence against the null hypothesis, which in this case means that there is a significant difference between the variables and that they are not associated.

In the table 3, significance means that there is a difference between the measured variables and that they have no association. In other words, the prevalence of all genes analyzed does not correlate with biofilm formation in CRA. This was observed in our

work, where several isolates presenting a low prevalence of biofilm genes produced biofilm in the CRA. The opposite is also true; isolates with a high prevalence of biofilm genes did not produce biofilm in the CRA test.

As previously reported, the *eno* and *bap* genes were the most prevalent in this study of NAS isolates. Similar results were found previously, where the *eno* gene was present in most isolates (Khoramian *et al.* 2015, Zuniga *et al.* 2015). The *eno* gene encodes α-enolase, which can bind to laminin and acts as a plasminogen receptor. Laminin plays a crucial role in the composition of the basement membrane of the vasculature. Therefore, adhesion to laminin may facilitate tissue invasion and dissemination of staphylococcal cells through the blood to various sites in the host (Kot *et al.* 2022).

Studies show that the *bap* gene is associated with mastitis (Piessens *et al.* 2012, Khoramian *et al.* 2015, Zuniga *et al.* 2015). The *bap* gene is responsible for initial adhesion in the biofilm formation process and is encoded by the Bap protein (biofilm-associated protein) (Cucarella *et al.* 2001, Crippa *et al.* 2024). In our study, the *bap* gene was present in both NAS isolates from clinically healthy (SCC <200,000 cells/mL) and diseased animals. This suggests the association of this gene with the host, independent of inflammation. However, it is important to highlight that staphylococcal biofilm can cause problems not only during the treatment of bovine mastitis but also represents a great risk for dairy production since the microorganism can persist in the dairy chain.

When analyzing the distribution by NAS species, we can see that the species that most presented genes responsible for biofilm formation and adhesion were *S. chromogenes*, *S. haemolitycus*, *S. epidermidis*, and *S. xylosus*. However, we can also observe some genes found in 13/18 (72%) species analyzed in this study.

It's important to note that few studies on NAS from clinical or subclinical mastitis in dairy cattle have been conducted. One study by Åvall-Jääskeläinen *et al.* (2018) observed that some of the genes were only found in *S. simulans*, while others were present in both *S. agnetis* and *S. chromogenes*. These differences align with the results of our present study. It appears that different NAS species may have their genotypic profile, which can influence the inflammatory response in the udder and should, therefore, be studied. In human infections, the *bbp* and *cna* genes are frequently found (Cruzado-Bravo *et al.* 2018).

Biofilm formation on stainless steel contact surface

As previously mentioned, 10 strains (8, 14, 263, 267, 283, 885, 891, 895, 897, and 942) were selected due to their high prevalence of adherence and biofilm genes. Of these 10, 4 were identified as *S. epidermidis*, 4 as *S. chromogenes*, 1 as *S. auricularis*, and 1 as *S. haemolyticus*. A positive control, ATCC 6538, was selected, and the negative control involved the absence of bacteria. Table 3 displays the genetic profile of each species obtained after conducting PCR.

Table 3. Distribution of biofilm genes and CRA test among isolates selected for biofilm formation analysis

Sample code	CRA	Species	Genotypic profile
8	-	*Staphylococcus epidermidis*	*ica*A, *bap*, *cna*, *clf*A, *eno*, *mec*A
14	-	*Staphylococcus epidermidis*	*ebps*, *clf*A, *eno*, *mec*A
263	-	*Staphylococcus chromogenes*	*ica*A, *ica*D, *bap*, *ebps*, *cna*, *clf*B, *clf*A, *eno*, *bbp*
267	-	*Staphylococcus chromogenes*	*ica*A, *ica*D, *bap*, *ebps*, *cna*, *clf*A, *eno*, *mec*A
283	-	*Staphylococcus auriculares*	*ica*A, *ica*D, *fnb*A, *ebps*, *cna*, *clf*B, *clf*A
885	-	*Staphylococcus epidermidis*	*ica*A, *bap*, *cna*, *eno*, *fib*, *mec*A
891	-	*Staphylococcus haemolyticus*	*ica*D, *fnb*A, *eno*, *fib*
895	-	*Staphylococcus epidermidis*	*ica*A, *ica*D, *bap*, *fnb*A, *clf*B, *eno*, *fib*, *mec*A
897	-	*Staphylococcus chromogenes*	*ica*D, *bap*, *fnb*A, *clf*B, *eno*, *fib*
942	-	*Staphylococcus chromogenes*	*ica*A, *ica*D, *ebps*, *clf*A, *eno*, *fib*

Biofilm quantification

The averages of the three repetitions referring to the bacterial counts of the isolates tested at the three times (24h, 48h, and 72h) can be seen in Table 4 and Figure 3. As we can see, most isolates tested for biofilm formation on stainless steel presented counts above 6 log10 CFU/cm^2 at all times, except isolate 263 at 24 hours, which presented a count value of 5.94 log10 CFU/cm^2. According to the literature, counts between 6 and 7 log10 CFU/cm2 are necessary to consider biofilm formation. Furthermore, lower results indicate the adhesion process (Marques *et al.* 2007, Cruzado-Bravo *et al.* 2018). The count showed normality (W= p> 0.05) for all isolates, with isolate 942 presenting the highest value and isolate 897 the lowest. The microbiological count between times showed normality using the Shapiro-Wilk test (W shown in Table 5; p>0.05) and did not differ between different times (24h, 48h, and 72h).

Table 4. Results of bacterial counts of the ten isolates tested for biofilm formation at 24h, 48h and 72h, normality using the Shapiro-Wilk test and Shapiro-Wilk W values for normality.

Sample code	**Species**	**Bacterial Count (log10 CFU/cm^2)**			**Normality (p-value)**	**W**
		24h	**48h**	**72h**		
8	*Staphylococcus epidermidis*	8	8.5	8.07	0.248	0.853
14	*Staphylococcus epidermidis*	7.09	7.35	7.46	0.561	0.948
263	*Staphylococcus chromogenes*	5.94	6.45	6.1	0.595	0.956
267	*Staphylococcus chromogenes*	7.04	7.62	6.1	0.741	0.982
283	*Staphylococcus auricularis*	6.89	9.1	7.19	0.240	0.850
885	*Staphylococcus epidermidis*	7.34	7.66	8.11	0.814	0.991
891	*Staphylococcus haemolyticus*	6.95	6.27	6.07	0.417	0.910
895	*Staphylococcus epidermidis*	7.25	6.87	7.31	0.241	0.850
897	*Staphylococcus chromogenes*	7.02	7.06	6.41	0.105	0.796
942	*Staphylococcus chromogenes*	7.32	7.49	7.13	0.939	0.999
Negative Control	Absence of bacteria	0.000	0.000	0.000	-	-
Positive Control	ATCC 6538	6.14	6.85	6.97	0.256	0.856

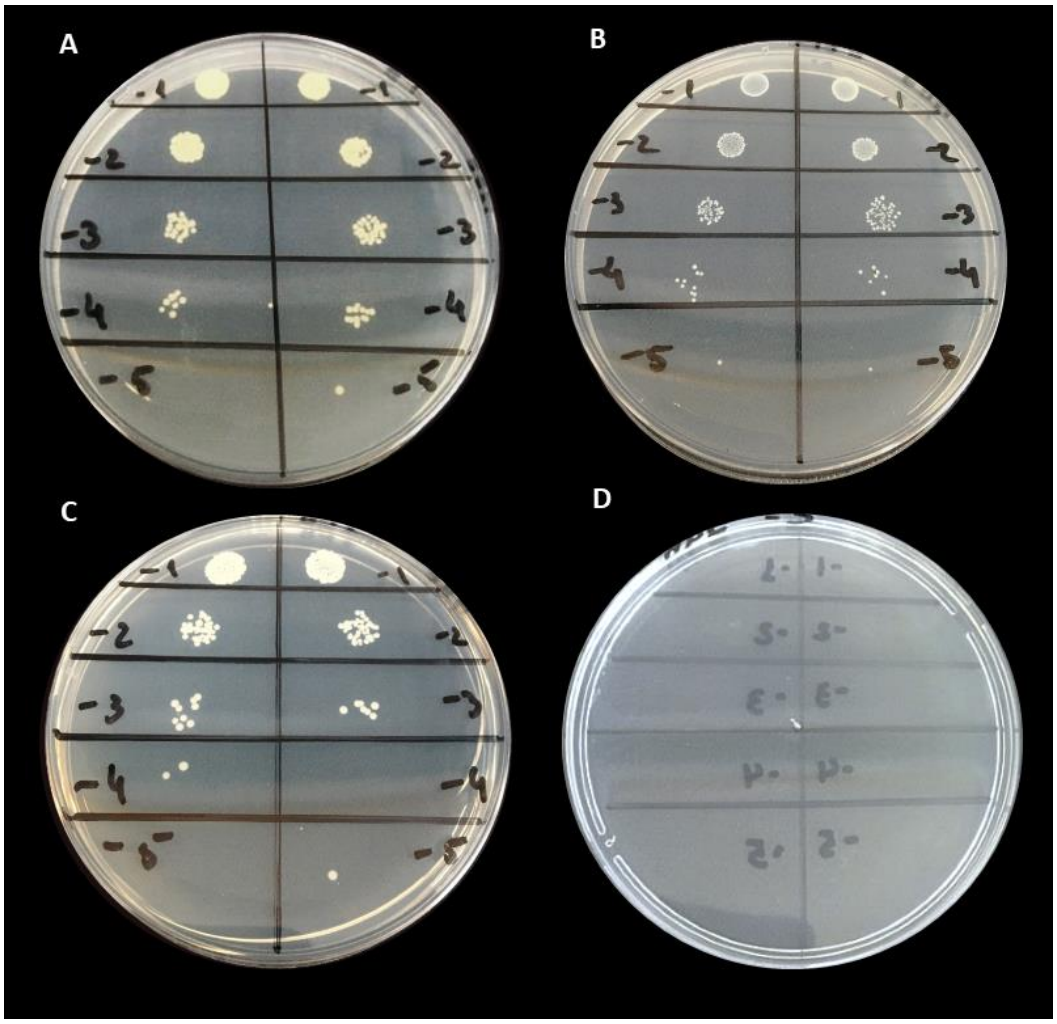

Figure 6. A: Isolate 897 after 48h, second repetition, growth for counting after the microdrop technique; B: Isolated 8 after 24h, third repetition; C: positive control (ATCC 6538) after 48h, third repetition; D: negative control (no growth) after 24h, first

After obtaining these results, we performed a linear regression analysis to determine the association of each gene with biofilm formation. As there were no significant differences between times (24h, 48h, 72h), we calculated the average count to compare the presence or absence of biofilm genes in the isolates. However, we did not find any gene significantly associated with biofilm formation ($p < 0.05$). The values of R^2 and F are provided in Table 5.

In Table 5, goodness-of-fit (R^2) values for the influence of each gene on biofilm colony counts are displayed. R^2 measures the percentage of variation in biofilm colony

counts that can be explained by the presence or absence of each gene. The results show that *ica*D explains 39% of biofilm formation, *bbp* explains 33%, *ica*A 12%, *eno*10%, *cna* and *clf*B 8.3%, *clf*A 4.1%, *fnb*A 3.8%, *fib* 2.3% and *ebps* 2%. The *fnb*B gene does not show any correlation with biofilm formation since this gene was not present in any of the isolates. It is important to note that an R^2 value of 1, which usually indicates a perfect explanation, indicates the opposite due to the absence of the *fnb*B gene in all isolates. The *ica*D and *bbp* genes show p values close to the significance threshold (<0.05), suggesting a possible association with biofilm formation Furthermore, the F value of both genes indicates that this is unlikely to occur by chance; therefore, the null hypothesis is rejected.

Table 5. Values given by linear regression to assess the correlation between biofilm formation and the presence of genes.

