



**UNIVERSIDADE ESTADUAL DE CAMPINAS  
FACULDADE DE ENGENHARIA DE ALIMENTOS**

**KAREN VANESSA MUNIVE NÚÑEZ**

**ANTIMICROBIAL POTENTIAL OF ESSENTIAL OILS AGAINST  
ANTIBIOTIC-RESISTANT, ENTEROTOXIN-PRODUCING, AND BIOFILM-  
FORMING *Staphylococcus aureus***

**POTENCIAL ANTIMICROBIANO DE ÓLEOS ESSENCIAIS CONTRA  
*Staphylococcus aureus* RESISTENTES A ANTIBIÓTICOS, PRODUTORES DE  
ENTEROTOXINAS E BIOFILME**

**CAMPINAS**

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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.

**Orientadora:** Profa. Dra. Nathália Cristina Cirone Silva

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

*To my beloved parents, Nicolás and Mirtha, and  
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*"Science knows no country because knowledge belongs to  
humanity, and is the torch that illuminates the world."*

Louis Pasteur



## RESUMO

O *Staphylococcus aureus* é uma das principais causas de mastite subclínica em vacas leiteiras, e o desenvolvimento de resistência aos antibióticos tem limitado as opções de tratamento eficazes. Este estudo avaliou a virulência, resistência antimicrobiana e alternativas terapêuticas baseadas em óleos essenciais (OEs) para isolados do tipo *spa* t605 de *S. aureus* provenientes de mastite bovina subclínica em São Paulo, Brasil. O estudo também visou investigar a presença de genes de virulência, identificar variações genéticas por meio de tipagem *spa* e *agr*, determinar perfis de resistência antibiótica fenotípica, identificar os principais constituintes químicos de oito OEs diferentes (*Citrus aurantium bergamia* - Bergamota, *Copaifera reticulata* - Copaíba, *Foeniculum vulgare* – Erva doce, *Zingiber officinale* - Gengibre, *Ocimum basilicum* - Manjeriço, *Luma chequena* – Arrayan, *Mentha spicata* - Hortelã-verde, e *Eugenia caryophyllus* – Cravo), e avaliar sua atividade antimicrobiana e citotoxicidade. A Concentração Inibitória Mínima (CIM) e a Concentração Bactericida Mínima (CBM) contra *S. aureus* inoculados em concentrações de  $10^3$  UFC.mL<sup>-1</sup> e  $10^7$  UFC.mL<sup>-1</sup> também foram realizadas. A cromatografia gasosa acoplada a um detector seletivo de massas (CG/DMS) analisou as composições químicas dos OEs. Os efeitos sinérgicos ou antagônicos dos OEs também foram investigados. A citotoxicidade foi avaliada usando o ensaio MTT em células Vero. Um total de 57 isolados de *S. aureus* foram examinados para 49 genes. Os *S. aureus* eram do tipo *spa* t605 e carregavam uma grande variedade de fatores de virulência, incluindo *icaD*, *fib*, *fnbA*, *clfA*, *bap*, *clfB*, *icaA*, *see* e *sed*. O gene de resistência antimicrobiana mais comum foi o *blaZ*, associado à resistência a beta-lactâmicos, seguido por *aac6'aph2'* e *ant4*, ambos ligados à resistência a aminoglicosídeos. O tipo *spa* t605 MSSA é endêmico em São Paulo, Brasil, e apresenta alta resistência à penicilina. O estudo descobriu que os OEs de manjeriço e bergamota foram eficazes contra *S. aureus* em  $10^3$  UFC.mL<sup>-1</sup>, enquanto os OEs de cravo, arrayan e hortelã-verde mostraram forte ação antimicrobiana em  $10^7$  UFC.mL<sup>-1</sup>, com o cravo apresentando os resultados mais promissores. As análises de CG/DMS revelaram os principais constituintes desses OEs: eugenol no cravo (86,25%), limoneno no arrayan (32,64%) e carvona na hortelã-verde (63,21%). O estudo também usou concentrações subinibitórias (sub-CIMs) para avaliar a formação de biofilme para os OEs de hortelã-verde + arrayan, hortelã-verde, arrayan e cravo. Os resultados mostraram redução na formação de biofilme; às 24 horas, a DO foi de 0,085, enquanto às 48 horas, reduziu para 0,078. Além disso, a DO diminuiu de 0,094 a 1/4 da CIM para 0,069 a 1/16 da CIM. Os OEs mostraram citotoxicidade em células Vero; o OE de cravo teve um IC<sub>50</sub> de 0,021. Apesar dos resultados antimicrobianos promissores, os testes de citotoxicidade levantam preocupações sobre a segurança dos OEs. O estudo contribui para uma compreensão mais profunda da virulência, variações genéticas e resistência a antibióticos de *S. aureus* e enfatiza o potencial dos OEs como alternativas naturais aos antibióticos para tratar *S. aureus* em mastite bovina, mas levanta preocupações sobre sua citotoxicidade; são necessárias investigações adicionais para otimizar formulações e avaliar a eficácia e segurança in vivo.

**Palavras-chave:** Genes de virulência; Resistência a antibióticos; Mastite subclínica; Tipagem *spa*; Citotoxicidade.

## ABSTRACT

*Staphylococcus aureus* is a major cause of subclinical mastitis in dairy cows, and the development of antibiotic resistance has limited effective treatment options. This comprehensive study evaluated the virulence, antimicrobial resistance, and therapeutic alternatives based on essential oils (EOs) for *spa* type t605 *S. aureus* isolates from subclinical bovine mastitis in São Paulo, Brazil. The study also aimed to investigate the presence of virulence genes, identify genetic variations through *spa* and *agr* typing, determine phenotypic antibiotic resistance profiles, identify major chemical constituents of eight different EOs (*Citrus aurantium bergamia* - Bergamot, *Copaifera reticulata* - Copaiba, *Foeniculum vulgare* - Fennel, *Zingiber officinale* - Ginger, *Ocimum basilicum* - Basil, *Luma chequeen* – Arrayan, *Mentha spicata* - Spearmint, and *Eugenia caryophyllus* – Clove), and assess their antimicrobial activity and cytotoxicity. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *S. aureus* isolates inoculated to  $10^3$  CFU.mL<sup>-1</sup> and  $10^7$  CFU.mL<sup>-1</sup> concentrations were also performed. Gas chromatography coupled to a mass selective detector (GC/MSD) analyzed the chemical compositions of EOs. The synergistic or antagonistic effects of selected EOs were also investigated. Cytotoxicity was assessed using the MTT assay on Vero cells. A total of 57 *S. aureus* isolates were screened for 49 genes. *S. aureus* were *spa* type t605 and carried a wide variety of virulence factors, including *icaD*, *fib*, *fnbA*, *clfA*, *bap*, *clfB*, *icaA*, *see*, and *sed*. The most common antimicrobial resistance gene was *blaZ*, associated with beta-lactam resistance, followed by *aac6'aph2'* and *ant4*, both linked to aminoglycoside resistance. The *spa* type t605 MSSA is endemic in São Paulo, Brazil, and is highly resistant to penicillin. The study found that basil and bergamot EOs were effective against *S. aureus* at  $10^3$  CFU.mL<sup>-1</sup>, while clove, arrayan, and spearmint EOs showed strong antimicrobial action at  $10^7$  CFU.mL<sup>-1</sup>, with clove showing the most promising results. GC/MSD analyses revealed the principal constituents of these EOs: eugenol in clove (86.25%), limonene in arrayan (32.64%), and carvone in spearmint (63.21%). The study also used sub-minimum inhibitory concentrations (sub-MICs) to assess biofilm formation for the EOs of spearmint + arrayan, spearmint, arrayan, and clove. Results showed a reduction in biofilm formation; at 24 hours, the OD was 0.085, while at 48 hours, it reduced to 0.078. Further, OD decreased from 0.094 at 1/4 MIC to 0.069 at 1/16 MIC. Tested EOs showed cytotoxicity on Vero cells; clove EO had an IC<sub>50</sub> of 0.021. Despite promising antimicrobial results, cytotoxicity tests raised concerns about the safety of these EOs. The study contributes to a deeper understanding of the virulence, genomic variations, and antibiotic resistance of *S. aureus*. The study emphasizes the potential of EOs as natural alternatives to antibiotics for treating *S. aureus* in bovine mastitis but raises concerns about their cytotoxicity; further investigations are needed to optimize formulations and assess in vivo efficacy and safety.

**Keywords:** Virulence genes; Antibiotic resistance; Subclinical mastitis; *spa* typing; Cytotoxicity.

## **SUMMARY**

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## **Thesis structure**

This thesis contains seven distinct chapters presenting the doctoral research findings offering insights into various aspects of antimicrobial potential, virulence factors, and resistance profiles concerning *S. aureus*.

**Chapter I:** The first chapter provides a comprehensive introduction, outlining the general and specific objectives of the research. This section sets the stage for the subsequent chapters.

**Chapter II:** This chapter provides a more detailed literature review examining existing research. It examines existing research on the efficacy of EOs as a potential solution for combating antibiotic-resistant isolates of *S. aureus* that produce enterotoxins and form biofilms.

**Chapter III:** This chapter provides a detailed overview, highlighting the findings related to the virulence and antimicrobial resistance gene profiles of the *S. aureus* isolates tested. The study identified prevalent virulence and resistance genes, offering valuable genetic insights that have implications for both bovine and human health.

**Chapter IV:** Explores the antimicrobial efficacy of five EOs against *S. aureus* isolates in a concentration of  $10^3$  CFU.mL<sup>-1</sup>, including the MIC and MBC results. The study also identifies their main chemical constituents, emphasizing the promising results of basil and bergamot EOs. Further, discuss the implications of using EOs to treat *S. aureus*-causing subclinical bovine mastitis.

**Chapter V:** This chapter delves into the synergistic effects of selected EOs as alternative treatments for biofilm-forming *S. aureus* isolates. The study employs MIC, MBC, and FIC index tests, as well as cytotoxicity assessments, to evaluate the

antimicrobial and antibiofilm activities of the EOs against *S. aureus* isolates at a concentration of  $10^7$  CFU.mL<sup>-1</sup>.

**Chapter VI:** This chapter serves as a comprehensive discussion that synthesizes the findings from the preceding chapters II, III, IV, and V. In addition, it summarizes the main findings for understanding the antimicrobial potential of EOs against *S. aureus*, comparing and contrasting the results with existing literature.

**Chapter VII:** The final chapter provides a succinct summary of the key findings, concludes with an overview of the overall contribution of the research to the field, and suggests directions for future research.

# **CHAPTER 1**

*Introduction and objectives*

## Introduction

Bovine mastitis is a highly prevalent disease within dairy herds. *Staphylococcus aureus* is one of the primary microorganisms responsible for subclinical mastitis (SM), which is a prevalent and economically significant disease characterized by inflammation of the mammary gland without clinical signs, resulting in reduced milk production and quality (Cheng & Han, 2020; Dyson et al., 2022; Gonçalves et al., 2023). *S. aureus* is an opportunistic pathogenic microorganism responsible for many infections (Jenul & Horswill, 2018). The emergence of antimicrobial resistance in *S. aureus* strains poses a significant challenge in diagnosing and treating SM mastitis (Bonsaglia et al., 2018; Cheng & Han, 2020; Tomanić et al., 2023). This pathogen has different virulence and contributes to the ability to establish and maintain infections, leading to persistent and recurrent cases of bovine mastitis (Zaatout et al., 2020).

Molecular studies are essential to assess the genetic diversity of pathogens. This can help us understand the sources, transmission routes, prognosis, and adaptation mechanisms of pathogens (McMillan et al., 2016). The virulence profile of *S. aureus* can vary depending on the strain. Some strains of *S. aureus* are more virulent than others and are more likely to cause disease (Moore-Lotridge et al., 2022; Zayda et al., 2020). The expression of virulence factors is rigorously controlled by a series of regulatory genes, which are influenced by the accessory gene regulator (*agr*). The *agr* gene complex regulates the expression of several genes involved in producing virulence factors. Their expression plays an essential role in the persistence of the pathogen causing mastitis (Rossi et al., 2021). Among the virulence factors, staphylococcal enterotoxins (SEs) have been shown to play an essential role in the pathogenesis of bovine mastitis. Most *S. aureus* strains isolated from this disease harbor one or more enterotoxin genes (Bonsaglia et al., 2018; Rall et al., 2014; Ren et al., 2020). SEs are also a significant cause of food



poisoning. Foods containing sufficient amounts of preformed SEs can cause human intoxication, characterized by nausea, vomiting, and diarrhea (Grispoldi et al., 2021).

Virulence factors, such as biofilm formation and antimicrobial resistance acquisition, have limited treatment for bovine mastitis (V. Silva et al., 2022; Zaatout et al., 2020). Biofilm production enhances the capacity of strains to adhere to and colonize the epithelial surfaces of the mammary gland (Archer et al., 2011; Campos et al., 2022). When bacteria form biofilms, it becomes difficult for antibiotics to penetrate the matrix. This can reduce sensitivity to antimicrobials (Zaatout et al., 2020). Furthermore, the onset of intramammary infection is influenced by another important virulence factor, such as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) genes (Zuniga et al., 2015). These proteins allow bacteria to adhere to surfaces in the mammary gland, which helps bacteria evade the immune system. Altogether, these virulence factors play an essential role in the initial attachment, recruitment, and colonization of the mammary gland by *S. aureus*, leading to the persistence of bovine mastitis infection (Aslantaş & Demir, 2016; Zaatout et al., 2020; Zuniga et al., 2015).

Methicillin-susceptible *Staphylococcus aureus* (MSSA) refers to a strain of *S. aureus* that displays susceptibility to methicillin, a type of antibiotic. However, MSSA can become resistant to methicillin and other antibiotics through horizontal gene transfer and mutations, making it difficult to treat. When MSSA acquires the *mecA* or *mecC* gene, which encodes for methicillin resistance, a new methicillin-resistant *Staphylococcus aureus* (MRSA) lineage may emerge (Bal et al., 2016; Moore-Lotridge et al., 2022; Silva et al., 2013). These new MRSA lineages can establish infections in animals, including cattle. They can also spread to humans, where they can cause serious diseases, such as pneumonia and bloodstream infections (Turner et al., 2019).

Penicillins and other  $\beta$ -lactam antibiotics are the drugs of choice for treating mastitis (Gonçalves et al., 2022). However, conventional antibiotics can be ineffective against staphylococcal infections, as the pathogen can adapt to different stress conditions due to its genotypic and phenotypic plasticity (Erickson et al., 2017). This has led to the emergence of antimicrobial resistance, and the control of staphylococcal has become a significant challenge in the dairy industry (da Silva Abreu et al., 2021; Zaatout et al., 2020). As such, there is a pressing need to develop new treatment approaches to combat these infections effectively (Lopes et al., 2020).

There is a growing interest in finding treatments that use natural compounds against diseases (Ni et al., 2021; Tomanić et al., 2023). Essential oils (EOs) are a complex mixture of volatile molecules. These mixtures contain many bioactive molecules, such as alkaloids, monoterpenes, and flavonoids (Ni et al., 2021; Tavares et al., 2022). Historically used for medicinal benefits (Ni et al., 2021), they promise to improve milk quality and treat bovine mastitis, particularly against *S. aureus* (Cheng & Han, 2020; Lopes et al., 2020). However, there is currently limited research on the topic, emphasizing the need for further studies to establish the safety and effectiveness of EOs in dairy cows (Caneschi et al., 2023; Fratini et al., 2014; Lopes et al., 2020; Tomanić et al., 2023).

Notably, EOs vary in antimicrobial activity due to their major components and can have synergistic interactions with trace elements (Bagheri et al., 2020; Chouhan et al., 2017), thereby contributing to combating microorganisms that may cause illnesses (Álvarez-Martínez et al., 2021; Caneschi et al., 2023). Studies have shown that some EO blends have been found to enhance antimicrobial efficacy (Caneschi et al., 2023; Milagres de Almeida et al., 2023). When it comes to combining different antimicrobial compounds, it is crucial to consider the potential interactions, which can be either synergistic, additive,

or antagonistic. Thus, it is essential to consider these possibilities when combining antimicrobials (Chouhan et al., 2017).

The cytotoxicity of EOs is an important consideration when using them as an alternative to antibiotics to treat *S. aureus* infections since some EOs are more cytotoxic than others, and it is necessary to ensure that the benefits outweigh the risks (García-Salinas et al., 2018; Ribeiro et al., 2020). Cytotoxicity varies depending on the EO, concentration, and cell type being exposed. By testing the cytotoxicity of EOs in vitro, researchers can identify EOs that are safe to use and determine the optimal concentration and exposure time for therapeutic use (Surowiak et al., 2022). Some methods can be used to test the cytotoxicity, including in vitro tests, such as the MTT assay (Brożyna et al., 2021). This method measures the metabolic activity of cells, where cells that are alive reduce the yellow tetrazolium salt to a purple formazan product. The amount of formazan produced is proportional to the number of living cells. The more MTT is reduced, the more metabolically active the cells are. One way to perform this test is by using Vero cells, a type of monkey kidney cell commonly utilized in cell culture studies. They are relatively easy to grow and maintain and sensitive to various toxins (Vijayarathna & Sasidharan, 2012).

Based on the abovementioned reasons, this study is relevant in the dairy cattle industry to identify natural compounds that may have therapeutic potential and assist in treating and managing this disease. Therefore, given the growing failure rate in conventional antimicrobial treatments and to find efficient alternatives, the search for new biologically active compounds against mastitis in dairy cows, strategic treatment, control, and disease prevention is essential. The study evaluated the efficacy of various EOs against *S. aureus* isolated from cow's milk with SM. Alongside evaluating their

antimicrobial properties, the study examined the cytotoxicity of these EOs on Vero cells to determine their safety and viability as treatment options for SM caused by *S. aureus*.

## Objectives

### General objective

- Evaluate the antimicrobial potential of EOs against antibiotic-resistant *S. aureus* isolates producing enterotoxins and biofilm.

### Specifics objectives

- To investigate *S. aureus* isolates for the presence of genes encoding enterotoxins, specifically *sea*, *seb*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*.
- To detect the genes that encode for biofilm formation (*icaA*, *icaD*, and *bap*) and encoding MSCRAMMs (*fnbA*, *fnbB*, *fib*, *clfA*, *clfB*, *cna*, *eno*, *bbp*, and *ebps*).
- To verify the genetic variations of *S. aureus* isolates using the *spa* and *agr* typing techniques.
- Determine the phenotypic antibiotic resistance profiles of *S. aureus* isolates by the disk diffusion test using nine antimicrobial drugs: penicillin (PEN, 10 µg), oxacillin (OXA, 1 µg), ceftiofur (CEF, 30 µg), gentamicin (GEN, 10 µg), tobramycin (TOB, 10 µg), erythromycin (ERY, 15 µg), tetracycline (TET, 30 µg), clindamycin (CLI, 2 µg), and chloramphenicol (CHL, 30 µg)
- To perform the genotypic resistance profile of isolates, considering the resistance to penicillin (*blaZ*), oxacillin and ceftiofur (*mecA* and *mecC*), gentamicin (*aac6'aph2'*), tobramycin (*ant4*), erythromycin and clindamycin (*ermA*, *ermB*, *ermC*, *ermF*, *ermT*, *msrA*, *cfr*, *vgaC*, *lnuA/A'*, *lnuB*, *lsaB*, and

*lsaE*), and tetracycline (*tetK*, *tetL*, and *tetM*), antiseptic (QAC) resistance (*qacA/B* and *qacC*)

- To identify the major chemical constituents of EOs of *Citrus aurantium bergamia*, *Copaifera reticulata*, *Foeniculum vulgare*, *Zingiber officinale*, *Ocimum basilicum*, *Luma chequen*, *Mentha spicata*, and *Eugenia caryophyllus* using gas chromatography coupled to a mass selective detector (GC/MSD).
- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) to evaluate the antimicrobial activity of the EOs of *Citrus aurantium bergamia*, *Copaifera reticulata*, *Foeniculum vulgare*, *Zingiber officinale*, and *Ocimum basilicum* against *S. aureus* inoculated to a final concentration of  $10^3$  CFU.mL<sup>-1</sup>.
- To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) to evaluate the antimicrobial activity of the EOs of *Luma chequen*, *Mentha spicata*, and *Eugenia caryophyllus* against *S. aureus* inoculated to a final concentration of  $10^7$  CFU.mL<sup>-1</sup>.
- To investigate the synergistic or antagonistic effects of the EOs (*Luma chequen*, *Mentha spicata*, and *Eugenia caryophyllus*) against *S. aureus* isolates inoculated to a final concentration of  $10^7$  CFU.mL<sup>-1</sup>.
- To investigate the effects of the EOs (*Luma chequen*, *Mentha spicata*, and *Eugenia caryophyllus*) in the biofilm formation of *S. aureus* isolates.
- To analyze the cytotoxicity of EOs (*Luma chequen*, *Mentha spicata*, and *Eugenia caryophyllus*) on Vero cells using the MTT assay.

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# **CHAPTER 2**

*Literature review*

### *Staphylococcus aureus*

The *Staphylococcus* genus comprises 66 valid species, according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) and documented by the DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) available at <http://www.bacterio.net>, with *Staphylococcus aureus* being the most pathogenic species. It is differentiated from other close family members by its low G + C content of DNA. This pathogen is a Gram-positive coccus, non-spore-forming, nonmotile bacteria that appear microscopically as grape-like clusters. It can grow in a wide range of temperatures, pH, and water activity. It is a catalase-positive, facultative anaerobe that grows abundantly under aerobic conditions. It is highly salt-tolerant (up to 20% NaCl) and relatively resistant to drying and heat (Bhunias, 2018).

*S. aureus* is identified as one of the leading pathogens causing foodborne outbreaks in Brazil (A. C. da Silva et al., 2020) and one of the most studied causative agents of bovine mastitis (Campos et al., 2022). This commensal pathogenic microorganism is responsible for many types of infections (Jenul & Horswill, 2019). It is capable of causing diseases ranging from minor skin conditions to life-threatening illnesses such as sepsis, pneumonia, endocarditis, osteomyelitis, and lethal pneumonia (Cheung et al., 2021; Y. Guo et al., 2020). The risk of infection is increased when unhygienic practices are performed during milking; these include contaminated equipment, unsanitary environments, and the lack of trained personnel in dairy operations (Zaatout et al., 2019).

*S. aureus* is classified into two categories based on its sensitivity to antibiotics: Methicillin-Sensitive *Staphylococcus aureus* (MSSA) and Methicillin-Resistant

*Staphylococcus aureus* (MRSA) (Y. Guo et al., 2020). MSSA refers to a strain of *S. aureus* that displays susceptibility to methicillin, a type of antimicrobial. However, MSSA has the potential to develop resistance to methicillin and other antibiotics through mechanisms like gene mutations and horizontal gene transfer, making it difficult to treat. The acquisition of specific genes like *mecA* or *mecC*, which confer methicillin resistance, can lead to the evolution of a new MRSA strain (Bal et al., 2016; Moore-Lotridge et al., 2022; Silva et al., 2013). MRSA lineages are resistant to various antibiotics, limiting treatment options. It was initially associated with healthcare settings but has emerged in the community, spreading rapidly among healthy individuals (A. C. da Silva et al., 2020). It has been a subject of increasing concern due to its high morbidity and mortality rates and its potential for zoonotic transmission. It is alarming, given the bacterium's resistance to commonly used antibiotics and its capacity for human transmission (Khanal et al., 2022).

### **Epidemiology of *S. aureus***

Understanding the epidemiology of *S. aureus* is crucial for effective prevention and treatment strategies. A significant concern in the epidemiology of this pathogen is the increasing prevalence of antibiotic resistance. Based on recent data reported by the European Center for Disease Prevention and Control and the World Health Organization (ECDC, WHO, 2023), *S. aureus* accounted for 17.2% of the isolates across 16 countries. The MRSA situation varies significantly across Europe. Of the 44 countries that reported data on *S. aureus*, 11 countries (25%) had MRSA percentages below 5%, while 13 countries (30%) reported MRSA percentages equal to or above 25%. When considering only the 13 countries in both 2020 and 2021, there was an overall increase in the number of isolates reported in 2021. This trend was consistent across all pathogens, suggesting

that AMR is a growing issue that includes MRSA. This data emphasizes the need for ongoing surveillance of MRSA across countries and country-specific interventions.

Rates of MRSA infections have declined in U.S. hospitals over the past 1–2 decades. However, despite the decrease, MRSA remains a significant issue in that country, with 323,700 infections reported in 2017 (Kadri, 2020). Another in-depth analysis of the trends and epidemiology of MSSA bloodstream infections was conducted in Switzerland over 13 years from 2008 to 2021. The research reveals significant epidemiological trends in *S. aureus* bloodstream infections, with a 30% increase from 19.7 to 25.6 cases per 100,000 inhabitants. Specifically, MSSA infections increased by 37%, from 17.8 to 24.4 cases per 100,000 inhabitants. In contrast, MRSA cases decreased from 1.9 to 1.2 per 100,000 (Renggli et al., 2023).

The presence of *S. aureus*, especially MRSA, in food and food-producing animals represents a potential risk to public health, especially in Brazil, where *S. aureus* is one of the leading pathogens causing foodborne outbreaks and is found with high frequency in certain cases (A. C. da Silva et al., 2020). A comprehensive meta-analysis by Khanal et al. (2022) provides valuable insights into the epidemiology of this pathogen. It reveals a prevalence rate of 3.81% for MRSA in dairy farms worldwide. There are geographical disparities in prevalence rates, with Asia showing the highest at 4.89% and South America the lowest at 1.33%. This geographical disparity may be attributed to different farming practices, antibiotic usage, and healthcare systems across these regions.

Brazil is a major producer of bovine milk, with the southern and southeastern regions being the largest milk-producing regions. However, subclinical mastitis is a significant problem in the Brazilian dairy industry, with high epidemiological indices reported in several studies (Busanello et al., 2017; R. P. de Oliveira et al., 2022;

Gonçalves et al., 2023). Furthermore, in this country, *S. aureus* is one of the leading pathogens causing foodborne outbreaks (A. C. da Silva et al., 2020). A recent study conducted over six years, from January 2014 to December 2019 in Southern Brazil, examined the epidemiology and 30-day mortality rates of adult patients with MRSA bacteremia. The study found that 22% of 928 *S. aureus* bloodstream infections were MRSA isolates. Most of these infections were hospital-acquired, and the 30-day mortality rate was 31.2%. The prevalence of this pathogen in Brazil highlights the need for targeted interventions and empirical treatment strategies to manage this public health concern effectively (Riche et al., 2023).

Notably, the presence of *S. aureus* in bovine dairy herds varies significantly across different regions and is influenced by multiple factors, including herd management practices and antimicrobial resistance. A recent study in New South Wales, Australia, did not find the pathogen in bulk tank milk samples, suggesting effective antimicrobial stewardship in the region (Rowe et al., 2023). In contrast, research from Italian dairy farms revealed a more concerning prevalence of *S. aureus*, among other pathogens, in milk samples from healthy cows, emphasizing the need for targeted interventions at the farm level (Magro et al., 2023). Additionally, a study from Ireland reported a 6% prevalence of the pathogen at the quarter-level in late-lactation grazing dairy cows, suggesting that even in grazing systems, *S. aureus* remains a significant etiological agent for intramammary infections (Valdecabres et al., 2023). These studies underscore the complex epidemiology of *S. aureus* in dairy herds, suggesting that regional factors, including management practices and antimicrobial use, may play a significant role. Consequently, effective region-specific, evidence-based interventions are needed to manage its prevalence.

### **Subclinical bovine mastitis**

Bovine mastitis is a prevalent disease among dairy cows that can cause significant economic losses, and *S. aureus* is one of the primary microorganisms responsible for subclinical mastitis (Zaatout et al., 2020). The impact of mastitis on dairy farms is significant, leading to reduced milk production, increased culling rates, and decreased milk quality (J. L. Gonçalves et al., 2018; Sharun et al., 2021). Mastitis can be classified into two categories: clinical mastitis, which shows evident symptoms, and subclinical mastitis, which lacks noticeable symptoms (Adkins & Middleton, 2018; Tomanić et al., 2023). This disease, especially in its subclinical mastitis (SM) form, is highly prevalent in dairy herds. Due to the silent development of the disease, there is a tendency to evolve into chronic infections, with *S. aureus* being the most harmful agent that causes this type of mastitis, which is widely distributed in dairy herds in several countries (Reyher et al., 2012; Schmidt et al., 2017). Cows with SM caused by *S. aureus* present high SCC ( $>200 \times 10^3$  cells mL<sup>-1</sup>), but no visual changes in milk are observed (J. L. Gonçalves et al., 2018). However, it can cause human intoxication since bacteria can be eliminated through a thermal process, but enterotoxins may remain, increasing the possibility of foodborne poisoning (Campos et al., 2022; Grispoldi et al., 2021).

The most commonly used method for treating mastitis in cows involves intramammary administration of antibiotics. Other routes like intramuscular and intravenous administration are options (Caneschi et al., 2023). In addition to antibiotic treatment, supportive and symptomatic therapies are crucial. These therapies aim to reduce local inflammation in the mammary gland, thereby enhancing the effectiveness of the antibiotics by improving blood flow in the mammary gland, which helps in faster recovery (Tomanić et al., 2023).

### **Antimicrobial resistance**

Antimicrobials are commonly administered to ensure health and productivity in livestock farming. Data suggest that antimicrobial use in food-producing animals will increase by 67% by 2030, with nearly double the increase in Brazil, Russia, India, China, and South Africa (Van Boeckel et al., 2015). Mastitis in cows is the most common reason for antibiotic use in dairy production, and the emergence of antimicrobial resistance in *S. aureus* strains poses a significant challenge in diagnosing and treating subclinical bovine mastitis (Bonsaglia et al., 2018; Cheng & Han, 2020; Tomanić et al., 2023). Preventive measures must be adopted to control the transmission of *S. aureus* in subclinical bovine mastitis. However, infection with this pathogen often does not respond well to treatment with conventional antibiotics, leading to the emergence of multi-resistant staphylococci and the presence of residues of these agents in milk that can negatively impact human health (Abreu et al.; Zaatout et al., 2020).