Gene	p-value	R^2	F
*ica*A	0.311	0.1276	1.17
*ica*D	0.0525	0.3927	5.172
bap	0.9374	0.000821	0.006575
*fnb*A	0.5893	0.03802	0.3162
ebps	0.6802	0.02235	0.1829
cna	0.4184	0.08338	0.7277
*clf*B	0.4194	0.08305	0.7246
*clf*A	0.5735	0.04127	0.3444
eno	0.3729	0.1002	0.8908
fib	0.6778	0.0227	0.1858
*fnb*B	-	1	-
bbp	0.0816	0.3313	3.964

It is important to highlight that among the 10 NAS isolates selected for analysis of biofilm formation on stainless steel in this study, none produced biofilm in the CRA analysis. However, an essential piece of information is that 5 (50%) of the 10 NAS isolates presented the *mec*A gene (which confers resistance to methicillin) in a previous PCR analysis. In addition to these 5 NAS isolates used in this analysis, another 10 NAS positives for the *mec*A gene tested in CRA did not produce biofilm in this test. This can

be explained by the fact that methicillin resistance represses the production of polysaccharide-type biofilms (a type of biofilm analyzed by the CRA test) and favors the formation of protein-type biofilms (Mccarthy *et al.* 2015).

Studies have shown that expression of the *fnb*A and *fnb*B genes in methicillin-resistant *Staphylococcus aureus* (MRSA) was associated with biofilm formation in BHI medium with glucose, where the pH was slightly reduced to acidic conditions. Furthermore, both genes were associated with biofilm formation due to intercellular aggregation and not initial binding (Hernández-Cuellar *et al.* 2023)

Although in this study there was no statistically significant association between genes and biofilm formation, this may be related to other factors. For example, studies have reported biofilm formation independent of the *ica* operon in *S. epidermidis* and *S. aureus* (Mccarthy *et al.* 2015). Furthermore, studies demonstrate that biofilms formed by MRSA, independent of PIA, are evident and are mainly involved with an adhesin protein(s) regulated by *Sar*A and *Agr*, in addition to presenting extracellular DNA (eDNA) as one of the main components of the biofilm's extracellular matrix (Avila-Novoa *et al.* 2021). While methicillin-sensitive *Staphylococcus aureus* (MSSA) isolates are more associated with biofilm formation dependent on the PIA mechanism (Pozzi *et al.* 2012, Hernández-Cuellar *et al.* 2023).

Chajęcka-Wierzchowska *et al.* (2023) evaluated 85 NAS isolates from ready-to-eat food for biofilm formation using the CRA test and the Microtiter Plate (MTP) method and found different results; 62.4% of the NAS tested formed biofilm in the MTP method and 35.3% formed biofilm in the CRA test. Furthermore, the authors did not find any statistically significant correlation between the MTP method, the CRA test, and the presence of the *ica*ADBC operon. Furthermore, the *eno* gene was also the most prevalent among the NAS analyzed in this study.

The *ar*IRS, *hla*, *rbf*, *sig*B, *tca,* and *trap* genes have also been reported in several studies as genes involved in *S. aureus* biofilm formation (Avila-Novoa *et al.* 2021). These genes were not researched in this study.

In addition to genetic factors that may not favor the pathogen in biofilm formation, the composition of the medium used for biofilm formation tests may also favor or not this formation. Medium with added glucose or NaCl; for example, researchers observed that in *S. aureus* the deletion of *ica* did not affect biofilm formation in medium with glucose, but with the addition of NaCl, this same isolate lost its ability to form biofilm (Vergara-Irigaray *et al.* 2009, Hernández-Cuellar *et al.* 2023).

Regardless of which factors are essential for biofilm formation, NAS-carrying genes responsible for biofilm formation and resistant to methicillin (MRNAS) are of concern since they are isolated from a food source. Biofilm-producing NAS can persist in the dairy environment or the dairy industry and transmit virulence factors through the exchange of genetic material between species (Turchi *et al.* 2020).

Resistant and virulent microorganisms are no longer restricted to the hospital environment, and NAS proves to be as worrying as *S. aureus*, the main species of the genus. Furthermore, the damage is not restricted to human and animal health; studies demonstrate that biofilm development can occur during the handling and storage of raw milk in tanks, negatively affecting the quality of dairy products by producing thermoresistant lipolytic enzymes, causing sensory and functional (Flint *et al.* 2020).

Conclusion

Staphylococcal biofilm can cause problems not only during the treatment of bovine mastitis but also poses a significant risk to dairy production and human health, as the microorganism can persist in the dairy chain. Therefore, resistant NAS (MRNAS) in dairy products is possible and should be controlled. In addition to the genes involved in

biofilm formation and adhesion studied here, other genes associated with this process should be analyzed to understand biofilm formation better. In addition, biofilm formation and composition may also be associated with different virulence and antibiotic-resistance genes. NAS is often reported as a single group. Still, with these results, improving knowledge about pathogenicity and the ability to carry genes responsible for biofilm formation individually among different NAS species is possible.

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Declaration of interest

Declarations of interest: none.

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CAPÍTULO 4

Why Non-*aureus Staphylococcus* (NAS) isolated from bovine milk should be a concern for the rise of superbugs

Why Non-*aureus Staphylococcus* (NAS) isolated from bovine milk should be a concern for the rise of superbugs

* Research Article to be Submitted
The Microbe

ABSTRACT

This work aimed to analyze the genetic profile of 14 methicillin-resistant *Staphylococcus* non-*aureus* (MRNAS) isolated from milk of cows with low and high somatic cell count (SCC) and milk of cows with clinical mastitis. The isolates were selected after identifying the *mec*A gene using conventional PCR from a previous analysis. Then, using whole genome sequencing (WGS), the genomes were analyzed for the presence of resistance genes and virulence genes, identification of the SCC*mec* type, presence of mobile genetic elements (MGEs) such as bacteriophages and plasmids, sequence type (ST) and phylogenetic analysis. The results showed the presence of twenty-three acquired resistance genes adc and twenty-nine virulence genes. The SCC*mec* type was identified in 9 (64%) of the 14 genomes analyzed. Plasmids and bacteriophages responsible for transferring resistance genes were also identified, containing genes such as those for resistance to Streptomycin, Erythromycin, Lantibiotics, Trimethoprim, and Lincosamide. The phylogenetic tree showed three distinct clades, having a diverse number of STs between the genomes, which, combined with WGS, allowed the efficient typing of the NAS genomes. These results show that the *S. epidermidis* species was more pathogenic than the others analyzed. Further, all NAS genomes presented virulence and resistance genes, in addition to MGEs responsible for the horizontal transfer of these genes between species, which is very worrying. Although studies point to NAS as a secondary pathogen of mastitis, their control is limited for several reasons presented in the results of this study.

Keywords: WGS; resistance genes, virulence genes; MGEs, plasmids; bacteriophages;

1. Introduction

Non-*aureus Staphylococcus* (NAS) were long considered minor contaminants and pathogens. Today, they are defined as opportunists and are frequently isolated from the milk of healthy cows and cows with mastitis, especially subclinical mastitis. NAS is a group of bacteria with more than 70 species (https://lpsn.dsmz.de/genus/staphylococcus) that are also found in milking environments, in addition to being found in the human and animal microbiota (Turchi et al., 2020).

Among the NAS species most frequently isolated from cattle herds, the species *S. haemolyticus*, *S. chromogenes*, *S. epidermidis*, *S. warneri*, *S. cohnii*, *S. simulans*, *S. hominis*, *S. capitis* and *S. xylosus* are the more prevalent (Traversari et al., 2019). And although their role as mastitis-causing agents is sometimes mitigated, several studies show that NAS isolated from cow's milk carry several virulence and resistance genes (Khazandi et al., 2018; Taponen and Pyörälä, 2009; Thorberg et al., 2009).

Antimicrobials' continuous and erroneous use contributes to increased resistance among microorganisms, a global concern (Dorneles et al., 2019). Methicillin-resistant non-*aureus Staphylococcus* (MRNAS) is within this concern and alert, as reported in several studies (Crippa et al., 2024).

Methicillin resistance was first reported in *Staphylococcus aureus* (MRSA), and this resistance is mediated by the *mec*A gene, which, except for the antibiotics ceftaroline and ceftobiprole, confers resistance to the entire class of β-lactam drugs. Although the first reports of methicillin resistance appeared in MRSA, recent studies point to a species of NAS (*S. sciuri* and *S. fleurettii*) as being responsible for the evolutionary origin of *mec*A in MRSA (Lakhundi and Zhang, 2018; Miragaia, 2018). Furthermore, there is evidence that NAS acts as a reservoir for genes that facilitate the evolution of *S. aureus*

as a successful pathogen (Otto, 2013). These genes can be transferred through mobile genetic elements (MGEs), such as bacteriophages and plasmids, which contribute to this evolution and increase in antimicrobial resistance and virulence (Pfeifer and Rocha, 2024)

The discriminatory power of whole genome sequence (WGS) allows large-scale and genomic epidemiological studies to be conducted. Furthermore, by using WGS, it is possible to identify emerging strains or strains responsible for outbreaks within the community (Lagos et al., 2022). Aiming at all of this, genomic studies, including the complete sequencing of NAS genomes isolated from cattle, allow us to deepen our knowledge about NAS's pathogenic potential, especially MRNAS, and better detail the genetic profile of the different species within this diverse group. Therefore, this work aimed to analyze MRNAS isolated from milk of cows with low and high somatic cell count (SCC) and milk of cows with clinical mastitis.

2. Material and methods

Fourteen MRNAS isolates were selected from the Food Microbiology Laboratory II bacteria collection, Faculty of Food Engineering, State University of Campinas (FEA/UNICAMP), Brazil (unpublished data). There were eight *S. epidermidis*, five *S. chromogenes,* and one *S. warneri,* and these isolates were selected based on the presence of the *mec*A gene, previously detected by PCR and after analysis of the antimicrobial susceptibility profile. These isolates came from milk cows with low and high somatic cell count (SCC) and milk cows with clinical mastitis (Table 1).

Table 1. Results of the susceptibility profile against antimicrobials tested according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022)

Isolate	Species	Genome Size	Mastitis	GEN	OXA	CEF	TET	ERY	CLI	TOB	CHL	PEN
B1	*S. epidermidis*	2438936	Clinic	S	S	S	S	R	R	S	S	R
B2	*S. epidermidis*	2544491	Clinic	R	R	R	R	R	R	R	R	R
B3	*S. epidermidis*	2538550	Clinic	R	R	S	R	R	R	R	R	R
B5	*S. epidermidis*	2439496	High SCC	S	R	S	S	S	S	S	S	S
B6	*S. epidermidis*	2424284	High SCC	S	R	S	R	S	S	S	S	R
B7	*S.chromogenes*	2307992	High SCC	S	R	S	S	S	S	S	S	S
B8	*S.chromogenes*	2308992	High SCC	S	S	S	I	S	S	S	S	S
B9	*S.chromogenes*	2315073	Low SCC	S	S	S	S	S	S	S	S	S
B10	*S. epidermidis*	2489760	Low SCC	S	S	S	S	R	S	S	S	R
B11	*S.chromogenes*	2478072	High SCC	S	S	S	S	R	S	R	S	S
B12	*S. epidermidis*	2548581	Clinic	S	S	S	S	S	S	S	R	S
B13	*S. epidermidis*	2545267	Low SCC	S	R	R	S	R	S	S	S	S
B14	*S. warneri*	2558275	High SCC	S	S	S	S	R	S	S	S	S
B15	*S.chromogenes*	2312698	Low SCC	S	S	S	S	S	R	S	S	S

R: Resistant/ I: Intermediate resistance/ S. Sensitive
Antibiotic: GEN: Gentamicin (10 µg), OXA: Oxacillin (1 µg), CEF: Cefoxitin (30 µg), TET: Tetracycline (30 µg), ERY: Erythromycin (15 µg), CLI: Clindamycin (2 µg), TOB: Tobramycin (10 µg), CHL: Chloramphenicol (30 µg) and PEN: Penicillin (10 µg).