It is essential to analyze the antimicrobial resistance profile of a microorganism before implementing an effective treatment. However, many producers do not perform the necessary diagnosis and treat the animals inappropriately, which can lead to the acquisition of resistance to antimicrobials (Adkins & Middleton, 2018; Tomanić et al., 2023). Therefore, an urgent need is to explore alternative treatments that can effectively combat mastitis without exacerbating antibiotic resistance (Lopes et al., 2020; Tomanić et al., 2023).

There is a risk of exchanging clones between humans and bovines since they adapt to the host and acquire virus factors to colonize and generate new infections (Veh et al., 2015). The organism's ability to develop resistance to antibiotics, such as methicillin and oxacillin, has made MRSA a major threat to public health (A. C. da Silva et al., 2020).



The association between antiseptic resistance genes and resistance to different antibiotics may be attributed to both groups of genes on the same plasmid or to the selection of resistant strains. Acquired resistance is caused by genes that confer resistance to biocides, such as phenolic compounds and quaternary ammonium compounds (QACs) (El Sayed Zaki et al., 2019). QACs are considered good antimicrobials because they can break the microbial cell membrane (Wassenaar et al., 2015; Zhang et al., 2018). The prevalence of *qacA* among *S. aureus* clinical isolates varies depending on the geographic location, ranging from 10% to 80% (El Sayed Zaki et al., 2019).

Conventional treatments for mastitis typically involve the use of antibiotics. Each case of mastitis should be individually evaluated before initiating treatment. The choice of the most suitable antibiotic should be based on the farm's history and the sensitivity profiles of pathogens isolated from previous mastitis cases (Langoni et al., 2017). However, the overuse and misuse of antibiotics have led to the emergence of antibiotic resistance in *S. aureus*, making it challenging to treat mastitis effectively. As a result, the efficacy of traditional treatment options has been compromised (da Silva Abreu et al., 2021; Sharun et al., 2021). Various drug classes are available for the treatment of MRSA. Glycopeptides, such as vancomycin and teicoplanin, are commonly used as first-line treatments for invasive infections in Europe, Asia, and South America (David & Daum, 2017).

Moreover, penicillins and other  $\beta$ -lactam antibiotics (e.g., ceftiofur) remain the most frequently used drugs for mastitis. According to a recent study, more than 50% of cows diagnosed with their first case of clinical mastitis received ceftiofur as the antibiotic of choice (Gonçalves et al., 2022). Furthermore, newer agents, such as cephalosporins (ceftobiprole in Europe and ceftaroline in the USA) and lipoglycopeptides (oritavancin, dalbavancin, and telavancin), have also emerged. While the role of these newer agents in

treating MRSA is not yet fully established, their increasing use suggests potential effectiveness in managing invasive infections (David & Daum, 2017).

Understanding the dynamics of MSSA and MRSA can guide healthcare providers in implementing effective antimicrobial strategies for managing *S. aureus* infections. Notably, a study highlights the role of antibiotic stewardship measures in Swiss hospitals. These measures are associated with the consumption of antibiotics specifically targeted against MRSA, indicating that responsible antibiotic use may be a critical factor in controlling resistant strains. The study discusses the concept of "fitness cost" as a bacteriological explanation for the decline of MRSA. This suggests bacterial resistance comes at a cost, potentially explaining why MRSA may not always dominate over MSSA (Renggli et al., 2023).

### **Pathogenesis and virulence of *S. aureus***

The evolution of *S. aureus* strains is driven by several factors, including horizontal gene transfer and dynamic mutations in the bacterial genome, which can lead to acquiring new virulence factors (Moore-Lotridge et al., 2022). This pathogen has different virulence factors and survival strategies to persist and defeat host defenses, such as the adhesion capacity and biofilm formation (Bhunia, 2008; Jenul & Horswill, 2019; Zhu et al., 2018). These virulence factors, such as coagulase, lipases, adhesins, nucleases, hemolysin, and toxins, contribute to the ability of *S. aureus* to establish and maintain infections (Cheung et al., 2021), leading to persistent and recurrent cases of bovine mastitis (Zaatout et al., 2020). These can include toxins, enzymes, and surface proteins and can vary from strain to strain, contributing to the severity of bovine mastitis (Campos et al., 2022).

The expression of virulence factors is rigorously controlled by a set of regulatory genes, which are controlled by the accessory gene regulator (*agr*). The *agr* gene complex

controls the expression of several genes involved in the production of virulence factors. The presence or absence of these factors plays an essential role in the persistence of the pathogen that causes mastitis (Rossi et al., 2021). Mutants in the Agr system are particularly beneficial in biofilm-associated infections due to their increased resistance to neutrophil attacks. These mutants are frequently isolated from chronic infections and bacteremia (Cheung et al., 2021).

The initiation of chronic mastitis caused by *S. aureus* begins with the adherence of the bacteria to the mammary gland epithelium. Subsequently, these bacteria produce biofilms, a significant virulence factor that enhances their capacity to adhere to and colonize the epithelial surfaces of the mammary glands (Archer et al., 2011; Campos et al., 2022). When bacteria like *S. aureus* form biofilms, it becomes difficult for antibiotics to penetrate the matrix. This can result in reduced sensitivity to antimicrobials, which can be a concern when dealing with mastitis infections (Zaatout et al., 2020). Furthermore, the onset of intramammary infection caused by *S. aureus* is influenced by another important virulence factor, such as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) genes (Zuniga et al., 2015). MSCRAMMs are proteins that allow bacteria to adhere to surfaces in the mammary gland. These proteins also help bacteria to evade the immune system. Altogether, these virulence factors play an essential role in the initial attachment, recruitment, and colonization of the mammary gland by *S. aureus*. This colonization can lead to the persistence of bovine mastitis infection (Aslantaş & Demir, 2016; Zaatout et al., 2020; Zuniga et al., 2015).

Other factors that influence biofilm formation include the polysaccharide intercellular adhesion (PIA), encoded by the *icaADBC* operon, an intercellular adhesion (*ica*) operon which contains *icaA* and *icaB* (N-acetyl glucosamine transferases), *icaC* (predicted exporter), and *icaD* (deacetylase) genes (Marques et al., 2021; Miao et al.,

2019; V. Silva et al., 2022). The *icaA* and *icaD* are essential factors in promoting intercellular adhesion and forming multilayered biofilms. The *icaC* gene transports *icaA* and *icaD* synthesized oligomers across the cell membrane. The absence of the *icaB* gene may reduce the efficiency in binding to the bacterial cell surface, leading to weak biofilm production (Miao et al., 2019; V. Silva et al., 2021). Studies assessing the presence of the *icaA* and *icaD* genes on milk samples of clinical or SM cases have reported that these genes are present at high frequencies (each 86.6%) (Aslantaş & Demir, 2016). Prior studies showed that the ability to form a biofilm in *S. aureus* strains depends on the expression of the *icaD* gene (Namvar et al., 2013).

The Bap protein is another surface protein of pathogenic bacteria that interacts with host proteins to facilitate bacterial attachment and penetration. This protein disrupts the internalization process guided by FnBP, a host protein that aids in the bacterial invasion of cells (Campos et al., 2022). Bap also plays a critical role in the initial attachment and recruitment of *S. aureus*. However, the *bap* gene, which is responsible for the production of the Bap protein, is present at a low frequency in cases of bovine mastitis caused by *S. aureus* (Aslantaş & Demir, 2016; Felipe et al., 2017; Zuniga et al., 2015), or it may be absent (Ren et al., 2020). In either case, biofilm formation can be attributed to the *ica*-dependent mechanism (Marques et al., 2021).

There is an association between the *bap*, *icaA*, and *icaD* genes and the genes encoding for MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). MSCRAMMs are specific staphylococcal surface proteins that play a crucial role in the initial attachment and colonization of the bacteria, as well as the intracellular internalization and initiation of biofilm (Klein et al., 2012). They provide a great capacity for adhesion and biofilm production in *S. aureus* strains derived from subclinical bovine mastitis, an essential characteristic for colonization and persistence of the pathogen

(Castilho et al., 2017). Prominent members include fibrinogen- and fibronectin-binding proteins such as clumping factors A and B (ClfA, ClfB) and fibronectin-binding proteins A and B (FnBPA, FnBPB) (Otto, 2018).

Among other virulence factors, *S. aureus* produces a variety of toxins, including enterotoxins (SEs), toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins (ETs), hemolysins, and leukocidins (Wu et al., 2016). Toxic shock syndrome (TSS) is a rare but severe condition caused by a potent toxin called TSS-1. This potent toxin leads to a severe condition that can result in multi-organ failure and death. It occurs in 0.006 cases per 100,000 people. TSS-1 is associated with food poisoning, but its prevalence is not yet well-known compared to SEs in humans, animals, or food (Babić et al., 2018). Other toxins produced by *S. aureus* can also promote infection. For example, alpha and beta hemolysins are toxins that can cause necrosis of the mammary gland tissue and lyse bovine erythrocytes to use hemoglobin as a source of iron. Furthermore, Leukotoxin LukMF' binds to neutrophils found in the bovine milk, causing them to release pro-inflammatory cytokines that can damage the mammary gland tissue (Campos et al., 2022).

### **Staphylococcal enterotoxins**

SEs have been shown to play an essential role in the pathogenesis of bovine mastitis. Most *S. aureus* strains isolated from this disease harbor one or more enterotoxin genes (Bonsaglia et al., 2018; Rall et al., 2014; Ren et al., 2020). SEs are particularly concerning as they are the primary cause of staphylococcal food poisoning. Improper food handling and storage conditions can lead to bacterial growth and toxin production, posing a significant risk to public health (Denayer et al., 2017). Foods containing sufficient amounts of preformed SEs can cause human intoxication, characterized by

symptoms such as nausea, vomiting, and diarrhea. The amount of toxins consumed and the individual's overall health determine the level of illness severity. Just a few micrograms of SEs can cause food poisoning in vulnerable adults, while children can experience the same effects with just 100 ng. Contaminated food can cause symptoms within 2-6 hours, with recovery typically within 24-48 hours (G. Abril et al., 2020; Grispoli et al., 2021; Wu et al., 2016).

*S. aureus* produces a wide range of enterotoxins with varying structure and biological activity (Fisher et al., 2018). Detection for food safety is limited due to the diversity of Staphylococcal enterotoxin variants (Hu et al., 2021). However, a reliable biological technique for detecting these enterotoxins is through DNA hybridization and PCR methods utilizing gene-specific nucleotide sequences as probes (Wu et al., 2016). Currently, 24 different SEs have been discovered and grouped into six serological groups (A, B, C, D, E, and H) encoded on different pathogenicity islands. These include SEs and SEI (SE-like toxins): SEA, SEB, SEC1-3, SED, SEE, SEG, SEH, SEI, SEIJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SES, SET, SEIU, SEIW, SEIV, SEIX, and SEIY. Among these, SEA and SEB are the most commonly produced enterotoxins by *S. aureus* (Fisher et al., 2018; Grispoli et al., 2021), and infectious bovine strains have been associated with SEB and SEE (Jung & Lee, 2022). Moreover, the most frequently involved enterotoxins in food poisoning outbreaks are SEA and SED (Fisher et al., 2018).

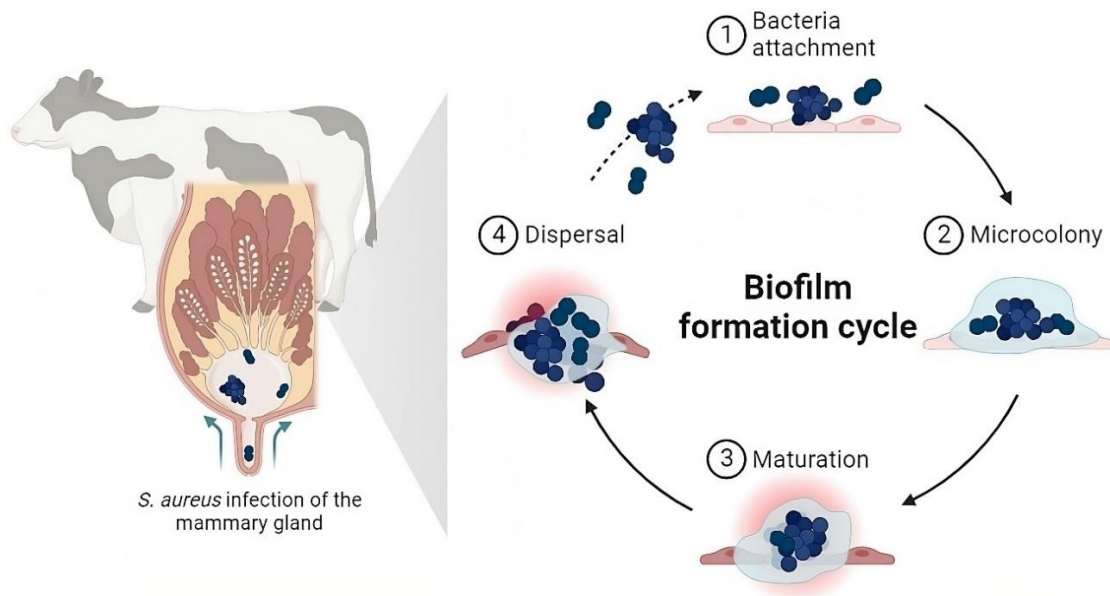
These enterotoxins are approximately 220–240 amino acids long and have similar molecular weights ranging from 25–30 kDa. They are highly stable proteins, resistant to various environmental conditions such as heat and low pH (Denayer et al., 2017). Only those enterotoxins that induce vomiting in monkeys are designated as SE. In contrast, those that do not induce vomiting or have not been tested in non-human primate models of emesis are classified as SEI antigens (Fisher et al., 2018). Enterotoxin serotype A

(SEA), SEB, SEC, and toxic shock syndrome toxin (TSST)-1 are highly effective at disrupting the immune response against the pathogen. By activating T cells and antigen-presenting cells (APCs) in an antigen-independent manner, they can evade the body's natural defenses, allowing the pathogen to thrive and spread (Hu et al., 2021).

### **Biofilm formation**

*S. aureus* originating from bovine sources is commonly characterized as a producer of biofilms (Campos et al., 2022; Felipe et al., 2017; V. Silva et al., 2021). A biofilm is a microbial community of cells embedded in a polymer extracellular matrix, which in *S. aureus* consists mainly of the PIA. Besides PIA, the biofilm also contains several MSCRAMMs proteins (Campos et al., 2022; Otto, 2018). Biofilms exhibit phenotypic and genetic traits distinct from planktonic cells, making them more resistant to antimicrobial treatments (V. Silva et al., 2022).

Biofilm formation is a multi-step process involving attachment, microcolony formation, maturation, and dispersal (Fig. 1). The molecular basis of biofilm formation in Staphylococci is a multifaceted subject involving specific genetic factors, complex regulatory networks, and interactions with the host. The interaction with host matrix proteins and immune defenses adds another layer of complexity to the biofilm formation. For instance, biofilms can evade the host immune system by forming a physical barrier, making it difficult for immune cells to penetrate and clear the infection (Otto, 2018; Scherr et al., 2014).



**Figure 1.** Biofilm formation cycle of *S. aureus*. Created with BioRender.com.

Biofilm development of *S. aureus* is a complex, multi-step process that involves various molecular and cellular mechanisms (Otto, 2018). The first stage is attachment to a surface. MSCRAMMs proteins facilitate the binding of staphylococcal cells to surfaces (François et al., 2023) and interact with host matrix materials like fibronectin, fibrinogen, and vitronectin (Otto, 2018).