The selected isolates were stored at -80°C and were reisolated on blood agar plates to select pure colonies. DNA extraction was performed using the DNeasy PowerFood Microbial Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The isolates of *Staphylococcus* spp. were subjected to PCR amplification of the 16S rRNA gene, followed by Sanger sequencing conducted at the Multiuser Laboratory of Genotyping and Sequencing of the Center for Molecular Biology and Genetic Engineering (CBMEG) of the State University of Campinas. The resulting sequences were analyzed with the

National Center for Biotechnology Information (NCBI) BLAST program to identify. (Wood et al., 1998).

The whole genome sequencing was carried out by the specialized company Novogene (Sacramento, CA, USA) with libraries assembled following the quality criteria (QC Library), where the genomic DNA was fragmented to a size of 350 bp, and the selected fragments were then polished at the ends, A-tailed and connected with the full-length adapter. Sequencing was performed on Illumina NovaSeq (PE150) equipment (Illumina Inc. San Diego, CA).

The quality control of the reads was carried out using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the assembly of the genomes was carried out using Unicycler (Wick et al., 2017) (https://github.com/rrwick/Unicycler). Afterwards, the assembly quality was checked using the Quast software (Gurevich et al., 2013) (https://github.com/ablab/quast). Next, a contamination check analysis was performed using Kraken2 (Wood et al., 2019) (https://github.com/DerrickWood/kraken2). Genome annotation was done using the Prokka software (Seemann, 2014) (https://github.com/tseemann/prokka) and the acquired resistance genes were identified by ABRicate (https://github.com/tseemann/abricate) and CARD (https://card.mcmaster.ca/analyze/rgi) (Jia et al., 2017; Zankari et al., 2012). Virulence gene analysis was conducted using the VFDB (Chen et al., 2016) (https://cge.food.dtu.dk/services/VirulenceFinder/) and Victors databases (Sayers et al., 2019) (https://hegroup.org/victors).

Using Center for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.org/services/), multilocus sequence typing (MLST), mobile genetic elements (MGE) and plasmids (PlasmidFinder 2.1) were also identified

(Wishart et al., 2023). Bacteriophages were also identified by VirSorter 2.2.4 and Phigaro 2.3.0 through the Proksee platform (Grant et al., 2023) (https://proksee.ca/).

The phylogenetic tree was generated by the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) server (https://www.bv-brc.org/) using the codon tree method that selects BV-BRC PGFams (Global Protein Families) and analyzes aligned proteins and coding DNA from single-copy genes using the program RAxML. All parameters of the free model were estimated by RAxML, to construct a maximum likelihood (ML) phylogeny of the sequencing isolates. The ML estimate of 25 rate categories per site (per 100 bootstraps) and the probability of the final tree were evaluated and optimized under GAMMA (Olson et al., 2023). The tree was edited using iTOL (https://itol.embl.de/).

The frequency of gene presence was estimated using the PROC FREQ using the statistical software SAS Studio (version 9.3, SAS Institute Inc., Cary, NC). This Whole Genome Sequence project has been deposited at GenBank under the BioProject accession PRJNA1129503.

3. Results and discussion

3.1 Resistance genes

Fourteen NAS isolates were sequenced, eigth *S. epidermidis* (SE), five *S. chromogenes* (SC), and one *S. warneri* (SW). Twenty-three resistance genes were found in the 14 genomes analyzed, and the genes found, as well as the respective classes and resistance mechanisms conferred, are presented in Table 2.

Table 2. Acquired resistance genes identified through whole-genome sequencing in Non-*aureus* staphylococci (NAS) isolates.

Gene	AMR Gene Family	Drug Class	Resistance Mecanism	N	(%)	Isolate
AAC(6')-Ie-APH(2'')-	aminoglycoside bifunctional resistance protein	aminoglycoside antibiotic	antibiotic inactivation	2	14.3	B2 B3
ANT(4')-Ia	ANT(4')	aminoglycoside antibiotic	antibiotic inactivation	3	21.4	B2 B3 B12
*bla*Z	BlaZ beta-lactamase	Penam	antibiotic inactivation	6	42.8	B1 B2 B3 B6 B10 B13
*dfr*C	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	antibiotic target replacement	8	55.1	B1 B2 B3 B5 B6 B10 B12 B13
*Erm*C	Erm 23S ribosomal RNA methyltransferase	macrolide antibiotic, lincosamide antibiotic, streptogramin antibiotic, streptogramin A antibiotic, streptogramin B antibiotic	antibiotic target alteration	2	14.3	B2 B3
*fex*A	major facilitator superfamily (MFS) antibiotic efflux pump	phenicol antibiotic	antibiotic efflux	2	14.3	B2 B3
*Fos*Bx1	fosfomycin thiol transferase	phosphonic acid antibiotic	antibiotic inactivation	9	64.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*fus*C	Target protecting FusB-type protein conferring resistance to Fusidic acid	fusidane antibiotic	antibiotic target protection	1	7.1	B15
*gyr*B	aminocoumarin resistant gyrB	aminocoumarin antibiotic	antibiotic target alteration	5	35.7	B1 B5 B6 B10 B14

*mde*A	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic, aminoglycoside antibiotic, penam, tetracycline antibiotic, disinfecting agents and antiseptics	antibiotic efflux	2	14.3	B12 B13
*mec*A	methicillin resistant PBP2	Penam	antibiotic target replacement	14	100	B1B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
*mph*C	macrolide phosphotransferase (MPH)	macrolide antibiotic	antibiotic inactivation	3	21.4	B1 B12 B13
*msr*A	msr-type ABC-F protein	macrolide antibiotic, streptogramin antibiotic, streptogramin B antibiotic	antibiotic target protection	4	28.6	B1 B10 B12 B13
*nor*A	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic, disinfecting agents and antiseptics	antibiotic efflux	8	55.1	B1 B2 B3 B5 B6 B10 B12 B13
*nor*C	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic, disinfecting agents and antiseptics	antibiotic efflux	8	55.1	B1 B2 B3 B5 B6 B10 B12 B13
*qac*J	small multidrug resistance (SMR) antibiotic efflux pump	disinfecting agents and antiseptics	antibiotic efflux	2	14.3	B10 B14
*sdr*M	major facilitator superfamily (MFS) antibiotic efflux pump	fluoroquinolone antibiotic, disinfecting agents and antiseptics	antibiotic efflux	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
*sep*A	small multidrug resistance (SMR) antibiotic efflux pump	disinfecting agents and antiseptics	antibiotic efflux	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
tet(45)	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	1	7.1	B6
tet(K)	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	2	14.3	B2 B3
*van*T	glycopeptide resistance gene cluster, vanT	glycopeptide antibiotic	antibiotic target alteration	14	100	B1B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
*van*W	vanW, glycopeptide resistance gene cluster	glycopeptide antibiotic	antibiotic target alteration	1	7.1	B14
*van*Y	vanY, glycopeptide resistance gene cluster	glycopeptide antibiotic	antibiotic target alteration	2	14.3	B12 B14

Analyzing the isolates separately, the genomes that presented the highest number of resistance genes were B2 (*S. epidermidis*) and B3 (*S. epidermidis*) with 14 genes, B1 and B12 (*S. epidermidis*) with 13 genes, B10 (*S. epidermidis*) and B13 (*S. epidermidis*) with 12 genes, B6 (*S. epidermidis*) with 11 genes, B5 (*S. epidermidis*) and B14 (*S. warneri*) with 9 genes, B15 (*S. chromogenes*) with 5 genes and B7 (*S. chromogenes*), B8 (*S. chromogenes*), B9 (*S. chromogenes*) and B11 (*S. chromogenes*) with 4 genes found in their respective genomes. Similar results were found by Frey et al., (2013), where the detection of multiple resistance combinations in the analyzed NAS was observed. Several genes were associated with the *mec*A gene's presence in different NAS species analyzed, such as *S. epidermidis* (Frey et al., 2013).

We can observe that the isolates of the species *S. epidermidis* were those that presented a more significant number of resistance genes, which can, in part, be supported by the phenotypic analysis of susceptibility to the antimicrobials tested, as this species also proved to be more resistant when compared with the others. Only B12 isolate, *S. epidermidis*, showed resistance to only one of the antibiotics tested, chloramphenicol. Mediated by efflux pumps, expression of the *nor*A gene (present in the B12 genome) confers resistance to chloramphenicol and antibiotics from the fluoroquinolone group and resistance to biocides and dyes (Piddock, 2006). However, in the analysis of the B12 genome, several resistance genes were identified, including genes for resistance to aminoglycosides such as gentamicin and tobramycin that were tested, in addition to genes for resistance to beta-lactams such as oxacillin, cefoxitin and penicillin that were also tested, but which did not show phenotypic resistance.

The same can be observed for the other isolates; several resistance genes were present in genome analysis, but resistance was not observed in the phenotypic analysis. This can be explained by the fact that we tested only a few antimicrobials; that is,

phenotypic resistance was not observed in these tested antibiotics, but it could be observed in other antibiotics from the same classes. Furthermore, it is known that microorganisms may present the gene in its genome, which may not be expressed phenotypically. Bacteria have genes encoded in their chromosomes for efflux pumps. Some of these genes are expressed constantly (constitutively), while others are activated or overexpressed in response to certain environmental stimuli or the presence of an appropriate substrate (Reygaert, 2018). Other studies have also found NAS that carried resistance genes but did not show phenotypic resistance, suggesting that there may be novel resistance mechanisms in NAS (Argudín et al., 2015; Frey et al., 2013).

The presence of these 23 resistance genes is worrying, since we have genes responsible for resistance to antibiotics in the following classes: beta-lactams, macrolides, glycopeptides, tetracyclines, fusidic acid, fluoroquinolones and aminoglycosides. In addition, genes such as *qac*J, *nor*A, *nor*C are responsible for resistance to sanitizers (Hassanzadeh et al., 2020). This shows that *S. aureus* is not the only species of concern within the genus, as it is evident that NAS also presents a risk to both human and animal health.

3.2 Virulence genes

Twenty-nine virulence genes were found in the analysis of the 14 genomes. The distribution of these genes among the genomes, the products, and their respective functions can be seen in Table 3.

The *aro*A gene is among those found in this study, involved in biofilm formation, and related to sequence types (STs) found in bacterial isolates from animals, including cows (Souza et al., 2023). The *Ccp*A gene, which, when inactivated, is also related to biofilm formation, extracellular capsule production, and red blood cell lysis (DebRoy et al., 2021). Still, within the genes related to biofilm formation, the *ica*A, *ica*B, *ica*C, and

*ica*D genes were also found. Biofilm attachment and accumulation are encoded by the *ica* operon by producing an extracellular adhesin called polysaccharide intercellular adhesin (PIA). The different gene loci *ica*ABCD are associated with PIA and are involved in its coding (Mohammad, 2022; O'Gara, 2007).

MsrR belongs to the LytR-CpsA-Psr family of cell envelope-associated transcriptional regulators. It has been demonstrated that this protein is induced by agents that act on the cell wall, such as β-lactams, glycopeptides and lysostaphin. Thus, *msr*R influences methicillin resistance, which is in line with the findings of this study, in addition to affecting the synthesis of virulence factors (Rossi et al., 2003). Furthermore, Rossi and collaborators (2003) found that the inactivation of *msr*R reduced the transcription of *spa* (which encodes protein A) and improved the transcription of *hla* (which encodes the alpha-toxin).

Among the virulence genes found, we also highlight the *rec*A gene, which is an essential gene in processes of antigenic variation, biosynthesis of toxins, synthesis of virulence factors, and delaying the evolution of antibiotic resistance (Kiran and Patil, 2022). Furthermore, studies demonstrate that wild *recA-positive* strains are more efficient against bacterial agents and associated with virulence factors and horizontal gene transfer, which contribute to antibiotic resistance in bacteria (Kiran and Patil, 2022). The SA1062 phosphatase is transcribed by the *stp1* gene, a conserved cytoplasmic protein of 247 a.a. The *stp1* gene has already been reported as an important factor in vancomycin resistance, cell wall biosynthesis, and regulation of virulence genes, such as hemolysin (Cameron et al., 2012).