Once attached, the bacterial cells proliferate and form microcolonies. These small clusters of cells serve as the foundation of the biofilm. During this stage, the cells also produce an extracellular matrix of polysaccharides, proteins, and DNA. The matrix provides structural integrity to the biofilm and protects the bacterial cells from environmental stressors. It also helps with nutrient acquisition and protection against antimicrobial agents.

As the biofilm grows, it becomes thicker and more complex. During this maturation phase, the biofilm transforms into a three-dimensional structure comprising multiple bacterial clusters. The bacterial cells within the biofilm also change their metabolism and gene expression. Proteins like the accumulation-associated protein,



clumping factor A (ClfA), staphylococcal surface protein (SSP1), and biofilm-associated protein (Bap) play roles in this phase (François et al., 2023). These changes are thought to contribute to the increased antibiotic resistance observed in biofilm-associated infections (Otto, 2018).

The final stage of biofilm development is the dispersing of bacterial cells or clusters from the mature biofilm. This detachment can occur through various mechanisms, including enzymatic degradation of the matrix, shear forces, or the production of surfactants. Once detached, the bacteria can spread to other sites, potentially leading to the spread of infection (Otto, 2018).

### **Essential Oils**

The rising prevalence of antibiotic-resistant strains of *S. aureus* has diminished the effectiveness of traditional antibiotic treatments, particularly in the case of bovine mastitis. This has led to an intensified focus on alternative therapies, such as essential oils (EOs), which have shown promising antimicrobial properties (Ni et al., 2021; Tomanić et al., 2023). As a result, given their demonstrated efficacy against a broad spectrum of microorganisms and their safety and environmental friendliness, the scientific community is increasingly interested in exploring natural substances like EOs as viable options for combating various illnesses since these hold promise as potential substitutes for synthetic pharmaceuticals (Tariq et al., 2019).

EOs are volatile organic compounds that naturally originate from diverse parts of plants, such as leaves, flowers, stems, buds, roots, seeds, twigs, and even wood, commonly extracted from plant materials through physical methods like steam distillation, which is frequently used for commercial production (Tariq et al., 2019). These compounds are a complex mixture of volatile molecules, specific for each plant

with a wide range of bioactivities, that comprises alkaloids, monoterpenes, carotenoids, flavonoids, isoflavones, phenolic acids, and oxygen-containing, non-oxygenated terpene hydrocarbons, and aldehydes (Ni et al., 2021; Tavares et al., 2022). There are approximately 3,000 EOs known to date, of which 300 are commercially important, particularly in the food, medicine, textile, cosmetics, and agricultural industries (Tariq et al., 2019). Furthermore, these compounds have a long history of medicinal and therapeutic applications attributed to their pharmacological and psychological benefits (Ni et al., 2021). Recent studies have investigated the impact of EOs on pathogenic and spoilage microbial strains in food. The Food and Drug Administration (FDA) has also certified these compounds as non-toxic and safe for food preparation, which has led to increased consumer interest in EO-based herbal products (Tariq et al., 2019).

EOs can improve milk production and quality in the long term as a treatment for bovine mastitis. Some studies have shown that EOs can effectively combat *S. aureus*-causing bovine mastitis (Cheng & Han, 2020; Lopes et al., 2020). However, there is currently limited research on the topic (Caneschi et al., 2023), underscoring the need for additional investigations to establish the safety and effectiveness of EOs when administered to dairy cows. In light of this, studies on the antimicrobial activity of new molecules, such as EOs, are becoming increasingly important, particularly in developing new treatments against mastitis (Caneschi et al., 2023; Fratini et al., 2014; Lopes et al., 2020; Tomanić et al., 2023). In addition to their antimicrobial activity, EOs have also shown antiviral properties against a range of pathogenic viruses, including herpes simplex types 1 and 2 (HSV-1 and HSV-2), dengue virus type 2, influenza, poliovirus, Junin virus, and coxsackievirus B1 (Tariq et al., 2019).

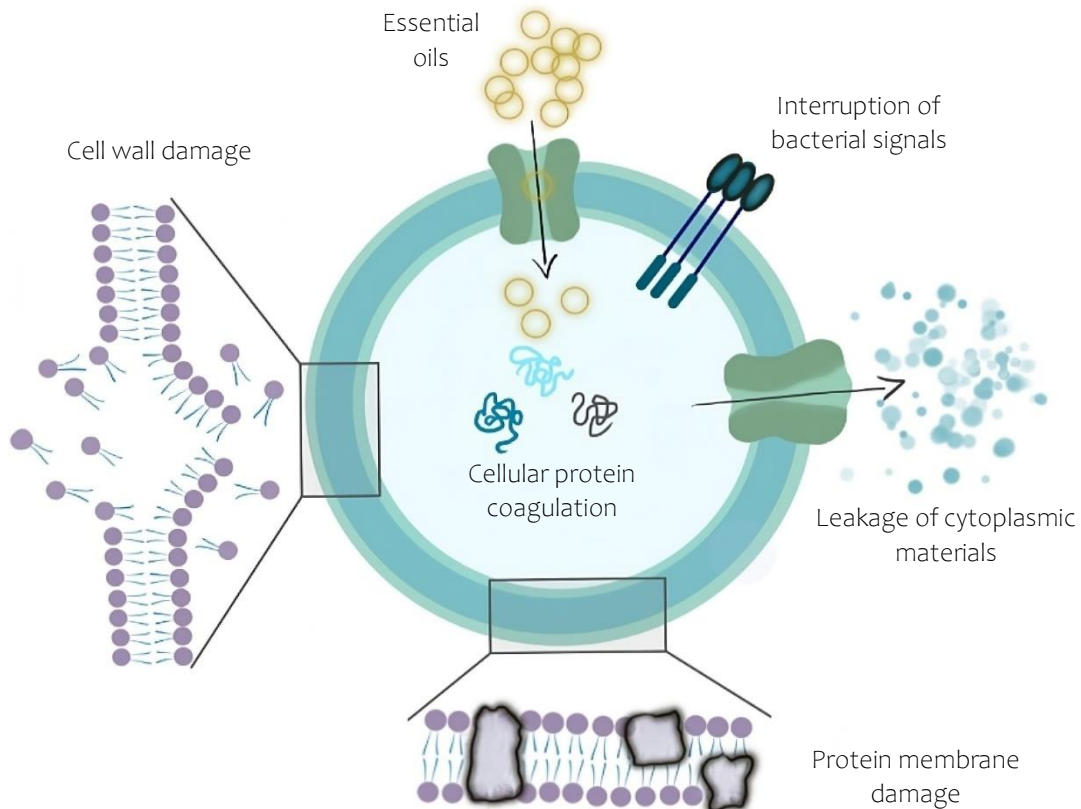
### **Antimicrobial effects of EOs**

The rising prevalence of antibiotic-resistant strains of *S. aureus* has diminished the effectiveness of traditional antibiotic treatments, particularly in the case of bovine mastitis. This has led to an intensified focus on alternative therapies, such as EOs, which have shown promising antimicrobial properties (Ni et al., 2021; Tomanić et al., 2023). As a result, the scientific community is increasingly interested in exploring natural substances like EOs as viable options for combating various illnesses.

The varying complexities of cell walls between Gram-positive and Gram-negative bacteria substantially influence their reactivity to different antimicrobial treatments. It is manifested through diverse outcomes in tests for inhibition zones, MICs, MBCs, and the ability to form biofilms (Patterson et al., 2019; Tariq et al., 2019). Gram-negative bacteria possess a complex cell wall with a lipopolysaccharide layer that causes the rejection of EOs, hindering their absorption and, consequently, their antimicrobial activity (Álvarez-Martínez et al., 2021; Ni et al., 2021). On the other hand, Gram-positive bacteria have a simpler, peptidoglycan-based cell wall that is less effective at blocking the entry of such compounds. Therefore, EOs are generally more effective against Gram-positive than Gram-negative bacteria (Caneschi et al., 2023; Patterson et al., 2019).

The effectiveness of EOs and their compounds against bacteria can vary depending on the microorganism. Multiple modes of action are involved in their antibacterial effects, making it crucial to understand their mechanisms. Detailed studies of EO components could help us better understand how they kill bacteria (Ni et al., 2021; Tariq et al., 2019). The lipophilic properties of EOs and their constituent elements facilitate their penetration through bacterial cell membranes and mitochondrial membranes. This destabilizes the cellular architecture, which compromises the membrane's integrity and makes it more permeable. Consequently, this disruption of the bacterial cell wall leads to the loss of many cellular functions, including but not limited

to membrane transport mechanisms, energy production, and the regulation of other metabolic activities (Sakkas & Papadopoulou, 2017; Tariq et al., 2019). Moreover, EOs exhibit remarkable antibacterial properties, which could be attributed to the synergistic effects between their main components (Ni et al., 2021). Fig. 2 illustrates the various mechanisms through which EOs may exhibit antibacterial properties.



**Figure 2.** Antibacterial mechanism of EOs action.

EOs can inhibit bacterial growth (bacteriostatic) or kill bacteria (bactericidal). Two main criteria are used to measure these effects: the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). MIC is the lowest concentration of an EO that inhibits the visible growth of a microorganism after incubation. MBC is the lowest concentration of an EO that kills 99.9% of the final inoculum after incubation for 24 hours. MIC and MBC can be determined using dilution

methods, either in broth or agar, allowing for accurate antimicrobial concentration calculations (Balouiri et al., 2016).

Identifying the main constituents of the EOs by gas chromatography coupled to a mass selective detector (GC/MSD) provides valuable information. Gaining insights into their chemical compositions can aid in optimizing their use and determining which specific compounds are primarily responsible for the antimicrobial effects of these EOs (Bagheri et al., 2020). Several studies have shown that the chemical constituents of EOs are abundant in aromatic compounds, which can be classified into two main structural groups: terpenoids, which include monoterpenes, sesquiterpenes, and diterpenes, and phenylpropanoids (Caneschi et al., 2023; Ni et al., 2021).

In the context of seeking natural antimicrobial solutions, a study by Ben Jemaa et al. (2017) that explored the efficacy of thyme EO (*Thymus capitatus*) and its nanoemulsion in preserving the quality of milk contaminated with Gram-positive bacteria, including *S. aureus*, *B. licheniformis*, and *E. hirae*. The study found both the EO and its nanoemulsion to be effective in inhibiting bacterial growth. Similarly, a more recent study by Milagres de Almeida et al. (2023) corroborated the antibacterial efficacy of *Thymus vulgaris* EO against *S. aureus*. These highlight the promise of some EOs as natural solutions for combating bacteria.

### **Synergism**

A single compound does not usually cause the biological efficacy of EO. Instead, they result from the combined effects of various chemical components. This phenomenon is known as synergy (Tariq et al., 2019). Notably, although the antimicrobial properties of EOs can vary based on their main components, they are more effective when these elements work together, exhibiting enhanced activity when combined synergistically

(Bagheri et al., 2020; Chouhan et al., 2017), thereby contributing to combating microorganisms that may cause illnesses (Álvarez-Martínez et al., 2021; Caneschi et al., 2023). Studies have shown that certain blends of EOs, carefully chosen based on their profiles, have been demonstrated to enhance their antimicrobial efficacy and, in some instances, create a more potent antimicrobial than if they were used separately (Caneschi et al., 2023; Milagres de Almeida et al., 2023). However, complex interactions of chemical constituents in each EO can affect synergistic activity, and the blend proportions may play a significant role (Burt, 2004). According to Vieira-Brock, Vaughan, and Vollmer (2017), blending orange, patchouli, peppermint, and clary sage EOs was found to be as effective as individual EOs for inhibiting *S. aureus*, indicating no additive effect.

When using antimicrobial compounds together, there are three possible outcomes: synergy (more significant effect than individual compounds), additive effect (equal effect to individual compounds), or antagonistic effect (decreased effect). Thus, it is essential to consider these possibilities when combining antimicrobials (Chouhan et al., 2017).

### **Bioactive properties of chemical compounds present in EOs**

EOs are typically complex mixtures of hundreds of individual aroma compounds. Most of the EOs that have been studied for their antimicrobial activity are derived from herbs and spices commonly used in foods. The chemical composition and biological properties of plant-derived EOs have been the subject of comprehensive research in previous review literature (Calo et al., 2015; Chouhan et al., 2017; Masyita et al., 2022; Ni et al., 2021; Tariq et al., 2019). However, there is a growing focus on their specific effectiveness against *S. aureus*, particularly in bovine mastitis. Previous research indicates that certain chemical components of EOs may offer hopeful solutions for treating *S. aureus*-related bovine mastitis (Bagheri et al., 2020; Caneschi et al., 2023;

Fratini et al., 2017; Lopes et al., 2020). Chemical compounds of selected EOs with bioactive properties supported by previous research are summarized in Table 1.

**Table 1.** Main constituents of EOs with bioactive properties.

EO	Scientific name	Family	Bioactive compounds	Reference
Clove	<i>Eugenia caryophyllus</i>	Myrtaceae	Eugenol, <i>trans</i> -caryophyllene, $\alpha$ -humulene, $\delta$ -cadinene	(Milagres de Almeida et al., 2023)
Bergamot	<i>Citrus bergamia</i>	Rutaceae	Linalool acetate, Limonene, Linalool, $\gamma$ -terpinene	(Pedonese et al., 2017)
Spearmint	<i>Mentha spicata</i>	Lamiaceae	Carvone, Limonene, $\beta$ -bourbonene	(Shahbazi, 2015)
Basil	<i>Ocimum basilicum</i>	Lamiaceae	Estragole, Linalool, Eugenol, Eucalyptol	(da Silva et al., 2021)
Arrayan	<i>Luma chequen</i>	Myrtaceae	$\alpha$ -pinene, 1,8-Cineole, Linalool, Limonene	(M. J. Gonçalves et al., 2006)
Black pepper	<i>Piper nigrum</i>	Piperaceae	<i>trans</i> -caryophyllene, Limonene, Sabinene, $\alpha$ -pinene	(Milagres de Almeida et al., 2023)
Fennel	<i>Foeniculum vulgare</i>	Apiaceae	<i>trans</i> -anethol, Fenchone, Methyl-chavicol	(Roby et al., 2013)
Ginger	<i>Zingiber officinale</i>	Zingiberaceae	Curcumene, Zingiberene, $\alpha$ -pinene	(Shukla et al., 2019)
Copaiba	<i>Copaifera</i> spp.	Fabaceae	$\beta$ -caryophyllene, $\alpha$ -copaene, $\alpha$ -humulene	(de Faria et al., 2017)
Eucalyptus	<i>Eucalyptus</i> spp.	Myrtaceae	1,8-cineole, Cryptone, $\alpha$ -pinene	(Elaissi et al., 2012)
Oregano	<i>Origanum vulgare</i>	Lamiaceae	Thymol, Carvacrol, $\alpha$ -terpinene, Limonene	(Aguirre et al., 2013)
Tea tree	<i>Melaleuca alternifolia</i>	Myrtaceae	Terpinen-4-ol, $\gamma$ -Terpinene (21.1%), $\alpha$ -Terpinene	(Corona-Gómez et al., 2022)



Monoterpenes are a class of terpenes widely distributed in nature and commonly found in EOs. Their diversity in structure and function suggests a broad spectrum of applications, particularly against pathogens like *S. aureus*. Terpenes and terpinenes have been associated with antimicrobial and antioxidant activities and represent the most active phytochemicals studied, with properties for treating or preventing diseases, followed by polyphenols (such as phenolic acids and flavonoids) (Álvarez-Martínez et al., 2021; Falleh et al., 2020; Masyita et al., 2022). These compounds, constituting up to 90% of most EO compositions, exhibit diverse chemical and biological properties (Sakkas & Papadopoulou, 2017). Several EOs, including black pepper, ginger, and tea tree, are rich in these compounds (Corona-Gómez et al., 2022; Milagres de Almeida et al., 2023; Shukla et al., 2019).