In addition to the virulence genes presented, the genomes of isolates B12 and B14 presented the *hld* virulence gene in an acquired form. δ-hemolysin is a peptide that causes erythrocyte lysis and acts against cells and organelles, being encoded by the *hld* gene

(Verdon et al., 2009). Its secretion mechanisms are not yet completely understood since the genetic composition of delta toxin (δ) is encoded by polarized RNAIII molecules, whose transcriptional function is associated with a set of virulence factors, such as alpha toxin, enterotoxins, and toxic shock syndrome, as well as surfaces proteins that inhibit transcription, including surface protein A (Spa). Unlike alpha (α), beta (β), and gamma hemolysins (PVL) hemolysins, which have their secretion mechanisms better elucidated (Motamedi et al., 2018).

Furthermore, in a study conducted by Nasaj et al., (2020) the *hld* gene showed a significant association with resistance to the antibiotic erythromycin, corroborating this study's findings. The genome of isolate B14 presented the *hld* gene, and in the phenotypic analysis of susceptibility to antibiotics, total resistance to the antibiotic erythromycin was determined. This reinforces that not only are antimicrobial resistance genes important and related to resistance but virulence genes also have a significant role in antimicrobial resistance mechanisms.

Table 3. Virulence genes identified through whole-genome sequencing in Non-*aureus* Staphylococci (NAS) isolates.

Gene	Product	Function	N	(%)	Isolate
*aro*A	3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19)	Biosynthesis and transport of aromatic compounds	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
asd	Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	Intracellular survival and replication	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*ccp*A	Catabolite control protein A	Metabolism, bacterial transport and amino acid biosynthesis	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*cit*B	Aconitate hydratase (EC 4.2.1.3)	Crucial role in the tricarboxylic acid (TCA) cycle	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*clf*A	Adhesin of unknown specificity SdrC	Host cell invasion; Adherence	2	14.3	B2 B3
*clp*P	ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92)	Bacterial growth and survival; Stress protein	8	57.1	B1 B2 B3 B5 B6 B10 B12 B13
*clp*X	ATP-dependent Clp protease ATP-binding subunit ClpX	Bacterial growth and survival	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
*din*G	DinG family ATP-dependent helicase YoaA	Helicase activity, ATP Synthase	8	57.1	B1 B2 B3 B5 B6 B10 B12 B14
fbp	Fructose-1,6-bisphosphatase, Bacillus type (EC 3.1.3.11)	Gluconeogenesis	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*fem*B	tRNA-dependent lipid II-GlyGlyGly glycyltransferase (EC 2.3.2.18) tRNA-dependent lipid II-GlyGlyGlyGly glycyltransferase (EC 2.3.2.18) @ FemB, factor involved in methicillin resistance	Methicillin resistance regulator	2	14.3	B14 B15
*ica*A	Polysaccharide intercellular adhesin (PIA) biosynthesis N-glycosyltransferase IcaA (EC 2.4.-. -)	Adherence, Biofilm formation	3	21.4	B1 B10 B12
*ica*B	Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase	Adherence, Biofilm formation	3	21.4	B1 B10 B12
*ica*C	putative poly-beta-1,6-N-acetyl-D-glucosamine export protein	Adherence, Biofilm formation	4	28.6	B1 B10 B11 B12
*Ica*D	Poly-beta-1,6-N-acetyl-D-glucosamine synthesis protein IcaD	Adherence, Biofilm formation	3	21.4	B1 B10 B12
lip	Triacylglycerol lipase (EC 3.1.1.3)	Secretion system, Type VI secretion system	4	28.6	B7 B8 B9 B11
*lys*A	Diaminopimelate decarboxylase (EC 4.1.1.20)	Cell lysis	1	7.1	B14
*mgr*A	Transcriptional regulator MgrA (Regulator of autolytic activity)	Regulation of surface protein expression	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
msrA	Peptide-methionine (S)-S-oxide reductase MsrA (EC 1.8.4.11)	MULTISPECIES: ABC-F type ribosomal protection protein Msr(A)	1	7.1	B14
*msr*R	Peptide methionine sulfoxide reductase regulator MsrR	Membrane-associated protein, Synthesis of virulence factors	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14

*opp*D	Oligopeptide ABC transporter, ATP-binding protein OppD (TC 3.A.1.5.1)	Nutritional uptaking, environmental sensing, drug resistance, and virulence	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
*pur*L	Phosphoribosylformylglycinamidine synthase, synthetase subunit (EC 6.3.5.3)	Metabolism, bacterial transport, and amino acid biosynthesis	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*pyr*AA	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	Pyrimidine/arginine synthesis	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*rec*A	RecA protein	Homologous recombination processes, DNA damage repair	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
SA1062	Protein serine/threonine phosphatase PrpC, regulation of stationary phase	Regulation of virulence genes (hemolysins), vancomycin susceptibility, and cell wall biosynthesis	8	57.1	B1 B2 B3 B5 B6 B10 B12 B14
SA1453	Iron-sulfur cluster regulator IscR	Transcriptional regulation	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15
SAHV_0924	Hypothetical NagD-like phosphatase	Metabolism and Survival	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
SAOUHSC_01373	tRNA-dependent lipid II-Gly glycyltransferase (EC 2.3.2.17) @ tRNA-dependent lipid II-GlyGly glycyltransferase (EC 2.3.2.17) @ FemA, factor essential for methicillin resistance	Encodes the methicillin resistance factor FemA	9	69.3	B1 B2 B3 B5 B6 B10 B12 B13 B14
*sdr*D	Antiadhesin Pls, binding to squamous nasal epithelial cells	Adherence, MSCRAMMs	1	7,1	B10
*trp*B	Tryptophan synthase beta chain (EC 4.2.1.20)	Tryptophan biosynthesis	14	100	B1 B2 B3 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15

3.3 Mobile genetic elements (MGEs) and SCCmec typing

The identification of MGEs from the 14 genomes analyzed was carried out using MobileElementFinder. The results showed that the B1 genome presented a total of 182 MGEs, the B2 227, the B3 224, the B5 191, the B6 192, the B7, B8 and B9 genomes 113, the B10 182, the B11 125, the B12 221, B13 223, 14 163 and B15 125 MGEs. Figure 1 shows the main categories and the distribution of MGEs in each genome within these categories.

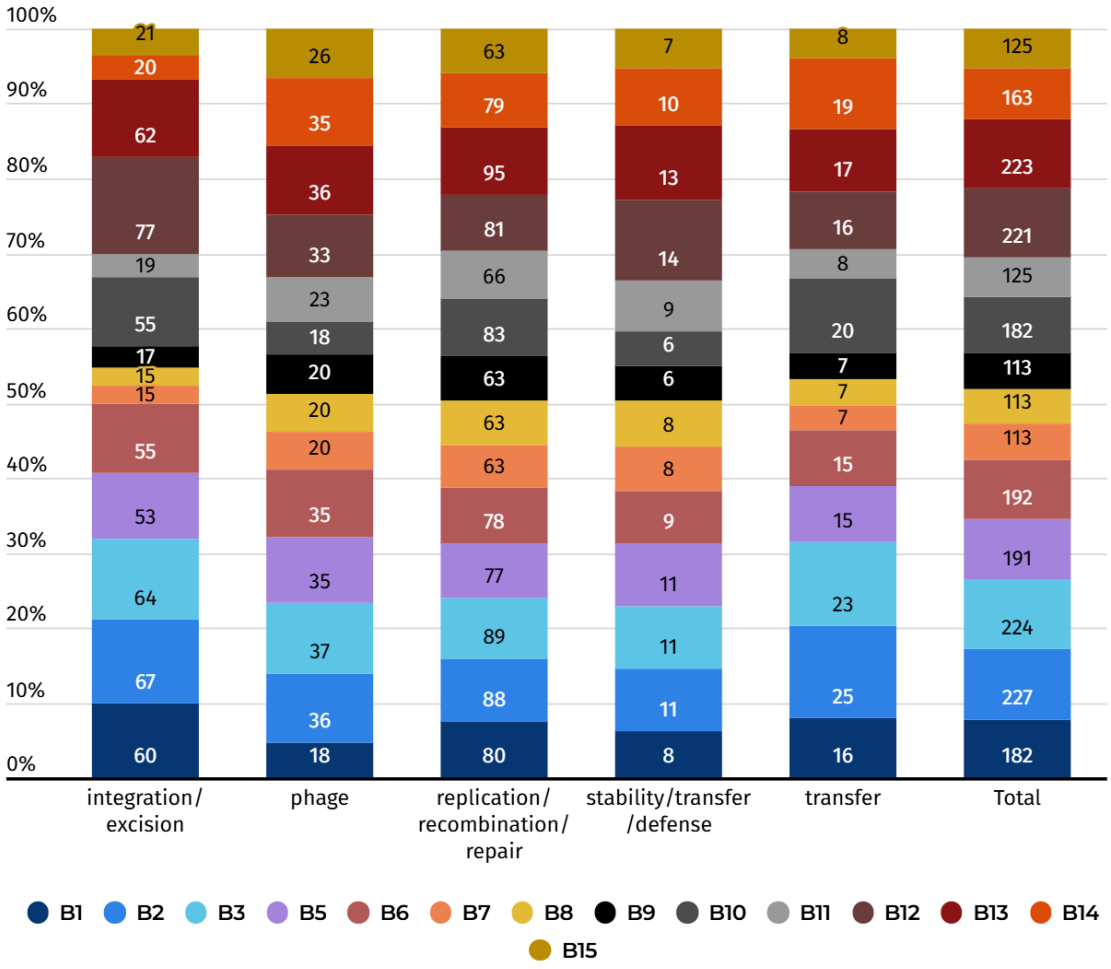


Figure 1. Distribution of the main categories of MGEs in each genome analyzed

The central genome of bacteria comprises genes present in all isolates, which are genes related to metabolism and DNA replication. However, in pathogens, a vast part of the genetic diversity occurs within the accessory genome, where mediators of resistance and virulence are located. MGEs comprise this accessory genome in pathogens,

composed of plasmids, bacteriophages, pathogenicity islands, and chromosomal cassettes acquired via horizontal transfer (Turner et al., 2019). MGEs are small segments of DNA, ranging from ~5–500 kb, whose function is to encode enzymes and proteins that regulate DNA movement within genomes or between bacterial cells (Frost et al., 2005). In this topic, we will highlight the MGEs related to the presence of the *mec*A gene previously identified by PCR and later confirmed in complete genome sequencing.

The MGE belonging to the transfer category is called mobileOG_000024562|*mec*A| and was identified in all genomes. It is known that the staphylococcal *mec* chromosomal cassette (SCC*mec*) is an MGE and is responsible for the horizontal transfer of the *mec*A gene, thus conferring resistance to methicillin (Turner et al., 2019). In methicillin-resistant *Staphylococcus aureus* (MRSA), at least three different *mec* genes have been identified, three different allotypes of the *mec*A gene: *mec*A1, and *mec*A2, *mec*B, and four different allotypes of the *mec*C gene: *mec*C, *mec*C1, *mec*C2 and *mec*C3 (Tchamba et al., 2023). The detailed results of the SCC*mec* elements of the genomes analyzed in this work are presented in Figure 2.

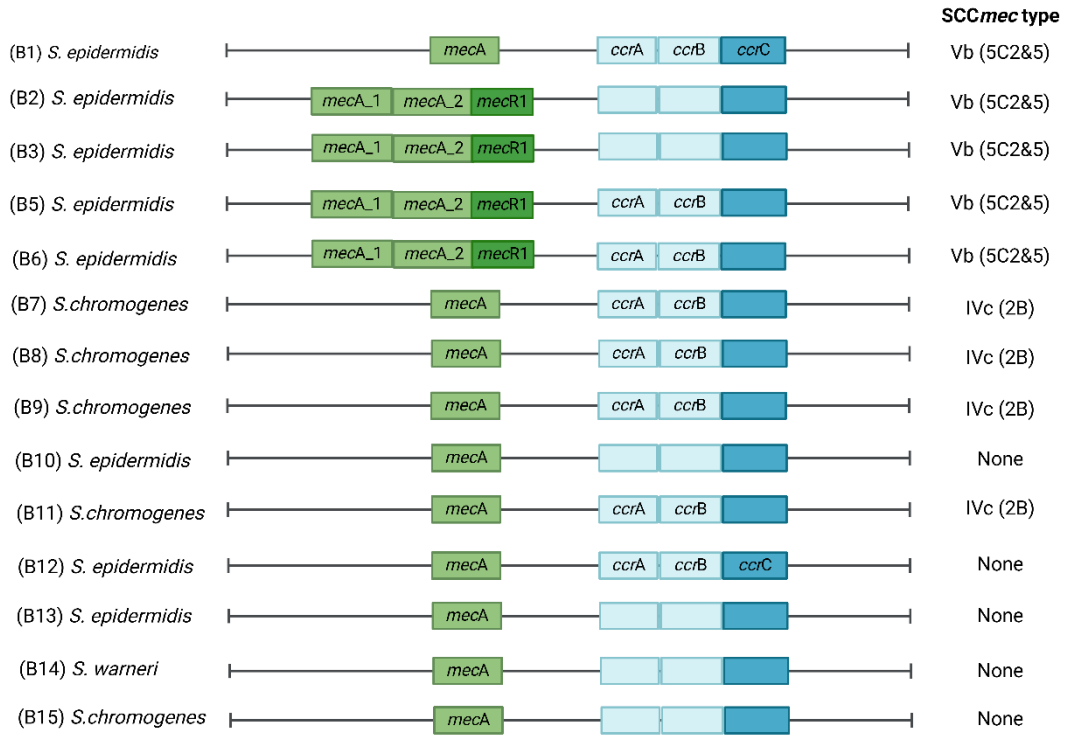


Figure 2. Distribution of the elements that comprise the SCC*mec* complex found in the analyzed genomes and SCC*mec* type identified. Figure created in https://BioRender.com

As can be seen in Figure 2, the B2, B3, B5, and B6 genomes presented the *mec*A_1 allotypes; *mec*A_2, and the inducing regulatory gene *mec*R1 (Miragaia, 2018). The SCC*mec* complex is also composed of chromosomal cassette recombinases (*ccr*), the so-called *ccr* complex, responsible for the mobility of the MGE SCC*mec* (Miragaia, 2018). In this work, the *ccrs* MGEs (integration/excision category) were identified in 8/14 (57%) analyzed genomes (Figure 2). SCC*mec* types are classified based on combinations of the *ccr* and *mec* genetic complexes. Some studies in the literature present SCC*mec* found in NAS, and some are part of the 14 types already reported in MRSA; however, some are not part of these 14 types (Tchamba et al., 2023).

Tchamba et al., (2023) analyzed the SCC*mec* type (by SCC*mec*Finder) of nine NAS isolates from dairy cattle, and only 1 (11%) was identified, one being S. *epidermidis*

type IVa(2B). In our study, 9 (64%) NAS had the SCC*mec* type identified, with type IVa (2B) identified in 4 NAS, type IVc (2B) also identified in 4 NAS, and type Vb (5C2&5) identified in 1 NAS (Figure 2).

In a review article on NAS and SCC*mec* types, Saber et al., (2017) show that *S. epidermidis* is the species that presents the greatest diversification of SCC*mec* types, as it is a species well-distributed in sources of humans and animals. On the other hand, the authors report the species *S. chromogenes* to be only associated with humans and being type IV. Several studies did not have the type of SCC*mec* identified in the species *S. epidermidis*, *S. chromogenes*, and *S. warneri*, as in this study (Saber et al., 2017).

There is strong evidence for transferring SCC*mec* elements from *S. epidermidis* to *S. aureus*, as evidenced by the high DNA sequence homology between SCC*mec* elements from *S. aureus* and *S. epidermidis*. Furthermore, SCC*mec* type IV of *S. epidermidis* shows 98–99% homology with SCC*mec* IVa of *S. aureus*. Furthermore, SCC*mec* type IV was also observed first in *S. epidermidis* (in 1970) and later in *S. aureus*. In addition, methicillin resistance is much more widespread in *S. epidermidis* than in *S. aureus* (Otto, 2013).

Studies on SCC*mec* typing in NAS isolated from animals and food sources are scarce. Still, they are very important since NAS isolated from food and animals are reservoirs of virulence and resistance genes and can transfer these genes to other species and spread infections. Furthermore, horizontal transfer of SCC*mec* genes between NAS can also occur between animals (Rolo et al., 2017; Saber et al., 2017). Although studies point to NAS as a secondary pathogen of bovine mastitis, their control is necessary. NAS-carrying resistance genes can transfer these genes to MRSA and other species of *Staphylococcus* sp (Rolo et al., 2017).

3.4 Bacteriophages

VirSorter and Phigaro software found bacteriophages in all genomes except the B1 genome. Some bacteriophages were identified entirely and others partially, and in this study, all bacteriophages found belonged to the *Siphoviridae* Family. Detailed information is presented in Table 4.

Phages (bacteriophages) belong to the order *Caudovirales*, and based on morphology, they are divided into three families: *Podoviridae* (with very short tails), *Siphoviridae* (with long non-contractile tails), and *Myoviridae* (with contractile tails) (Moineau and Tremblay, 2017).

Table 4. Name and distribution of bacteriophages found in the genomes, as well as their respective sizes, percentage of viral gene, percentage of non-viral gene and taxonomy

Isolate	Seqname	Length	Viral gene %	Nonviral gene %	Taxonomy
B2	15||full	55685	64.4	14.0	*Siphovirus*
	16||full	50101	59.1	45.0	
B3	14||full	51820	69.1	15.0	
	15||0_partial	29235	35.4	25.0	
B5	13||full	93919	63.1	13.8	
B6	5||full	169449	45.7	25.4	
B7	1||0_partial	70668	48.6	16.8	
B8	1||0_partial	77987	47.8	18.6	
B9	1||0_partial	77987	47.8	18.6	
B10	9||0_partial	29349	26.9	15.4	
	16||0_partial	17986	18.2	18.2	
B11	1||0_partial	78208	47.8	17.7	
	8||0_partial	13180	10.7	17.9	
B12	9||full	40941	80.0	33.0	
	24||full	7901	50.0	16.7	
	4||0_partial	12741	26.3	53.0	
B13	2||full	208962	30.6	28.8	
	35||full	7899	50.0	16.7	
B14	9||full	41991	70.1	0	
	2||0_partial	36488	20.9	16.4	
	3||0_partial	32294	23.6	16.4	
B15	2||0_partial	108620	34.8	25.4	
	4||0_partial	13752	18.8	21.9	

Oliveira et al., (2019) compared the genomes of 205 phages that infect different species of staphylococci and divided them into four clusters based on the average content

of shared genes. The vast majority of *Staphylococcus* sp phages belonging to the *Siphoviridae* family, as compared by the authors, belonged to cluster B, and this cluster exclusively presents phages that encode virulence genes. Hosts such as *S. epidermidis* and *S. warneri* were found among these phages as in this study. However, unlike what was found in our analysis, no phage was found in the *S. chromogenes* host by the authors.

Phages are important within the *Staphylococcus* genus and responsible for the horizontal transfer (transduction) of resistance and virulence genes. It can even transform bacteria into a pathogenic phenotype if genes that encode toxins or other virulence factors are present in their genome. Furthermore, phages contribute to better genomic plasticity, favoring faster adaptation of pathogens to the host (Deghorain et al., 2012; Göller et al., 2021; Moineau and Tremblay, 2017).

3.5 Plasmids

The identification of plasmids was carried out using PlasmidFinder-2.1, with plasmids identified in the B2, B3, B6, B7, B8, B9, B10, B11 and B14 genomes. Seven different plasmid families were identified, namely *rep*7a, *rep*10, *rep*40, *rep*39, *rep*13, *rep*5d and *rep*US35. In this study, some identified plasmids related to resistance to Streptomycin, Erythromycin (Figure 3), Lantibiotics, Trimethoprim, and Lincosamide. Detailed identification information can be seen in Table 5.

Plasmids can be divided into two groups: (1) low copy number plasmids (LCPs), which are often conjugative large but have a low copy number; (2) high copy number plasmids (HCPs), but which are generally small, and unlike LCPs, lack a functional conjugative system, having their mobilization done by other plasmids or through a conjugative apparatus (Rodríguez-Beltrán et al., 2021). In *Staphylococcus*, the replication initiation protein called RepA_N (highly conserved) is responsible for encoding the majority of conjugal and non-conjugal multiresistant plasmids (Schumacher et al., 2014)

Among *Staphylococcus* species, resistance genes are linked to MGEs as plasmids. Plasmids are fundamental for the propagation of these genes and the faster evolution of multidrug-resistant strains. This propagation of genes occurs through horizontal transfer mechanisms between bacterial cells by conjugation, mobilization, and/or phage-mediated mechanisms (Kwong et al., 2017). In addition, the transfer of virulence and resistance to antibiotics carried by plasmids has been frequently described in *S. aureus*. There is also evidence of interspecies transfer. This is the case for a self-transmissible plasmid transferred between NAS and *S. aureus* (Otto, 2013).

Table 5. Plasmids identified in the genomes, the percentage of identification, the gene products and information about the contigs.

Isolate	Plasmid	Identity (%)	Gene products	Contig	Position in contig	Plasmids Notes	Accession number
B2	*rep*7a	100.0	Streptomycin resistance	43 length=2594 depth=16.53x	1426..2370	CDS4(pS194)	NC005564
	*rep*7a	100.0	Amino acid ABC transporter permeasse	41 length=4447 depth=2.38x	3752..4290	ORF(pKH1)	SAU38656
	*rep*10	100.0	Erythromycin resistance	45 length=2481 depth=10.93x	1202..1678	*rep*L(pDLK1)	GU562624
	*rep*40	99.12	Lantibiotics resistance	31 length=18728 depth=2.97x	388..1641	*rep*A(SAP106A)	GQ900454
B3	*rep*7a	100.0	Streptomycin resistance	53 length=1101 depth=21.53x	234..1101	CDS4(pS194)	NC005564
	*rep*7a	100.0	Amino acid ABC transporter permeasse	55 length=613 depth=16.54x	150..613	ORF(pKH1)	SAU38656
	*rep*40	99.12	Lantibiotics resistance	30 length=18729 depth=2.76x	17088..18341	*rep*A(SAP106A)	GQ900454
	*rep*10	100.0	Erythromycin resistance	44 length=2482 depth=13.03x	1202..1678	rep*L(pDLK1)	GU562624
B6	*rep*39	99.9	Lantibiotics resistance	20 length=14908 depth=2.60x	5251..6207	*rep*A(SAP016A)	GQ900381
	*rep*7a	100.0	Essential for plasmid replication	22 length=4395 depth=2.89x	10..954	*rep*C(Cassette)	AB037671
B7	*rep*13	97.14	Lincosamide; nucleotidytransferase; *lnu*(A) gene; *rep* gene; replication	10 length=2490 depth=15.25x circular=true	429..1268	*rep*(pLNU9)	AM399082
B8	*rep*13	97.14	Lincosamide; nucleotidytransferase; *lnu*(A) gene; *rep* gene; replication	10 length=2490 depth=12.84x circular=true	680..1519	*rep*(pLNU9)	AM399082
B9	*rep*13	97.14	Lincosamide; nucleotidytransferase; lnu(A) gene; *rep* gene; replication	11 length=2577 depth=9.75x	305..1144	*rep*(pLNU9)	AM399082
B10	*rep*13	99.68	Lincosamide; nucleotidytransferase; *lnu(*A) gene; *rep* gene; replication protein.	25 length=2514 depth=11.54x	863..1786	*rep*(pPI2)	AB125342
	*rep*5d	95.28	Trimethoprim resistance protein (*dfr*A), thymidylate; synthetase (*thy*E), and putative transposase (*tnp*) genes	24 length=6982 depth=11.43x circular=true	643..1278	*rep*(pJE1)	AF051916
	*rep*39	100.0	Hypothetical protein	18 length=22327 depth=2.62x	12479..13432	*rep*A(SAP110A)	GQ900465
	*rep*40	99.12	Lantibiotics resistance	14 length=40236 depth=2.72x	430..1683	*rep*A(SAP106A)	GQ900454
B11	*rep*13	97.14	Lincosamide; nucleotidytransferase; *lnu(*A) gene; *rep* gene; replication protein.	21 length=1832 depth=9.74x	693..1532	*rep*(pLNU9)	AM399082
B14	*rep*US35	98.62	Hypothetical protein	17 length=6125 depth=2.98x	139..1005	A28412072(pvSw2)	CP003671
	*rep*13	99.68	Lincosamide; nucleotidytransferase; *lnu(*A) gene; *rep* gene; replication protein.	22 length=2365 depth=12.75x	1122..2045	*rep*(pPI2)	AB125342