The mechanisms through which terpenes act can vary. Research has confirmed that limonene can effectively inhibit the growth of pathogenic bacteria such as *S. aureus* and *L. monocytogenes* (Han et al., 2019). This antimicrobial activity is likely attributed to compromised cell membranes, enhanced membrane permeability, and reduced metabolic function in the respiratory system, along with the disruption of cell morphology and bacterial cell wall integrity (Han et al., 2021). Moreover, this prevalent monoterpene has been identified in EOs from bergamot, spearmint, arrayan, and oregano (M. J. Gonçalves et al., 2006; Pedonese et al., 2017; Shahbazi, 2015).

Terpinen-4-ol, a primary monoterpene found in *Melaleuca alternifolia* EO, has been the subject of comprehensive studies focusing on its antimicrobial attributes, especially its efficacy against *S. aureus* (Corona-Gómez et al., 2022). Additional studies have reinforced its potent bactericidal properties against *S. aureus*, likely by impeding the bacterial cell wall synthesis. Moreover, it has displayed significant antibiofilm activity, inhibiting biofilm formation even at sub-inhibitory concentrations (Cordeiro et al., 2020).

Linalool is another monoterpene found in diverse EOs, such as bergamot and basil (W. M. F. da Silva et al., 2021; Pedonese et al., 2017). Recent studies have shown that linalool has potent antibacterial activity against a range of microorganisms, including *Pseudomonas fluorescens*, *Shigella sonnei*, and resistant strains of *Klebsiella pneumoniae* (F. Guo et al., 2021; Su et al., 2022; Yang et al., 2021). The mechanism of action of linalool against *P. fluorescens* involves membrane damage, bacterial metabolic and oxidative respiratory perturbations, interfering with cellular functions, and even causing cell death (F. Guo et al., 2021). Linalool has also been found to have synergistic effects with EOs. The addition of linalool to certain of these compounds may increase their antimicrobial potential against gram-negative and gram-positive bacteria, such as *S. aureus* (Aelenei et al., 2019; Herman et al., 2016).

Carvone is a dominant monoterpene in spearmint EO (Shahbazi, 2015) and demonstrates notable biological activities both in vitro and in vivo. It possesses potent antimicrobial properties against a variety of microorganisms, including MRSA. Moreover, it has shown efficacy in inhibiting biofilm formation by *S. aureus*. Its mechanism of action involves penetrating the bacterial cell membrane, thereby increasing cell permeability. In addition, this EO displays a range of other pharmacological effects, including antifungal, antiparasitic, anti-neuraminidase, antioxidant, anti-inflammatory, and anticancer activities (Bouyahya et al., 2021).

The sesquiterpene, *trans*-caryophyllene, is a bioactive compound found in various EOs, including those derived from clove, thyme, oregano, and black pepper (Milagres de Almeida et al., 2023). Its presence in multiple EOs and its unique properties make it worthy of discussion, particularly in antimicrobial activities against gram-positive and gram-negative bacteria (Ghavam et al., 2020; Sengun et al., 2021). The combination of *trans*-caryophyllene and linalool was found to be the most effective in inhibiting biofilm

formation in *S. aureus*. This combination decreased the expression levels of genes associated with initial cell adhesion and quorum sensing. Notably, *trans*-caryophyllene has been approved by the FDA and the European Food Safety Authority (EFSA) for use in cosmetics and as a flavor enhancer. Its low toxicity makes it an excellent candidate for further applications (Salinas et al., 2022).

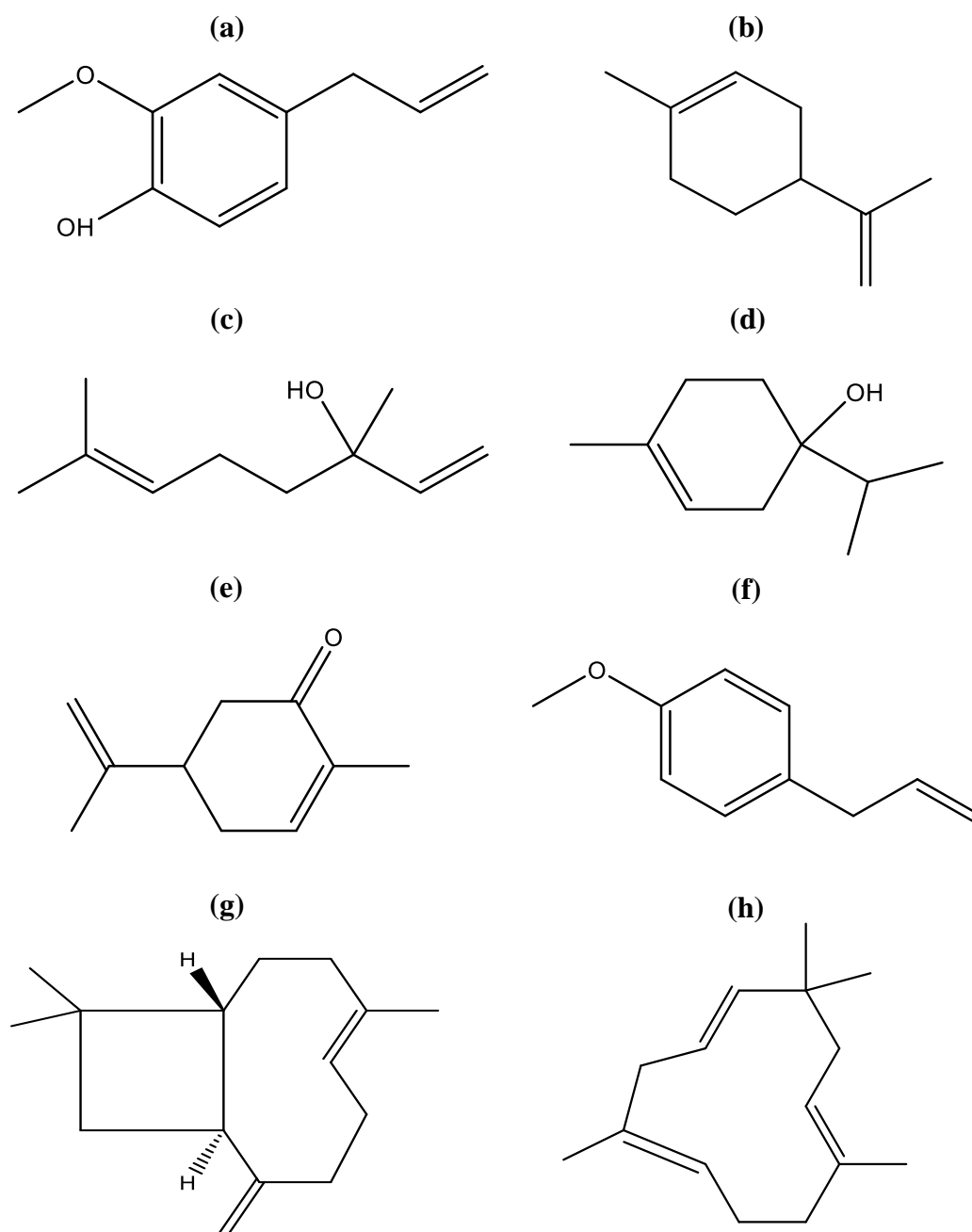
Both clove and basil EOs are abundant in eugenol, a phenolic compound known for its antimicrobial activity against *S. aureus* and other gram-positive bacteria (da Silva et al., 2021; Milagres de Almeida et al., 2023). The highly reactive hydroxyl groups of eugenol can form hydrogen bonds with a particular bacterial enzyme, interfering with its function and leading to cell membrane damage or failure (Guimarães et al., 2019). Previous research reported that eugenol exhibits antibacterial activity against MSSA and MRSA strains. Additionally, at sub-inhibitory concentrations, eugenol can inhibit biofilm formation. It does this by effectively eradicating pre-established biofilms, decreasing the expression of biofilms and enterotoxin-related genes, such as *icaD*, *sea*, and *sarA*, damaging the cell membrane, and causing the leakage of cell contents (Yadav et al., 2015). Interestingly, clove also contains  $\delta$ -cadinene, which is relatively rare and may offer additional therapeutic benefits. However, this sesquiterpene is relatively less studied. This metabolite is very abundant in some EOs, such as the one obtained from *Kadsura longipedunculata* (21.8%), and has presented some degree of cytotoxic activity against some human cell lines (Mulyaningsih et al., 2010).

Estragole, also known as methyl chavicol, is an organic compound that naturally occurs in various plants, including basil, tarragon, and fennel. Over the years, estragole has garnered attention for its potential therapeutic benefits, including its antimicrobial properties. A 2022 study by Galgano et al. revealed that the *Ocimum basilicum* EO, which contains about 73% estragole, exhibited bactericidal effects against *S. aureus* strains.

Other studies have also corroborated the antimicrobial potential of EOs containing estragole against this pathogen., demonstrating the importance of phenylpropene compounds for this activity (Bezerra et al., 2021; Mota et al., 2015).

$\alpha$ -humulene, found in EOs like sage, ginseng, and hops, exhibits a variety of biological activities, including antimicrobial properties. This compound has been identified as the primary compound in *Cordia verbenacea* EO and is known for its anti-inflammatory effects. Studies have shown its effectiveness against bacterial and fungal pathogens such as *S. aureus* and *Candida albicans* (Leite et al., 2021). Additional research on *Cannabis sativa* EO, which contains  $\alpha$ -humulene, also demonstrated moderate antibacterial and antibiofilm activities (Zengin et al., 2018).

The chemical compounds mentioned above underscore the multifaceted bioactive potential of EOs. As discussed, these compounds present a wide range of biological activities, including antimicrobial, anti-inflammatory, antioxidant, and even cancer-fighting properties. Figure 2 provides a comprehensive overview of the chemical structures of some representative bioactive compounds commonly found in diverse EOs.



**Figure 2.** Chemical structures of some representative bioactive compounds found in EOs. (a) Eugenol, (b) Limonene, (c) Linalool, (d) Terpinen-4-ol, (e) Carvone, (f) Estragole, (g) *trans*-caryophyllene, (h)  $\alpha$ -humulene. Chemical structures were drawn using ChemDraw 18.0 software.

### **Cytotoxicity of EOs**

Chemical compounds can have various cytotoxic mechanisms, including inhibition of key enzymes, cell cycle arrest, destruction of cell membranes, and prevention of protein synthesis. Cytotoxicity testing of EOs is of important consideration when using them as an alternative to antibiotics to treat *S. aureus* infections since some EOs are more cytotoxic than others, and it is necessary to ensure that the benefits outweigh the risks (Aslantürk, 2018; García-Salinas et al., 2018; Ribeiro et al., 2020). Cytotoxicity varies depending on the EO, concentration, and cell type being exposed. By testing the cytotoxicity of EOs in vitro, researchers can identify EOs that are safe to use and determine the optimal concentration and exposure time for therapeutic use (Surowiak et al., 2022). Available cytotoxicity assays evaluate alterations in lysosomal and mitochondrial functions, plasma membrane permeability, and ATP production. In addition, in vitro cellular assessment offers cost-effective and rapid evaluation approaches without needing animal models (Aslantürk, 2018).

Some methods can be used to test the cytotoxicity, including in vitro tests, such as the MTT assay (Brożyna et al., 2021). This method measures the metabolic activity of cells, where cells that are alive reduce the yellow tetrazolium salt to a purple formazan product. The amount of formazan produced is proportional to the number of living cells. The more MTT is reduced, the more metabolically active the cells are. One way to perform this test is by using Vero cells, a type of monkey kidney cell commonly utilized in cell culture studies. They are relatively easy to grow and maintain and sensitive to various toxins (Vijayarathna & Sasidharan, 2012). An irregular number of chromosomes characterizes this cell lineage. It has less or more than the typical 23 pairs of chromosomes. This characteristic allows the cells to be replicated through numerous division cycles without any function deterioration. Unlike typical mammalian cells, Vero

cells cannot produce interferon when exposed to viral infections. Nevertheless, these cells possess the interferon-alpha/beta receptor and can respond as expected when recombinant interferon is introduced into the culture medium (Modimola et al., 2022).

Previous research has explored the cytotoxicity activity of EOs by testing them on diverse cell lines (García-Salinas et al., 2018; Niksic et al., 2021; Pauzer et al., 2021). In addition, EOs have displayed promising results in other research fields. Particularly notable is their cytotoxic activity against cancer cells. Studies have shown that some EOs can selectively target cancer cells, promoting apoptosis or inhibiting proliferation while displaying reduced toxicity toward healthy cells. These findings have been observed in both in vitro and in vivo studies. However, it is essential to note that the dosage and formulation of EOs are critical factors that can impact their cytotoxic effects (Mohamed Abdoul-Latif et al., 2023).

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## CHAPTER 3

*Virulence and antimicrobial resistance genes profiles of spa type t605 methicillin-susceptible Staphylococcus aureus isolated from subclinical bovine mastitis*

**Virulence and antimicrobial resistance genes profiles of *spa* type t605 methicillin-susceptible *Staphylococcus aureus* isolated from subclinical bovine mastitis**

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Running Head: Virulence profile of MSSA

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## Abstract

**Aims:** *Staphylococcus aureus* is one of the most common pathogens associated with mastitis in dairy herds worldwide. This study evaluated the profile of virulence and antimicrobial resistance genes of *spa* type t605 methicillin-susceptible *Staphylococcus aureus* isolated from subclinical bovine mastitis in São Paulo, Brazil.

**Methods and results:** Fifty-seven *S. aureus* strains were screened by conventional PCR for 49 virulence genes. The most prevalent virulence genes detected were *icaD* (94.7%), *fib* (93%), *fnbA* (82.5%), *clfA* (80.7%), *bap* (78.9%), *clfB* (73.7%), *icaA* (66.7%), *see* (64.9%), and *sed* (61.4%). The *blaZ* (94.7%), *aac6'aph2'* (15.8%), and *ant4* (12.3%) genes were the most common antimicrobial resistance genes; however, *mecA* and *mecC* genes were not found. All methicillin-susceptible *S. aureus* (MSSA) were characterised through *spa* and *agr* typing. The *spa* type t605 was found in all isolates. By *agr* typing, the most prevalent were type II (56.1%). Antimicrobial resistance was determined by the disk diffusion method, and 93% showed resistance to at least one antibiotic. Penicillin resistance was the most prevalent (87.7%), followed by tetracycline (12.3%), oxacillin (10.5%), and gentamicin (10.5%) resistance.