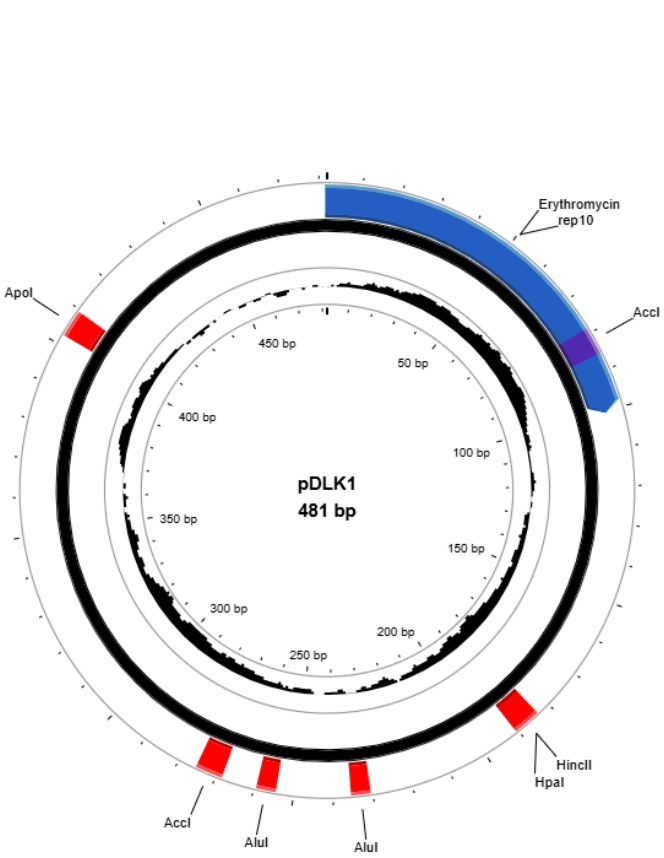


Figure 3. Schematic representation of pDLK1 plasmid (Created using PlasMapper 3.0)

3.6 Phylogenetic analysis and multi-locus sequence type (MLST)

Phylogenetic analysis shows three distinct clades: the *S. chromogenes* clade, the *S. warneri* clade, and the *S. epidermidis* clade (figure 4). A consistent relationship between species was observed within clades, and diversity was observed between species. The species *S. epidermidis* presented a greater diversity and prevalence of resistance and virulence genes. The B2 and B3 genomes showed high genetic similarity, which may indicate transmission between cows since these strains come from the same herd.

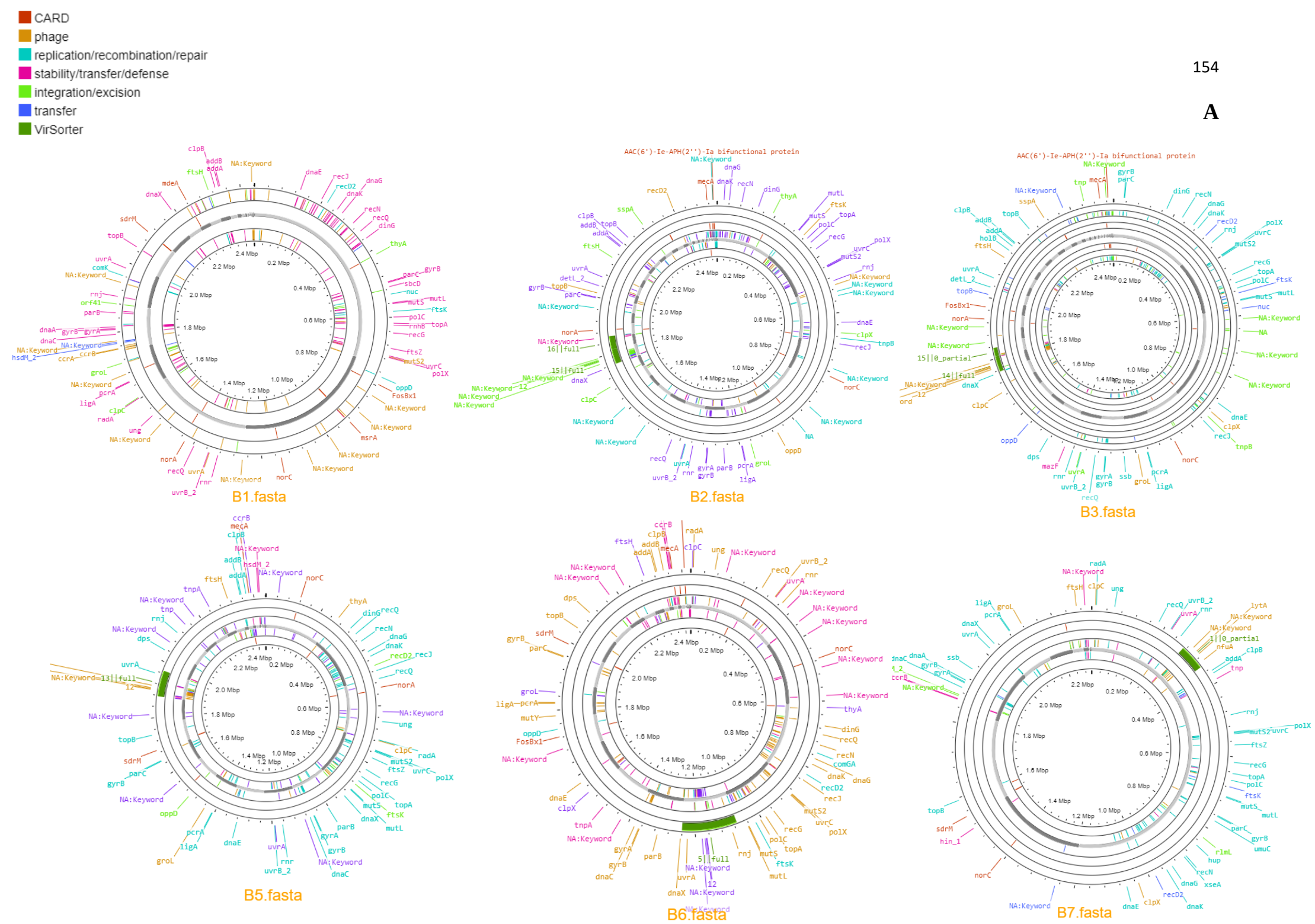
In total, 8 different sequence types (STs) were identified (figure 4). ST25 is the most prevalent among the *S. chromogenes* species (80%), followed by ST86 (20%). Among the *S. epidermidis* species, STs were more diverse, with two strains presenting ST6 (25%), two presenting ST59 (25%), one presenting ST1012 (12.5%), one presenting ST509 (12,5%), one presenting the ST628 (12.5%), and one presenting the ST35 (12.5%). Identifying the ST of the B14 genome (*S. warneri*) was impossible.

According to information from the Public Database for Molecular Typing and Microbial Genome Diversity (PubMLST) (Jolley et al., 2018), there is information on isolating STs 6 and 35 from human sources in Brazil and other countries worldwide. ST59 presents data on isolations from cases of bovine mastitis and human isolates in Brazil and other countries worldwide, and ST509 presents isolation data only in Brazil and isolated from human sources. Regarding STs 628 and 1012, the present data only show information on these STs isolated from humans in other countries, such as Estonia and the United Kingdom. Regarding the STs identified in the species *S. chromogenes*, according to the results found in the pubMLST database, ST25 presents isolation data in Canada and the USA, being isolated from cow's milk with subclinical bovine mastitis, and ST86 presents isolation data only in Canada and from a milk sample from the mammary gland.

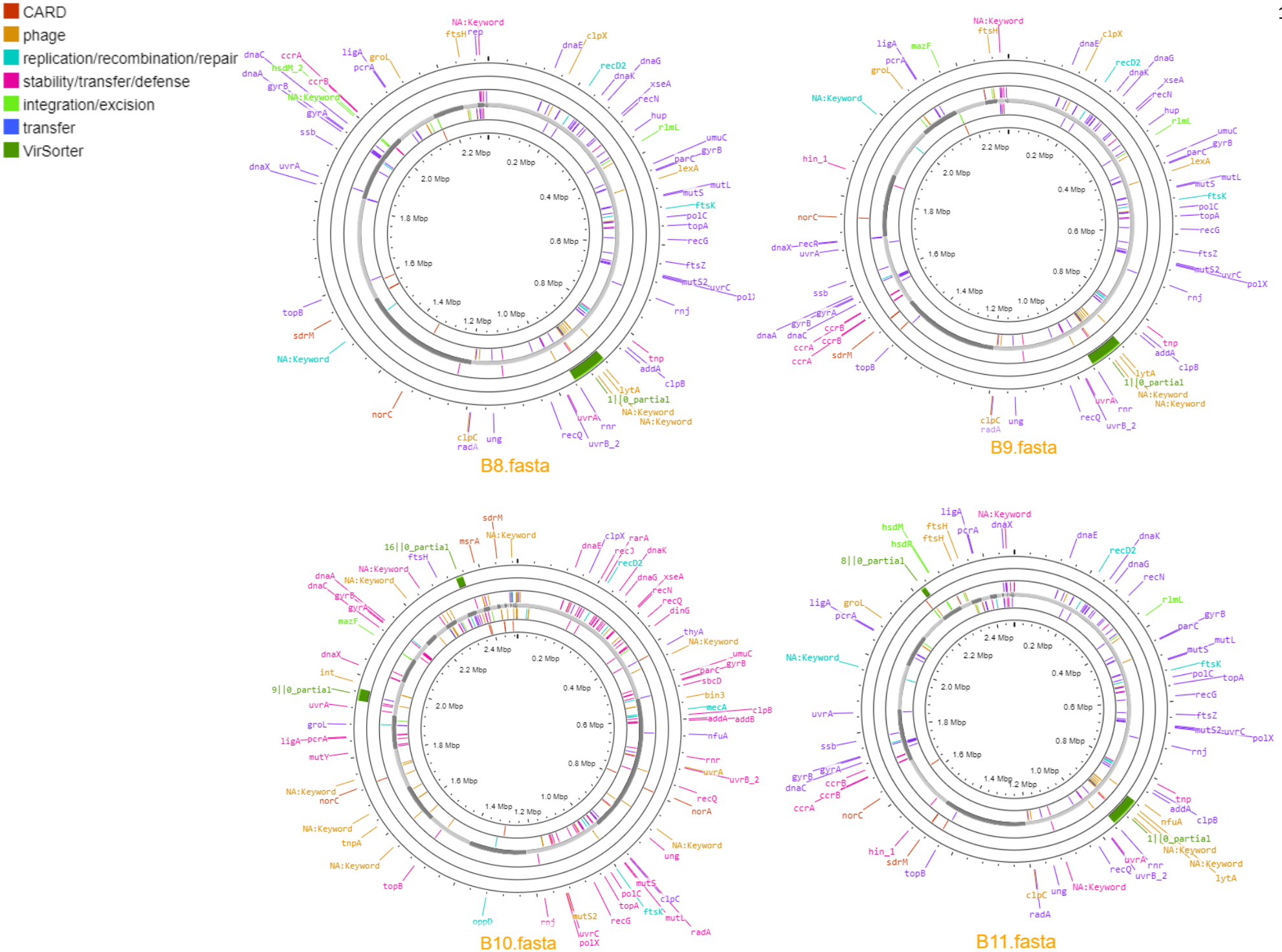
A high genetic diversity was observed between the different NAS genomes analyzed, which is reflected in the STs. Figure 5 (A, B, and C) shows an overview of the genetic diversity of all genomes. Similar genomes presented the same STs, and ST combined with WGS efficiently typed the different genomes. Although the B1 and B10 genomes show similarities in the distribution of virulence genes, the STs are different since these genomes belong to different herds. The B7, B8, and B9 genomes presented the same ST. It belonged to the same herd, while the B11 genome, despite presenting the same ST, belongs to another herd and presents the *ica*C virulence gene differently from the other genomes of the same clade. The B12 genome belonged to the same herd as the B1, B2, B3, B5, and B6 genomes and was the only one identified with ST1012. The genetic difference of this strain is evident compared to the others from the same herd; this shows that the MLST analysis was efficient in strain typing.

Figure 4. Phylogenetic tree of the 14 NAS generated by BV-BRC using the Codon Tree method. Red squares indicate antimicrobial resistance genes, and blue squares indicate virulence genes. An unassigned ST appears unfilled below the STs. *ST: Sequence type.

A


CARD
phage
replication/recombination/repair
stability/transfer/defense
integration/excision
transfer
VirSorter
B1.fasta
AAC(6')-Ie-APH(2'')-Ia bifunctional protein
B2.fasta
AAC(6')-Ie-APH(2'')-Ia bifunctional protein
B3.fasta
B5.fasta
B6.fasta
B7.fasta

B


CARD
phage
replication/recombination/repair
stability/transfer/defense
integration/excision
transfer
VirSorter
B8.fasta
B9.fasta
B10.fasta
B11.fasta

C

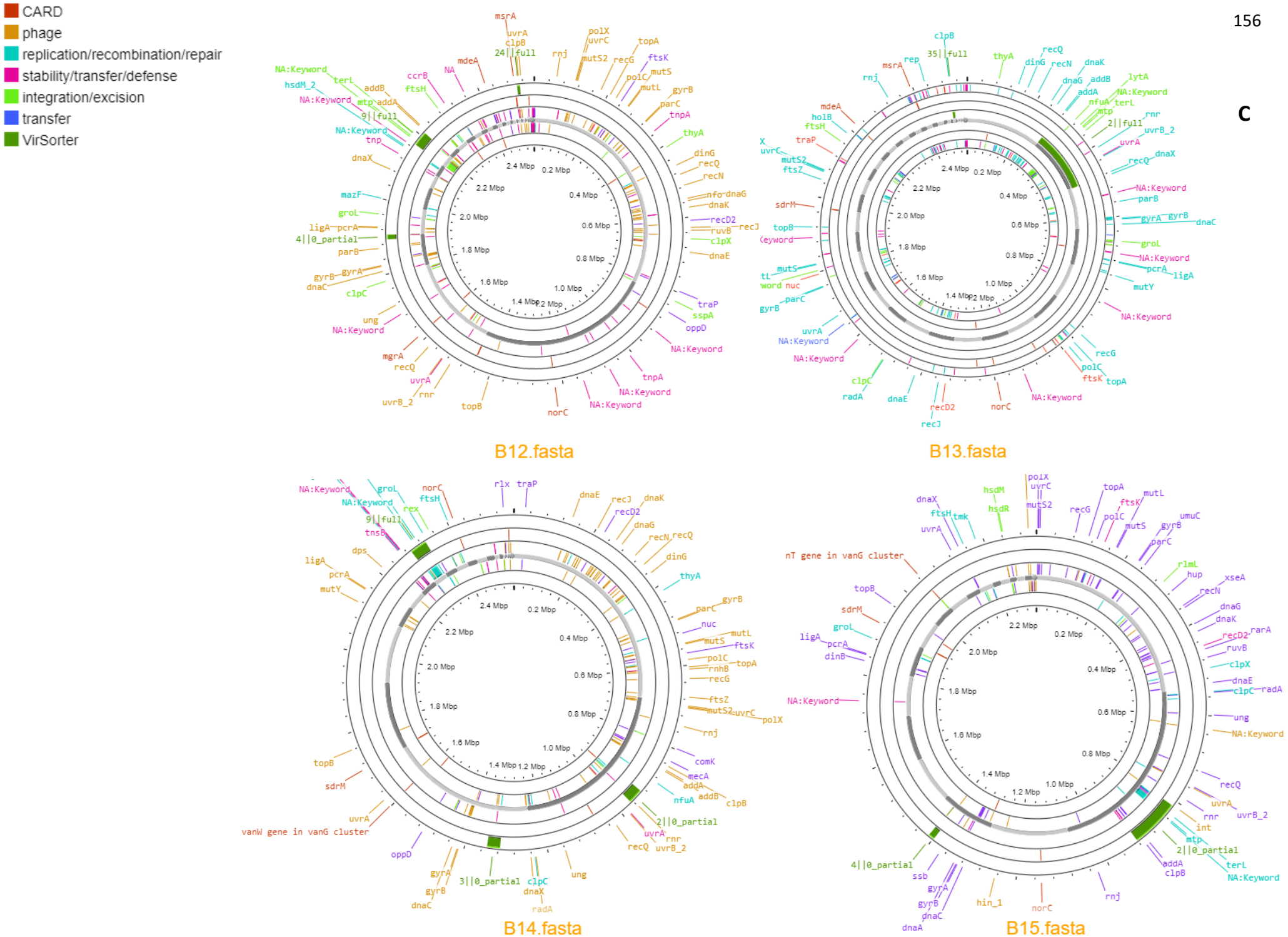

CARD
phage
replication/recombination/repair
stability/transfer/defense
integration/excision
transfer
VirSorter
B12.fasta
B13.fasta
B14.fasta
B15.fasta

Figure 5. Genome map of the 14 NAS constructed with ProKsee. **A:** Genomes B1 to B7. **B:** Genomes B8 to B11. **C:** Genomes B12 to B15.

4. Conclusion

This work showed that MRNAS presented resistance genes to several other classes of antibiotics in addition to beta-lactams. Furthermore, virulence genes related to various functions have been identified, contributing to an increase in their pathogenicity. It was possible to identify the SCC*mec* type of most of the NAS analyzed, helping to contribute to the epidemiology of MRNAS isolated from food sources and their relationship with other MRNAS clones. The genomes analyzed presented many MGEs related to phages and plasmids, contributing to the spread of resistance genes between species. STs contributed to good typing of the analyzed genomes, especially in combination with WGS. It is also important to highlight that these multiresistant isolates come from food sources and that transmission of these microorganisms can occur among milk consumers, which contributes to the increase in antimicrobial resistance among humans and animals.

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DISCUSSÃO GERAL

Esse estudo analisou 1,468 amostras de leite de vacas com baixa contagem de células somática (CCS), amostras com alta CCS e amostras de leite de vacas com mastite clínica de cinco estados brasileiros, sendo eles São Paulo (n=356), Santa Catarina (n=268), Pará (n=301), Paraíba (n=285) e Goiás (n=258). No total, 309 NASM foram isolados e identificados, sendo identificadas 18 espécies diferentes das quais: *S. chromogenes* (N=181), *S. simulans* (N=26), *S. haemolyticus* (N=25), *S. epidermidis* (N=14), *S. xylosus* (N=14), *S. warneri* (N=9), *S. equorum* (N=9), *S. cohnii* (N=7), *S. saprophyticus* (N=7), *S. hyicus/agnetis* (N=5), *M. sciuri* (N=4), *S. pasteuri* (N=2), *S. succinus* (N=1), *S. auricularis* (N=1), *S. caprae* (N=1), *S. capitis* (N=1), *S. kloosii* (N=1), *S. arlettae* (N=1). De forma geral, *S. chromogenes* foi a única espécie isolada em todos os estados, sendo também a espécie mais prevalente. Porém, algumas espécies foram identificas apenas em amostras pertencentes a um único estado, como é o caso de *S. capitis* e *S. kloosii* identificados apenas no estado da Paraíba, *S. auricularis* e *S. arlettae* identificados apenas no estado de Santa Catarina, *S. succinus* e *S. cohnii* identificados apenas no estado de São Paulo e *S. caprae* identificado apenas no estado do Pará.

Na Itália, em um estudo realizado por Addis et al. (2024), os autores avaliaram 17.213 amostras de leite de vacas com mastite clínica, subclínica e amostras compostas de vários animais com objetivo de caracterizar NASM também por MALDI-TOF MS. Como resultado, os autores isolaram 2,195 NASM, sendo identificadas 20 espécies diferentes. A prevalência das espécies variou de acordo com o tipo de amostra, mas de forma geral, as espécies mais prevalentes foram *S. chromogenes, S. haemolyticus, S. epidermidis, M. sciuri e S. xylosus.* Esses resultados são similares aos encontrados nessa tese. Os autores destacam a importância desse tipo de estudo para a expansão do conhecimento e da epidemiologia de NASM nos rebanhos.

Estudos envolvendo as diferentes características das fazendas também são importantes, uma vez que a prevalência de NASM como causadores de mastite ou de IMI é influenciada por essas características. Diferenças entre rebanhos foram observadas quanto ao isolamento de diferentes espécies NASM, sendo algumas encontradas no ambiente e outras no leite. Além disso, os reservatórios primários entre espécies NASM podem variar (PIESSENS et al. 2011). Esse estudo associou o isolamento de NASM a práticas de manejo adotadas nas fazendas, porém devido às limitações da coleta de dados essa análise foi feita considerando NASM como um grupo e não considerando as diferentes espécies. De qualquer forma, foi possível associar o isolamento de NASM principalmente a ordenha do tipo canalizada, ao uso de luvas, a baixa CCS, a fazer a CCS regularmente, a prática de alimentar os animais imediatamente após a ordenha, a manutenção periódica dos equipamentos de ordenha, entre outros. Essas variáveis citadas contribuem para que as infecções causadas por NASM e outros patógenos sejam evitadas, pois estão relacionadas a práticas de controle da mastite bovina (SANTOS & FONSECA, 2019). O que pode explicar a presença de NAS associadas a essas variáveis é o fato dessas espécies estarem disseminadas no ambiente de ordenha ou por algumas espécies, como por exemplo *S. chromogenes*, estarem adaptadas ao hospedeiro. Além disso, estudos sugerem que NASM podem persistir nos rebanhos, podendo causar reinfecção, além de causar IMI crônicas ao longo da lactação, principalmente em vacas multíparas (CONDAS et al., 2017; De BUCK et al., 2021).

Como já reportado na literatura (RALL et al., 2014; DA SILVA CÂNDIDO et al., 2020; DA SILVA ABREU et al., 2020), genes de resistência e de virulência também foram encontrados em diferentes espécies NASM identificadas nesse estudo. NASM podem causar intoxicações alimentares devido à presença de genes de enterotoxinas, reforçando a importância de não considerar a pesquisa de enterotoxinas estafilocócicas

nos alimentos apenas quando houver a presença de *S. aureus* ou a presença de estafilococos coagulase positiva com contagens acima de 10^5 UFC.mL.