**Conclusion:** Our study confirmed the *spa* type t605 as endemic, carrying a wide variety of virulence factors and high-level penicillin resistance. The profile seems to be associated with the colonisation of MSSA and its persistence in subclinical mastitis.

**Significance and Impact of the Study:** The MSSA characteristics found here evidence its potential risk to human and bovine health and emphasise the need for continuous surveillance to control its presence. These findings improve the comprehension of its pathogenesis, genomic variations, and antibiotic resistance, which can help to enhance therapeutic recommendations and optimise bovine mastitis treatment.

**Keywords:** MSSA; MSCRAMMs; Antibiotic resistance; *spa* typing; Mastitis

## Introduction

Subclinical mastitis (SM) represents the most prevalent type of bovine mastitis, and *S. aureus* is one of the main etiologic agents causing contagious intramammary infections (Ruegg, 2017; Côté-Gravel & Malouin, 2019; Dyson *et al.*, 2022). The risk of *S. aureus* infection stems from unhygienic practices during milking; it includes contaminated equipment, unsanitary environments, and the lack of trained personnel in dairy operations (Zaatout *et al.*, 2019). Cows with SM caused by *S. aureus* present high SCC ( $>200 \times 10^3$  cells mL<sup>-1</sup>), but no visual changes in milk are observed (Gonçalves *et al.*, 2018); however, it can cause human intoxication since bacteria can be eliminated through a thermal process, but enterotoxins may remain, increasing the possibility of foodborne poisoning (Grispoldi *et al.*, 2021; Campos *et al.*, 2022). In this regard, *S. aureus* has been associated with 9.4% and 7.8% of food poisoning cases concerning milk and dairy products, respectively, with 120,584 cases reported by the Brazilian Ministry of Health between 2009 and 2018 (BRASIL, 2019).

Molecular studies are important to evaluate the genetic diversity of pathogens and contribute to understanding sources, routes of transmission, prognosis, and adaptation mechanisms (McMillan *et al.*, 2016). *S. aureus* represents a significant issue for bovine udder health. Its adaptation to the tissue has been associated with persistence and specific genetic and phenotypic traits (Schmidt, Kock & Ehlers, 2017; Santos *et al.*, 2020). In addition, this pathogen has various virulence factors and survival strategies to persist and defeat host defences, such as adhesion capacity and biofilm formation (Bhunja, 2008; Zhu *et al.*, 2018). The expression of these virulence factors is rigorously controlled by a series of regulatory genes influenced by the accessory gene regulator (*agr*). Their presence or absence plays an important role in the persistence of the pathogen causing mastitis (Rossi *et al.*, 2021). Overall, mastitis can be reduced in the dairy industry by



identifying the pathogenesis and virulence factors present in the pathogens (Khan *et al.*, 2013).

Among the virulence factors, various studies have revealed that staphylococcal enterotoxins (SEs) play an important role in the pathogenesis of bovine mastitis since most *S. aureus* strains isolated from this disease harbour one or more enterotoxin genes (Rall *et al.*, 2014; Bonsaglia *et al.*, 2018; Ren *et al.*, 2020). Additionally, it is important to highlight that these toxins are considered a major cause of food poisoning since foods containing sufficient amounts of preformed SEs can cause human intoxication. (Grispoldi *et al.*, 2021).

The onset of the intramammary infection caused by *S. aureus* is influenced by diverse virulence factors, such as biofilm formation and MSCRAMMs (microbial surface components recognising adhesive matrix molecules) genes (Zuniga *et al.*, 2015). Of these genes, *bap* (encoding biofilm-associated protein), *ica* (encoding intercellular adhesion gene cluster), *clfA* and *clfB* (encoding clumping factors A, B), *fnbA* and *fnbB* (encoding fibronectin-binding protein A, B), *ebps* (encoding elastin binding protein), *eno* (encoding laminin-binding protein), *cna* (encoding collagen adhesin), *fib* (encoding fibrinogen-binding protein) and *bbp* (encoding bone sialoprotein-binding protein) play an important role in the initial attachment, recruitment and colonisation of mammary glands; consequently, it is associated with the persistence of bovine mastitis infection (Zuniga *et al.*, 2015; Aslantaş & Demir, 2016; Zaatout, Ayachi & Kecha, 2020).

The large quantity of antimicrobials used in dairy cattle is a potential source for the emergence and dissemination of resistant bacteria, which is the main reason for the restrictions on using critically important antimicrobials (CIAs) in veterinary medicine (Zaatout *et al.*, 2020). They are called CIAs because they can treat potentially serious bacterial infections in humans. Therefore, the use of "highest priority" CIAs in veterinary

medicine (e.g., a broad-spectrum  $\beta$ -lactam antibiotic, 3<sup>rd</sup> generation cephalosporin as ceftiofur) has already been restricted by legislation in their use (Koops, Ekström & Armstrong, 2018). Penicillins and other  $\beta$ -lactam antibiotics (e.g., intramammary ceftiofur that represented 52.8% of first cases of treated clinical mastitis) remain the drugs of choice most frequently used for treating mastitis (Gonçalves *et al.*, 2022). Although methicillin is not used to treat cattle, methicillin-resistant *S. aureus* (MRSA) is regarded as a superbug that poses a concern to human health, and the emergence of methicillin resistance is a marker for the emergence of universal resistance to other  $\beta$ -lactam medicines (e.g., as ceftiofur also used to treat human infections). Unfortunately, acquired resistance remains a notable concern; in this regard evaluating the antimicrobial resistance profiles becomes essential to guide management decisions and implement prudent and effective antimicrobial usage in dairy cattle (Ruegg, 2017; Zaatout *et al.*, 2020). The pathogen adapts to different stress conditions due to its genotypic and phenotypic plasticity, which explains the variations in its susceptibility to antimicrobial compounds (Erickson, Otoupal & Chatterjee, 2017). Genes that confer less susceptibility to phenolic and quaternary ammonium compounds (such as benzalkonium chloride and cetrимide) are called *qacs*. Quaternary ammonium compound (QAC) sanitisers can break the microbial cell membrane and are considered efficient antimicrobial agents (Wassenaar *et al.*, 2015; Zhang *et al.*, 2018). MSSA constantly evolves through horizontal gene transfer and mutations. When the *mecA* or *mecC* gene carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) is acquired by MSSA, new MRSA lineages may emerge, and these could establish infections, affecting the health of animals, including cattle (Silva *et al.*, 2013; Bal *et al.*, 2016; Moore-Lotridge *et al.*, 2022). Furthermore, assessing the risk of MSSA exposure for those involved in milking processes and for

consumers of raw dairy products is crucial due to the potential exchange between human and bovine bacterial reservoirs (Sheet *et al.*, 2019).

Analyses concerning the evolution and genomic variation of *S. aureus* from SM could provide information to formulate surveillance measures that decrease or eliminate this pathogen and reduce the potential risks to public health. From this perspective, the present study aimed to screen the virulence gene profiles and determine the antibiotic resistance of *spa* type t605 MSSA strains isolated from cows with chronic SM in the State of São Paulo – Brazil.

## **Material and methods**

### ***Bacterial strains***

A total of 57 viable *S. aureus* were recovered from a bacterium library in the Milk Quality Research Laboratory of the University of São Paulo that had been cryopreserved at -80°C from two companion studies of subclinical mastitis cases (Gonçalves *et al.*, 2018, 2020) without taking into account the study design or exclusion criteria prementioned in both studies (e.g., contralateral quarters).

Briefly, six commercial dairy herds in the Midwest area of São Paulo State, Brazil, were enrolled in the companion studies during a nine-month sampling period (Feb – Oct 2014) (Gonçalves *et al.*, 2018, 2020). Cow selection (stage 1, three samplings; timeframe 6 weeks) was designed to identify cows with evidence of chronic SM and to disregard cows with previous episodes of clinical mastitis. All lactating Holstein cows (n = 647) with four functional quarters and milk yield  $\geq 10$  kg/day per cow were selected. Dairy cows with 2 out of 3 biweekly consecutive SCC testing  $> 200,000$  cells mL<sup>-1</sup> and culture-positive results were selected for quarter milk sampling. Mammary quarter milk samples (stage 2, three samplings; timeframe, 6 weeks) were collected during the three subsequent consecutive sampling occasions from all selected cows (n = 117) at intervals of 15 days.

Twenty-two cows were excluded from the study because they lacked the third sampling, had clinical mastitis (n = 11), were dried-off (n = 7), or were culled from the herd (n = 4). A total of 57 *S. aureus* were isolated from three biweekly aseptic mammary quarter milk samples (n = 1,140 from 95 cows) using bacteriological culture analyses (Oliver *et al.*, 2004). Of 57 isolates, 26 *S. aureus* were isolated in herd 1, 20 were isolated in herd 2, 2 were isolated in herd 3, and 9 were isolated in herd 4. In two herds, no *S. aureus* was detected. Milk samples with more than one pathogen detected were not included. Quarters were considered infected with *S. aureus* when at least one colony of *S. aureus* was isolated from 10  $\mu$ L of milk plated ( $\geq 100$  CFU mL<sup>-1</sup>).

The matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF) method was used for the microbial identification of *S. aureus* isolates (Barcelos *et al.*, 2019). The strains were subjected to DNA extraction using the "InstaGene Matrix" kit (BIO-RAD) according to the manufacturer's instructions. The nuclease gene (*nucA*) was amplified to reconfirm *S. aureus* species using the method described by Sasaki *et al.* (2010).

#### ***Phenotypic Characterisation of Antibiotic Resistance***

The isolates were subjected to the disk diffusion test using Mueller-Hinton agar (MHA) plates (OXOID, USA) with disks impregnated with nine antimicrobial drugs: penicillin (PEN, 10  $\mu$ g), oxacillin (OXA, 1  $\mu$ g), cefoxitin (CEF, 30  $\mu$ g), gentamicin (GEN, 10  $\mu$ g), tobramycin (TOB, 10  $\mu$ g), erythromycin (ERY, 15  $\mu$ g), tetracycline (TET, 30  $\mu$ g), clindamycin (CLI, 2  $\mu$ g), and chloramphenicol (CHL, 30  $\mu$ g; all from Laborclin, Paraná, Brazil). These antibiotics were selected based on their human and veterinary use. The zones of inhibition (halos) were measured according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2021).

#### ***PCR assays for the detection of virulence-associated genes***

The PCR assays were performed according to the reference protocols cited in Supplementary Table S1, with modifications. All the 49 virulence genes investigated were those responsible for: enterotoxin production (*sea*, *seb*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej*); resistance to penicillin (*blaZ*), oxacillin and ceftiofur (*mecA* and *mecC*), gentamicin (*aac6'aph2'*), tobramycin (*ant4*), erythromycin and clindamycin (*ermA*, *ermB*, *ermC*, *ermF*, *ermT*, *msrA*, *cfr*, *vgaC*, *lnuA/A'*, *lnuB*, *lsaB*, and *lsaE*), and tetracycline (*tetK*, *tetL*, and *tetM*); antiseptic (QAC) resistance (*qacA/B* and *qacC*); biofilm formation (*icaA*, *icaD*, and *bap*); and encoding MSCRAMMs (*fnbA*, *fnbB*, *fib*, *clfA*, *clfB*, *cna*, *eno*, *bbp*, and *ebps*).

Each PCR was carried out with a final volume of 25  $\mu$ L containing: 1X PCR Buffer (Promega Corp., Madison, USA), 0.2 mM of each deoxynucleotide (dNTP, Invitrogen, Carlsbad, CA), 1.5 to 4 mM MgCl<sub>2</sub> (25 mM; Promega Corp.) depending on the gene, 0.4  $\mu$ M of the respective primers (Invitrogen, or Sigma, St. Louis, MO; Exxtend, São Paulo, Brazil), 2.5 U of GoTaq Hot Start Polymerase (Promega Corp., Madison, USA), 50 ng of template DNA, and sterilised ultrapure Milli-Q water (Merck KGaA, Darmstadt, Germany).

PCR for all reactions was performed in a thermocycler (Thermo Fisher Scientific, Waltham, MA). The conditions included: initial denaturation at 94 to 95 °C for 2 to 5 min for all primers, followed by different amplification cycles (25 to 40) of denaturation (94 to 95 °C for 30 s to 1 min), annealing (Supplementary Table S1), and extension at 72 °C for 1 min. The final extension step was performed at 72 °C for 10 min.

Positive controls from the collection of the Laboratory of Microbial Toxins at the Department of Food Science and Nutrition at UNICAMP (Campinas, Brazil) were included in each PCR reaction. We used Ultrapure water (Merck KGaA, Darmstadt, Germany) as the negative control. PCR products were analysed by electrophoresis in a

1.5% (w/v) agarose gel with 0.5X TBE buffer (Promega Corp., Madison, WI) and stained with SYBR® Safe (Invitrogen, Carlsbad, CA). A 100 bp molecular weight DNA ladder (New England Biolabs, Ipswich, MA, USA) was used to validate the length of the products and then visualised using a UV Gel Documentation system (Uvitec, Cambridge, UK).

### ***Molecular typing***

The *agr* system groups were classified based on the hypervariable domain of the *agr* locus (I, II, III, and IV) through amplification using the oligonucleotide primers described by Shopsin *et al.* (2003). For *spa* typing, DNA amplification was performed according to the protocol from the official Ridom website, [www.ridom.com](http://www.ridom.com). The samples were sequenced using the Sanger method, and DNA sequencing data were analysed using the *spa* typing database in order to determine the repeat profile and *spa* type of each isolate (<http://spa.ridom.de/repeats.shtml> and <http://spa.ridom.de/spatypes.shtml>). Description of PCR primers used for *agr* and *spa* typing are shown in Supplementary Table S1.

### ***Statistical analyses***

Statistical analysis of the genes was performed using Cochran's Q test, after multiple comparisons using the McNemar (Bonferroni) procedure, using the XLSTAT 2020 software for Microsoft Excel® (Microsoft®, WA, USA). Because of the dichotomous variables, a gene's presence was equal to one (1), and its absence was zero. A significance level of 5% was considered; therefore, all results with a *p*-value <0.05 were considered statistically significant.

## **Results**

All *S. aureus* isolates were identified at the species level with a score > 2.0 by MALDI-TOF MS. Regarding the phenotypic antibiotic resistance, 7% showed no

resistance to any antibiotics, and 93% showed resistance to at least one antibiotic (Table 1). Penicillin resistance was the most prevalent (87.7%), followed by tetracycline (12.3%), oxacillin (10.5%), gentamicin (10.5%), and erythromycin (3.5%) resistance. In addition, 5.3% exhibited intermediate resistance to tobramycin and 1.8% to erythromycin. Resistance to clindamycin was observed in one isolate (1.7%). Eight antibiotic resistance profiles were detected; penicillin (68.4%) was the most abundant profile, followed by penicillin and oxacillin (7%).