Nesse estudo, diversos NAS apresentaram diferentes genes de biofilme e todos os NASM selecionados formaram biofilme em superfície de aço inoxidável. Além de alguns isolados NAS também carregarem genes responsáveis pela resistência a sanitizantes. A formação de biofilme é um problema em todas as etapas da produção de leite, desde o ambiente de ordenha, funcionando como uma barreira e não permitindo a passagem do antibiótico até sua efetiva ação contra os patógenos que causam mastite, além de diminuir a susceptibilidade dos sanitizantes utilizados em processos de sanitização dos tetos, independente da presença de genes de resistência. Até mesmo na indústria leiteira, onde pode ocorrer a formação de biofilme em tanques e equipamentos, ocasionando o desprendimento de células provenientes de biofilmes maduros e que podem ser uma fonte de contaminação cruzada na indústria de alimentos (TURCHI et al., 2020; ZHANG et al., 2021).

Esse estudo possibilitou caracterizar as 18 espécies identificadas frente ao perfil de resistência aos antimicrobianos, o que contribui para ampliar o conhecimento individual entre as espécies e traçar estratégias contra NASM causadores de mastite ou IMI. NASM MDR foram identificados nesse estudo, assim como *Staphylococcus* não-*aureus* resistentes à meticilina (MRNAS). Esses resultados servem como um alerta para que o potencial dessas espécies não seja ignorado e nem negligenciado, pois há indícios do aumento de NAS MDR com o passar dos anos (NOBREGA et al., 2018; CRIPPA et al., 2024).

Os resultados obtidos nesse estudo mostram uma disseminação de MRNAS em diferentes rebanhos, que pode ser explicada pela diversificação de fontes apontadas como reservatório NASM, como por exemplo leite, úbere, cama dos animais, mão do

ordenhador, luvas, equipamentos de ordenha, pele do teto etc (PIESSENS et at., 2011; DE VISSCHER et al., 2014). Ou seja, MRNAS podem se disseminar também fora do ambiente de ordenha, uma vez que esses isolados podem ser veiculados pelo leite, pelos ordenhadores e por alimentos preparados com essa matéria prima onde MRNAS estão presentes. Para que esse aumento de NASM MDR não continue ocorrendo, a iniciativa de Saúde Única é uma abordagem recomendada. Essa abordagem abrange práticas envolvendo saúde humana, animal e ambiental e que visa mitigar o uso indiscriminado de antibióticos na pecuária leiteira. Sabemos que os antibióticos utilizados para tratar a mastite são utilizados muita das vezes de forma profilática, contribuindo para o aumento de isolados resistentes. Além disso, estima-se que mais de 70% dos antibióticos consumidos em todo o mundo sejam usados em animais (PANCHAL et al., 2024).

Nesse trabalho, 54% dos NASM foram isolados de amostras de leite de vacas com alta CSS e 35% foram isolados de leite de vacas com baixa CSS, ou seja, amostras de leite que macroscopicamente aparentavam estar normais, mas que apresentam NAS resistentes a antibióticos. Assim, por meio da ingestão de leite cru ou pasteurizado inadequadamente ou pelo consumo de produtos lácteos derivados de leite, os consumidores de produtos lácteos são expostos a essas bactérias resistentes a antibióticos. A presença de NAS resistentes a antibióticos e NASM portadores de genes de virulência em amostras de “leite saudável”, ou seja, livre de infecção por mastite, é um problema grave que implica tanto na saúde humana como animal (PANCHAL et al., 2024).

Para que fosse possível aprofundar mais ainda os estudos sobre o papel de NASM na saúde do úbere, isolados MRNAS foram selecionados para uma análise do perfil de resistência e virulência através do sequenciamento completo do genoma (WGS). O WGS mostrou isolados NASM altamente patogênicos, uma vez que eram portadores de diversos genes de resistência e virulência, além de apresentarem diversos elementos

genéticos móveis (MGEs), como por exemplo plasmídeos e bacteriófagos, responsáveis pela transferência horizontal desses genes entre outras espécies, o que apresenta mais um motivo para o controle de NASM nos rebanhos, já que além de patogênicos, podem tornar patogênicos outros isolados via transferência de genes por MGEs (OTTO, 2012; SMITH & ANDAM, 2021). O WGS também possibilitou classificar *S. epidermidis* como a espécie mais patogênica quando comparada com *S. chromogenes*, visto que a análise dos genomas mostrou uma maior prevalência de genes associados a essa espécie. Além disso, foi possível obter a informação de que isolados NASM da mesma espécie e do mesmo rebanho, porém provenientes de amostras de leite diferentes, podem ser bastante distintas geneticamente.

CONCLUSÃO GERAL

Os resultados desse trabalho permitiram expandir o conhecimento sobre as espécies NASM que estão disseminadas nos diferentes rebanhos brasileiros. Apesar da diversidade de espécies, a prevalência de *S. chromogenes* se mostrou altamente superior.

Houve uma prevalência maior de isolados NASM em alguns estados e tanto a geografia quanto as características das fazendas, como práticas de manejo, podem ter influenciado nesse isolamento.

NASM são resistentes a antibióticos utilizados tanto na medicina veterinária quanto na medicina humana, além de apresentar isolados MDR. *S. epidermidis*, *S. chromogenes* e *S. warneri* foram identificados como MRNAS e tanto NAS MDR quanto MRNAS estavam presentes em sua grande maioria em amostras de leite consideradas aptas para comercialização.

NASM são capazes de formar biofilme em aço inox, além de serem portadores de diversos genes responsáveis pela formação de biofilme, o que contribui para o aumento da resistência aos antimicrobianos nas espécies NASM. Além disso, diferentes espécies NAS desse estudo apresentaram genes de produção de enterotoxinas na análise por PCR, evidenciando também o potencial dessas espécies em causar intoxicação alimentar.

A análise completa do genoma propiciou conhecer melhor o potencial patogênico de MRNAS. Os genomas analisados mostraram genes de resistência a diversas classes de antibióticos, além de diversos genes de virulência que não deixam dúvidas de que espécies NASM podem causar doenças em animais e consequentemente em humanos. O uso de antibióticos na pecuária deve ser controlado, pois espécies que antes não eram consideradas um problema de saúde pública estão se tornando um problema.

Boas práticas agrícolas e boas práticas de manejo devem ser adotadas nas fazendas a fim de diminuir a disseminação de NASM no ambiente de ordenha e consequentemente

NASM causadores de IMI e causadores de mastite. A presença de NASM em amostras de leite com baixa CCS ou alta CCS podem não causar problemas visíveis no leite ou no animal, mas representam um risco de saúde pública visto os resultados obtidos nesse estudo.

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Anexo 1

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

UFG

C E RT I F I C A D O

Certificamos que o uso de material biológico de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) registrado sob protocolo **n° MB 057/21,** sob a responsabilidade de **Clarice Gebara Muraro Serrate Cordeiro** encontra-se de acordo com os preceitos da Lei n° 11.794, de 8 de outubro de 2008, do Decreto n° 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal de Goiás (UFG), em reunião de **09/08/2021**. Informamos que para esse tipo de autorização não há necessidade de entrega de relatório final.

- Finalidade: ( ) Ensino (X) Pesquisa Científica
- Tipo de material biológico: leite
- Espécie do material biológico: bovina
- Origem do material biológico: Laboratório de qualidade do leite – LQL/CPA/EVZ/UFG

Informamos que de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais em Atividades de Ensino ou de Pesquisa Científica (DBCA) vigente é responsabilidade do Pesquisador Responsável pela atividade manter documentação que evidencie a origem do material de forma inequívoca. Essa evidência pode ser dada por meio de nota fiscal de compra, recibo, fotografias ou documentos oficiais dos serviços de vigilância, dentre outros aplicáveis quando o material não for oriundo de uma atividade de ensino ou de pesquisa científica. Quando o material for obtido de animais incluídos em uma atividade de ensino ou de pesquisa científica manter o certificado de autorização pela CEUA pertinente.
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Dra. Liliana Borges de Menezes Leite
Coordenadora da CEUA/PRPI/UFG

Comissão de Ética no Uso de Animais/CEUA
Pró-Reitoria de Pesquisa e Inovação/PRPI-UFG, Alameda Flamboyant, Qd. K, Edifício K2, 1° andar, Prédio da Agência de Inovação, Parque Tecnológico, sala da CEUA, Campus Samambaia – Goiânia-GO, Fone: (55-62) 3521-1876.
Email: ceua.ufg@gmail.com

Anexo 2

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão
Cadastro nº A4784B5

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A4784B5**
Usuário:	**Nathalia Silva**
CPF/CNPJ:	**342.895.448-35**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**
Título da Atividade:	**Diversidade microbiana em leite de vacas saudáveis e com mastite de diferentes regiões do Brasil**

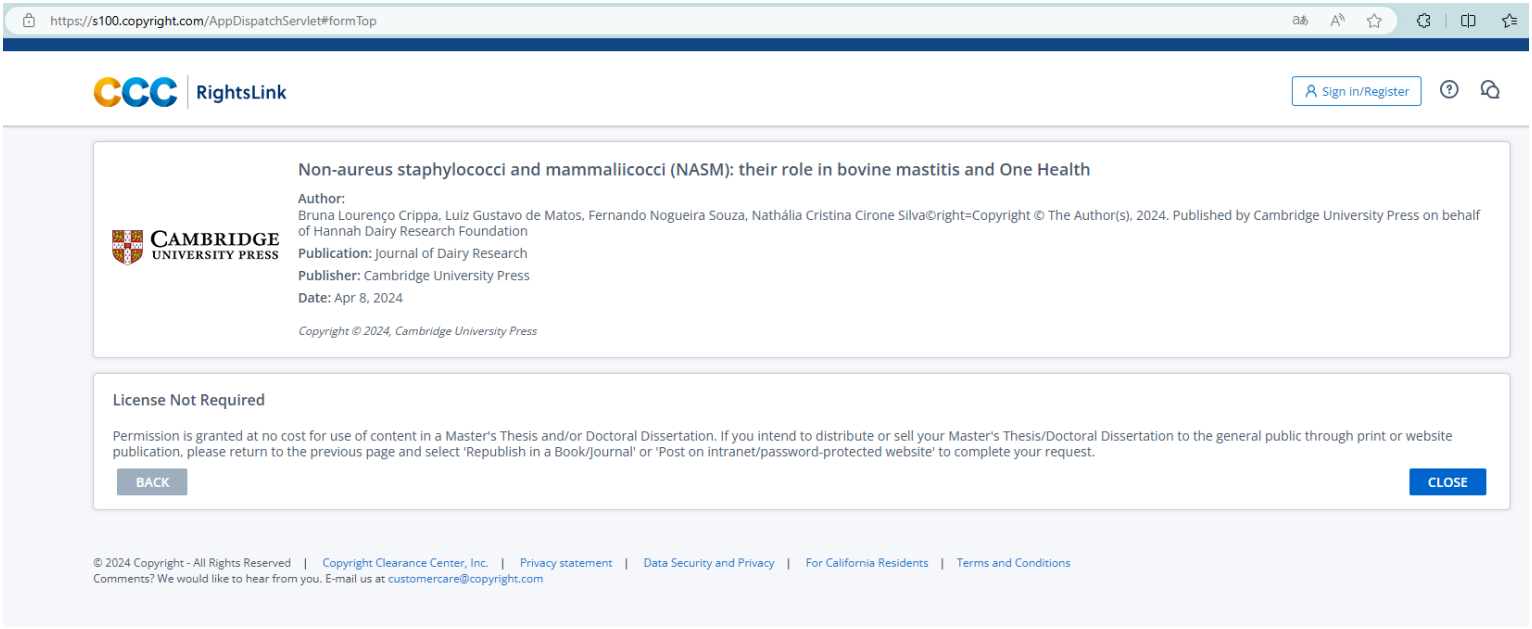
Equipe

Data do Cadastro:	**08/07/2022 16:38:05**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **15:12** de **17/03/2023**.

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

ANEXO 3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Non-aureus staphylococci and mammaliicocci (NASM): their role in bovine mastitis and One Health
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