The results in Table 1 show the total genotypic and phenotypic data of the 57 strains of *S. aureus* isolated from all chronic SM cases. All these data were subjected to statistical analysis by Cochran's Q test. Based on the PCR assay, the relative frequencies of virulence-encoding genes for *S. aureus* are shown in Table 2. Our results showed that 98.2% of isolates contained at least one biofilm-forming gene. There was no significant difference ( $P > 0.05$ ) in the occurrence of *icaA*, *icaD*, and *bap*. The most frequent biofilm formation gene detected was *icaD*, with 94.7% ( $n = 54$ ), followed by *bap* with 78.9% ( $n=45$ ), and *icaA* with 66.7% ( $n = 38$ ). The most frequent MSCRAMMS genes detected were *fib* (93%), *fnbA* (82.5%), *clfA* (80.7%), *clfB* (73.7%), and *eno* (63.2%). On the other hand, the less prevalent were *cna* (7%) and *ebps* (7%), while the *bbp* and *fnbB* genes were not detected. For enterotoxin genes, the frequencies were: *see* (64.9%), *sed* (61.4%), and *seh* (3.5%). Forty-seven isolates (82.5%) presented at least one enterotoxin-encoding gene. Only ten isolates (17.5%) did not have any enterotoxin genes. Enterotoxin genes *sea*, *seb*, *sec*, *sec*, *sei*, and *sej* were undetected.

The highest detection frequency for antibiotic resistance genes was *blaZ* (94.7%), followed by *aac6'aph2'* (15.8%) and *ant4* (12.3%). The lowest frequency was observed for *lnuA/A'* (1.8%). All isolates resistant to tetracycline had *tetL* (10.5%), while the *tetK* and *tetM* genes were undetected. No *mecA* and *mecC* (MRSA) positive strains were

identified. Only three isolates (5.26%) had no antibiotic-resistance genes. The antibiotic resistance genes *ermA*, *ermB*, *ermC*, *ermF*, *ermT*, *msrA*, *cfr*, *vgaC*, *lsaB*, *lsaE*, and *lnuB* were also not detected. Regarding the prevalence of antiseptic (QAC) resistance genes, it is indicated that no *S. aureus* carried the QAC resistance genes (*qacA/B* and *qacC*).

Concerning the gene patterns with different gene combinations, there was only one type of combination for enterotoxin genes (*sed*, *see*), representing 47.4% of the isolates. MSCRAMMs patterns showed that the most prevalent combination for these genes was *clfA*, *clfB*, *fnbA*, *eno*, *fib* (29 isolates, 50.9%), followed by the combination of *clfA*, *clfB*, *fnbA*, *fib* (9 isolates, 15.8%). Thus, we identified that 3.5% of the isolates did not present MSCRAMMs genes. Furthermore, high genetic diversity was detected in three isolates (5.3%), which showed 7 out of 9 genes (*clfA*, *clfB*, *cna*, *fnbA*, *fnbB*, *eno*, *fib*, and *ebps*). For biofilm formation patterns, the most prevalent combination was *icaA*, *icaD*, *bap*, and *fib* (33 isolates, 57.9%), followed by *icaD* and *bap* (10 isolates, 17.5%). Table 3 shows the gene combinations of the *S. aureus* isolates.

All isolates belonged to the *spa* type t605 (100%). Through *agr* typing, we determined that *agr* type II was the dominant type (n = 32, 56.14%) and that 5.26% (n = 3) were *agr* type III. The *agr* type I and IV were undetected, and 22 isolates (38.6%) were non-typeable.

## **Discussion**

Molecular studies are fundamental because they provide epidemiological information and knowledge regarding pathogenicity mechanisms, making it possible to understand the association of these mechanisms with the microorganism virulence profile (Veh *et al.*, 2015; Ren *et al.*, 2020). Our study demonstrated different virulence profiles for *S. aureus* strains involved in chronic SM. Knowledge regarding the genetic diversity and antimicrobial resistance of these *S. aureus* strains isolated from chronic subclinically



infected cows can help implement control measures for this disease in dairy herds. The results confirmed that an endemic type is found in Brazilian dairy herds and carries virulence genes, which may explain the difficulty in eradicating and controlling chronic SM. Our findings improved the comprehension of the pathogenesis of chronic *S. aureus* by tracking important virulence genes involved in mastitis and determining genotypic variations of the strains.

The pathogenesis of chronic SM caused by *S. aureus* begins with adherence, subsequent biofilm production, and bacterial adhesion to the epithelium of mammary glands. It is influenced by the polysaccharide intercellular adhesion (PIA), encoded by the *icaADBC*, an intercellular adhesion (*ica*) operon which contains *icaA* and *icaB* (N-acetylglucosamine transferases), *icaC* (predicted exporter), and *icaD* (deacetylase) genes (Miao *et al.*, 2019; Marques *et al.*, 2021; Silva *et al.*, 2022). From these in *S. aureus*, *icaA* and *icaD* are important factors in promoting intercellular adhesion and forming multilayered biofilms associated with biofilm formation, *icaC* seems to be involved in the transport of *icaA* and *icaD* synthesised oligomers across the cell membrane. Further, the absence of the *icaB* gene may reduce the efficiency in binding to the bacterial cell surface, leading to weak biofilm production (Miao *et al.*, 2019; Silva *et al.*, 2021). Studies assessing *icaA* and *icaD* on milk samples of clinical or SM cases reported that these genes were present at high frequencies (each 86.6%) (Aslantaş & Demir, 2016). Our results showed that 98.2% of strains present genes involved in biofilm formation, similar to Vasudevan *et al.* (2003), who found *icaA* and *icaD* genes in 35 (100%) *S. aureus* isolates from bovine mastitis cases. Prior studies showed that the ability to form a biofilm in *S. aureus* strains depends on the expression of *icaD* (Namvar *et al.*, 2013). In our study, this gene was detected in high frequency (94.7%), followed by the *icaA* (66.7%).

The Bap protein plays a crucial role in the initial attachment and recruitment of *S. aureus*. Many studies showed that the *bap* gene was present in SM mastitis at a low frequency (Zuniga *et al.*, 2015; Aslantaş & Demir, 2016; Felipe *et al.*, 2017) or was not present (Ren *et al.*, 2020). For both occurrences, biofilm formation may be attributed to the *ica*-dependent mechanism, which encodes the PIA production (Marques *et al.*, 2021). However, in this study, the *bap* gene was detected at a high rate (78.9%), which is consistent with the results reported by Salimena *et al.* (2016), who determined that this gene was present in 95.6% of the isolates from the milk of dairy herds in Brazil, which is the highest prevalence rate reported to date.

There is an association between the *bap*, *icaA*, and *icaD* genes and the genes encoding for MSCRAMMs. They provide a great capacity for adhesion and biofilm production, which is an important characteristic of the persistence of *S. aureus* (Castilho *et al.*, 2017). MSCRAMMs are proteins found on the surface of the bacteria that allow for the initial addition and colonisation of *S. aureus* and the intracellular internalisation and initiation of biofilm. These adhesins play a vital role in the onset of *S. aureus* infection (Klein *et al.*, 2012). Their presence may explain the current difficulties in controlling and eradicating chronic SM caused by *S. aureus* in dairy herds. Our findings show that biofilm and MSCRAMMs genes are important virulence factors that can help with the colonisation and persistence of *S. aureus*.

Some adhesins are important virulence factors in binding host cells, colonisation, and invasion, such as fibronectin-binding proteins (*fnbA* and *fnbB*) and clumping factors (*clfA* and *clfB*) (Aslantaş & Demir, 2016; Zhang *et al.*, 2018). Zhang *et al.* (2018) revealed that the most frequent adhesion genes were *clfA*, *clfB*, *fnbA*, and *fnbB*, showing that more than 85% of *S. aureus* isolated from bovine mastitis cases in China presented these genes. Similar to our findings, Zaatout *et al.* (2019) found a high diversity of MSCRAMMs

genes (12 out of 14 genes), such as *clfA*, *clfB*, *ebpS*, *eno*, *fnbA*, *fnbB*, and *bbp*. This diversity in virulence factors, and their different combinations, can cause changes in the level of pathogenicity and the spread of infections among dairy cows inside or across herds (Veh *et al.*, 2015). Furthermore, Gogoi-Tiwari *et al.* (2015) found a difference in the rates of *fnbA* and *fnbB* genes. They reported that 54.5% of isolates present the *fnbA* gene, while *fnbB* was found at a low frequency (1.3%) among Australian bovine *S. aureus* isolates. Another study evaluated the aforementioned genes of *S. aureus* isolated from SM cases and showed a difference in rates with higher frequency in *fnbA* (72.6%) compared to *fnbB* (5.7%) (Zuniga *et al.*, 2015). In another study with isolates from bovine mastitis in Argentinean dairy farms, *clfA* and *clfB* genes were present at high rates, comparable to our results (Felipe *et al.*, 2017). Kumar *et al.* (2011) and Zuniga *et al.* (2015) found the *eno* gene (gene encoding the laminin-binding protein) in 100% and 82.1% of *S. aureus* isolated from clinical and SM, respectively. The *eno* gene was also frequently detected in the isolates of our study (63.2%). On the other hand, we detected the *cna* gene at a low frequency (7%), and similar studies have demonstrated a lower percentage of this gene in *S. aureus* isolates from SM cases (Klein *et al.*, 2012; Xu *et al.*, 2015).

The major enterotoxin gene detected in our investigation was the *see* gene. Similarly, Fursova *et al.* (2018) reported that this gene was present in *S. aureus* strains from cows with high SM frequency (46.6%). Furthermore, another abundant enterotoxin gene was *sed*, detected in 35 of 57 (61.4%) isolates. Previous studies reported the gene encoding for SED, which is a common enterotoxin produced by *S. aureus* isolated from dairy products (Vitale *et al.*, 2015) and cows with clinical and SM (Costa *et al.*, 2018; Fursova *et al.*, 2018; Grispoli *et al.*, 2019; Ren *et al.*, 2020). Another study indicated variable rates of enterotoxigenicity for subclinical mastitic *S. aureus* isolates (Rall *et al.*,

2014). Liu et al. (2014) showed that SEH toxin plays an important role in the mastitis process because it can induce apoptosis of the bovine mammary epithelial cells. Our results showed that the *seh* gene was present but not dominant; likewise, Cândido et al. (2020) found this gene in 13.1% of isolates from dairy products. Another study found the *seh* gene from SM cases in high frequency (41.5%) (Ren et al., 2020). None of the isolates harboured the *sec* gene. Based on previous studies, this gene was rarely present in bovine mastitis, as reported by Cândido et al. (2020). We did not detect *sea* genes comparable to previous studies of *S. aureus* from dairy cows with clinical or SM (Costa et al., 2018; Zhang et al., 2018). Therefore, our results suggest that *S. aureus* isolated from milk samples of chronic subclinically infected cows has the enterotoxigenic potential of food poisoning, which is worrisome since SM may not be detected visually but can be easily distributed for human consumption (Hussein et al., 2018).

Due to the low recovery rates and the tendency to evolve into chronic infection processes of longer duration, treatment for cows subclinically infected with *S. aureus* is not commonly adopted; however, antimicrobial treatment could result in a higher bacteriological cure rate in young cows that had first contact with this pathogen (Mcdougall et al., 2022). Then again, the non-assertive antibiotic might cause variability in *S. aureus* resistance profiles, as evidenced by the results obtained in this study. Furthermore, there is a risk of exchanging clones among humans and animals since they adapt to the host and acquire virulence factors to colonise and generate new infections (Veh et al., 2015). In our study, *S. aureus* isolates harboured the  $\beta$ -lactamase gene *blaZ*, indicative of penicillin resistance, at an alarming frequency (94.7%). However, phenotypically we found that resistance to penicillin was 87.72%. These results are in line with previous studies from different parts of the world. In China, Xu et al. (2015) and Qu et al. (2019) detected a high prevalence of the *blaZ* gene, 95%, and 82.1%,

respectively, in *S. aureus* strains from dairy cows with SM. Aslantaş & Demir (2016) reported a higher rate (100%) of *blaZ* among *S. aureus* from mastitis cases in Turkey. In addition, Zaatout *et al.* (2019) showed their presence in all nine *S. aureus* strains from dairies in Algeria. Some studies corroborate the *blaZ* gene in dairies in Brazil, with a prevalence of 25.3% and 40% (Soares *et al.*, 2017; Abreu *et al.*, 2021). Our results revealed a divergence between the phenotypic and genotypic prevalence of *blaZ*, which is in agreement with earlier studies on bovine mastitis. Research conducted by Martini *et al.* (2017) found 88% of *blaZ*-positive isolates and 83.3% expressed phenotypical resistance to penicillin; likewise, Neelam *et al.* (2022) showed that 92.73% harboured the *blaZ* gene and 83.64% were resistant to penicillin. These results demonstrate that gene detection does not necessarily indicate that the gene has a detectable phenotype. Our findings are of great concern to bovine health, given the fact that  $\beta$ -lactams are commonly used antibiotics to treat mastitis diseases (Aslantaş & Demir, 2016). The extensive use of these antibiotics results in a bacterial adaptation by developing resistance to penicillin amongst *S. aureus* isolates (Abreu *et al.*, 2021). Therefore, the alarming prevalence of the *blaZ* gene in the current study is probably an indicator of the intensive use of penicillin to treat bovine mastitis in herds.

Remarkably, our study did not identify *mecA* and *mecC* resistance genes among the isolates, which would help to classify these strains as MSSA. Similar studies with no positive isolates for the *mecA* and *mecC* genes in raw milk and dairy products emphasised the need for significant attention toward MSSA; since they may be involved in the establishment and evolution of MRSA lineages, which may be derived through the acquisition of *mecA* or *mecC* genes (Silva *et al.*, 2013; Bonsaglia *et al.*, 2018). In a previous study conducted in Michigan, the US, very few MRSA (0.6%) were isolated from the milk of cows suspected of having an intramammary infection (Erskine *et al.*,

2002). Methicillin-resistant *S. aureus* was found in <1% of bulk tank milk samples collected from smaller organic and conventional dairy herds (n = 288 farms) in New York, Wisconsin, and Oregon (Cicconi-Hogan *et al.*, 2014) and 4% from 50 Minnesota farms (Haran *et al.*, 2012). Other studies showed that MRSA was detected in raw milk and dairy products (Aslantaş & Demir, 2016; Rodrigues *et al.*, 2017a; Rodrigues *et al.* 2017b; Ren *et al.*, 2020; Abreu *et al.*, 2021). Previous studies have demonstrated that MRSA strains hold different gene profiles, with more enterotoxin genes (Papadopoulos *et al.*, 2019), and are more resistant to antimicrobials (Zayda *et al.*, 2020) when compared to MSSA strains. Therefore, to control the contagious MSSA associated with mastitis in dairy herds, procedures must be implemented to prevent the spread of the pathogen (i.e., considering all milk production stages), including an optimal milking routine, teat disinfection, and segregation of infected cows.

In contrast, phenotypic analyses determined that six strains were resistant to oxacillin. The absence of the *mec* gene and phenotypic resistance to oxacillin may be associated with the excessive production of the  $\beta$ -lactamase by the *blaZ* gene (Scholtzek *et al.*, 2019). It should be noted that all strains that expressed resistance to oxacillin carried the *blaZ* gene, which may explain the phenotypic resistance to oxacillin. Another study suggests that the appearance of this type of strain, without the *mec* gene, is potentially attributed to previously unknown selection pressure, such as the use of antiseptics or disinfectants (Speck *et al.*, 2020). Another cow-level study found zero proportion of resistance to oxacillin of 357 bovine mastitis-causing *S. aureus* from 24 farms in North Carolina and Virginia farms (Anderson *et al.*, 2006). Further investigations targeting phenotypic and genotypic methicillin resistance mechanisms should be carried out to increase our understanding of the genetic basis of antimicrobial resistance in these strains.

We detected the *aac6'aph2'* gene associated with gentamicin resistance in nine strains, but only five were identified with the resistance phenotype. Although one strain had phenotypic resistance to gentamicin, it did not present the resistance genotype. These findings are consistent with other studies from dairies where the authors found strains with these characteristics (Abreu *et al.*, 2021; Szczuka *et al.*, 2022). Concerning the *ant4* gene responsible for resistance to tobramycin, three of the eight strains that possessed the gene showed intermediate phenotypic resistance. The other five did not express the phenotype. It is important to emphasise the alarming problem that could cause the rapid worldwide spread of these antibiotic resistance genes amongst the huge diversity of *S. aureus* isolates. It highlights the crucial point of using these antimicrobials that are commonly applied for mastitis treatment in Brazilian dairy herds.

Little is known about QAC resistance genes in *S. aureus* from SM cases. However, evaluating the potential resistance of *S. aureus* strains to QAC helps to use and control the effectiveness of these disinfectants. Unlike other studies that reported the presence of QAC-resistant genes in staphylococcal strains from bovines (Ergun *et al.*, 2017; Kotb & Gafer, 2020) and goat herds (Bjorland *et al.*, 2005), we did not observe the presence of these genes, which is supported by other findings similar to our study (Qu *et al.*, 2019; Abreu *et al.*, 2021). Zero detection of the QAC-resistant genes affords us more possibilities for controlling SM cases caused by *S. aureus* with disinfectants in Brazilian dairy cattle.

In our research, *agr* II was the most frequent type, followed by *agr* III. None of the isolates tested was *agr* type I or IV, and 22 were negative for all known types. These findings are important from an epidemiological point of view since other studies conducted in São Paulo State, Brazil, reported similarly the prevalence of *agr* type II. For instance, research performed by Bonsaglia *et al.* (2018) regarding *S. aureus* isolates from

cows with SM confirmed a high prevalence of *agr* type II (47.7%), followed by type III (20%); likewise, *agr* type IV was not detected. Cândido *et al.* (2020) also reported a high prevalence of *agr* type II (29%) and III (44.7%) in *S. aureus* isolates from organic and conventional dairies. Silva *et al.* (2013) identified *agr* types II (41.1%) and III (48.2%) in MSSA isolates from dairy cows with mastitis. Rodrigues *et al.* (2017b) identified *agr* type II in 88.1% of isolates of *Staphylococcus* spp. from mastitic milk. Other studies reported the presence of *agr* type II in *S. aureus* isolates from dairies (Kumar *et al.*, 2011; Schmidt *et al.*, 2017; Soares *et al.*, 2017). Our findings and previous studies infer that *agr* type II could be well adapted to dairy environments in São Paulo State, Brazil, and may cause chronic SM.

By molecular typing, we have shown that we only detected the presence of *spa* type t605; this *spa* type consists of two *spa* repeat successions (r07-r23). The Ridom *spa* server network database shows that t605 has a relative global frequency (0.09%) (<http://spaserver.ridom.de> - Data collected in January 2021). A study performed in Minas Gerais, Brazil, showed that the udder is the most important reservoir for *S. aureus* t605 and emphasised that t605 is widespread in dairy herds and can be responsible for the persistence of bovine intramammary infections (Santos *et al.*, 2020). Furthermore, other studies conducted in São Paulo State, Brazil, such as the study conducted by Bonsaglia *et al.* (2018), found a high frequency of *spa* type t605 (70.5%) in *S. aureus* isolates from cows with SM. Likewise, Silva *et al.* (2013) observed a predominance of *spa* type t605 (37.5%) in milk from cows subclinically infected. This *spa* type was also discovered in high frequency (83.3%) in *Staphylococcus* spp. from milk samples with mastitis (Rodrigues *et al.* 2017a), but a low frequency (32.9%) was reported in samples from dairies (Rodrigues *et al.*, 2017b). The high prevalence of *spa* type t605 suggests that these strains are well adapted to bovine herds in Brazil, indicating the presence of an endemic



*S. aureus* widely disseminated, which can invade and remain in the bovine mammary cells, causing chronic SM. These findings corroborate what other studies have reported by showing the persistence of *spa* type t605 (Veh *et al.*, 2015; Cândido *et al.*, 2020).

Some limitations of the study should be noted, such as the relatively small sample size that may not represent the entire population of *S. aureus* involved in bovine chronic SM cases. In addition, despite covering a broad spectrum of virulence factors, research on virulence genes, such as *icaB* and *icaC* of the *icaADBC* loci, were not included in this study. However, the results found here follow the trends observed in other studies.

We determined a great pathogenicity potential among MSSA. Our results suggest that *S. aureus* isolated from milk samples of chronic subclinically infected cows has the enterotoxigenic potential for food poisoning. The most predominant strains for enterotoxin genes were *see* and *sed*. A unique endemic *spa* type (t605) was detected in the Brazilian dairy herds evaluated. Our findings showed that biofilm and MSCRAMMs genes are important virulence factors that can help with the colonisation and persistence of *S. aureus*. Clearly, *agr* type II is well adapted to dairy environments, increasing the risk of causing the disease. Although no *mecA* and *mecC* (MRSA) positive strains were identified, we observed high phenotypic resistance levels to at least one antibiotic class, mainly in  $\beta$ -lactams, which are widely used in dairy herds. Further studies are needed to provide detailed insights to guide clinical decisions around disease management and develop effective preventive measures to control *S. aureus* causing chronic SM.

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### **Conflict of interest**

The authors declare no conflict of interest.

### Data Availability Statement

The authors confirm the data supporting the findings of this study are available within the article.

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**Author Contributions**

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**Table 1.** Genotypic and phenotypic profiles <sup>a</sup> of the 57 strains of *S. aureus* isolates from bovine subclinical mastitis

Strain	Genotypic data				Phenotypic data		
	Enterotoxin genes	Biofilm formation genes	MSCRAMMs genes	Antibiotic resistance genes	<i>agr</i> type	<i>spa</i> type	Antibiotic resistance
262	<i>seh</i>	<i>icaD</i>	<i>clfA, clfB, cna, fnbA, eno, fib, ebps</i>	<i>blaZ</i>	<i>agrIII</i>	t605	-
296	-	<i>icaD</i>	<i>clfA, clfB, cna, fnbA, eno, fib, ebps</i>	<i>blaZ, aac6'aph2'</i>	<i>agrII</i>	t605	PEN, OXA, TET
300	-	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ, aac6'aph2', tetL</i>	<i>agrII</i>	t605	PEN, ERY, CLI
313	-	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
315	-	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
316	<i>see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN
345	-	<i>icaD</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
354	-	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
356	-	<i>icaA, icaD, bap</i>	<i>fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
361	<i>sed</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
375	-	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
376	-	<i>icaA, icaD, bap</i>	-	-	-	t605	-
506	<i>sed</i>	<i>icaD</i>	<i>clfA, clfB, cna, fnbA, fib, ebps</i>	-	<i>agrIII</i>	t605	-
613	<i>sed</i>	<i>icaD</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
614	<i>sed</i>	<i>icaD</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN, OXA
615	<i>sed</i>	<i>icaA, icaD</i>	<i>clfA, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
616	<i>sed</i>	<i>icaA, icaD, bap</i>	-	<i>blaZ</i>	-	t605	PEN
661	-	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
663	<i>sed</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN, OXA
664	<i>sed</i>	<i>icaA, icaD, bap</i>	<i>clfA, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN
680	<i>see</i>	<i>icaA, icaD, bap</i>	<i>clfA, fnbA, eno</i>	<i>blaZ</i>	-	t605	PEN
693	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>fib</i>	<i>blaZ</i>	-	t605	PEN
697	<i>sed, see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
715	<i>sed, see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN, OXA
716	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1009	<i>sed, see</i>	-	<i>fib</i>	<i>blaZ</i>	-	t605	PEN
1010	<i>sed, see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN

1011	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1012	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN
1041	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1045	<i>sed, see</i>	<i>icaD</i>	<i>clfA, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1061	<i>see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN
1063	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1064	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN
1074	<i>sed, see</i>	<i>icaA, icaD</i>	<i>fnbA</i>	<i>blaZ, aac6'aph2'</i>	-	t605	PEN
1077	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1097	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1100	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ, lnuA/A'</i>	-	t605	PEN, ERY*
1101	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ</i>	-	t605	PEN
1111	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ</i>	-	t605	PEN
1177	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ</i>	-	t605	PEN
1185	<i>sed, see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ</i>	-	t605	PEN
1191	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN, OXA
1199	<i>sed, see</i>	<i>icaA, bap</i>	<i>fib</i>	<i>blaZ</i>	-	t605	PEN
1219	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>fib</i>	<i>blaZ, ant4</i>	-	t605	PEN
1261	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ, aac6'aph2', tetL</i>	<i>agrII</i>	t605	GEN, TET
1269	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	-	t605	PEN
1286	<i>sed, see</i>	<i>icaA, icaD, bap</i>	<i>fib</i>	<i>blaZ, ant4</i>	-	t605	GEN, TET
1288	<i>see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ, aac6'aph2', ant4, tetL</i>	<i>agrII</i>	t605	GEN, TET
1349	<i>sed, see</i>	<i>icaA, bap</i>	<i>fib</i>	<i>blaZ, aac6'aph2', tetL</i>	<i>agrII</i>	t605	PEN, TOB*, GEN, TET
1365	<i>see</i>	<i>icaA, icaD, bap</i>	<i>clfA, clfB, fnbA, fib</i>	<i>blaZ, ant4</i>	-	t605	PEN, OXA, ERY
1388	<i>see</i>	<i>icaA, icaD, bap</i>	<i>fib</i>	<i>blaZ, aac6'aph2', ant4, tetL</i>	<i>agrII</i>	t605	PEN, TOB*, GEN, TET
1393	<i>see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ, aac6'aph2', ant4, tetL</i>	<i>agrII</i>	t605	PEN, TOB*, GEN, TET
1395	<i>see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ, aac6'aph2', ant4</i>	<i>agrII</i>	t605	PEN
1423	<i>seh</i>	<i>icaD</i>	<i>clfA, clfB, cna, fnbA, eno, fib, ebps</i>	-	<i>agrIII</i>	t605	-
1425	<i>see</i>	<i>icaA, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ</i>	<i>agrII</i>	t605	PEN
1427	<i>see</i>	<i>icaD, bap</i>	<i>clfA, clfB, fnbA, eno, fib</i>	<i>blaZ, ant4</i>	<i>agrII</i>	t605	PEN

<sup>a</sup> CEF = cefoxitin, CLI = clindamycin, ERY = erythromycin, GEN = gentamicin, PEN = penicillin, OXA = oxacillin, TET = tetracycline, TOB = tobramycin; - = gene not detected.

\*Indicates intermediate resistance.

**Table 2.** Frequency of *S. aureus* genes detected from bovine subclinical mastitis using the Cochran's Q test.

Genes		No. of positive isolates	Frequency, %
Enterotoxins	<i>seh</i>	2	3.5 <sup>a</sup>
	<i>sed</i>	35	61.4 <sup>ab, b</sup>
	<i>see</i>	37	64.9 <sup>ab, b</sup>
Biofilm formation	<i>icaA</i>	38	66.7 <sup>ab, b</sup>
	<i>bap</i>	45	78.9 <sup>ab, b</sup>
	<i>icaD</i>	54	94.7 <sup>b</sup>
MSCRAMMs	<i>cna</i>	4	7 <sup>a</sup>
	<i>ebps</i>	4	7 <sup>a</sup>
	<i>eno</i>	36	63.2 <sup>ab, b</sup>
	<i>clfB</i>	42	73.7 <sup>ab, b</sup>
	<i>clfA</i>	46	80.7 <sup>ab, b</sup>
	<i>fnbA</i>	47	82.5 <sup>ab, b</sup>
	<i>fib</i>	53	93 <sup>b</sup>
Antibiotic resistance	<i>lnuA/A'</i>	1	1.8 <sup>a</sup>
	<i>tetL</i>	6	10.5 <sup>a</sup>
	<i>ant4</i>	7	12.3 <sup>a</sup>
	<i>aac6'aph2'</i>	9	15.8 <sup>a</sup>
	<i>blaZ</i>	54	94.7 <sup>b</sup>

<sup>a, b</sup> Statistically significant difference ( $p < 0.05$ ) indicated by different letters in the same column.

**Table 3.** Different gene combinations of *S. aureus* isolates from bovine subclinical mastitis.

Enterotoxin genes		MSCRAMMs genes		Antibiotic resistance genes		Biofilm formation genes	
Gene Patterns	N° of isolates (%)	Gene Patterns	N° of isolates (%)	Gene Patterns	N° of isolates (%)	Gene Patterns	N° of isolates (%)
<i>sed, see</i>	27 (47.4)	<i>clfA, clfB, fnbA, eno, fib</i>	29 (50.9)	<i>blaZ</i>	40 (70.2)	<i>icaA, icaD, bap</i>	33 (57.9)
		<i>clfA, clfB, fnbA, fib</i>	9 (15.8)				
		<i>fib</i>	8 (14.0)				
<i>see</i>	10 (17.5)	<i>clfA, clfB, cna, fnbA, eno, fib, ebps</i>	3 (5.3)	<i>blaZ, aac6'aph2', tetL</i>	3 (5.3)	<i>icaD, bap</i>	10 (17.5)
		<i>clfA, fnbA, eno, fib</i>	3 (5.3)				
<i>sed</i>	8 (14.0)	<i>clfA, fnbA, eno, fib</i>	3 (5.3)	<i>blaZ, ant4</i>	4 (7.0)	<i>icaD</i>	8 (14.0)
		<i>clfA, clfB, cna, fnbA, fib, ebps</i>	1 (1.8)	<i>blaZ, aac6'aph2', ant4, tetL</i>	3 (5.3)		
<i>seh</i>	2 (3.5)	<i>clfA, fnbA, eno</i>	1 (1.8)	<i>blaZ, lnuA/A'</i>	1 (1.8)	<i>icaA, bap</i>	3 (5.3)
		<i>fnba</i>	1 (1.8)	<i>blaZ, aac6'aph2', ant4</i>	1 (1.8)	<i>icaA, icaD</i>	2 (3.5)
Gene not detected	10 (17.5)	Gene not detected	2 (3.5)	Gene not detected	3 (5.3)	Gene not detected	1 (1.8)
Total	100		100		100		100

## SUPPLEMENTARY MATERIAL DATA

**Supplementary Table S1.** Primers and cycle conditions used for polymerase chain reaction detection of enterotoxins genes, biofilm formation genes, MSCRAMMs genes, antiseptic resistance genes, antibiotic resistance genes, *spa* and *agr* typing.

Genotype	Gene	Primer sequence (5'-3') <sup>1</sup>	Product size (bp)	Annealing (°C)	Cycles (n°)	Reference
Enterotoxin	<i>sea</i>	F: TTGGAAACGGTAAAACGAA R: GAACCTTCCCATCAAAAACA	120	54	30	Johnson et al., 1991
	<i>seb</i>	F: TCGCATCAAACGACAAACG R: GCAGGTACTCTATAAGTGCC	478	54	30	Johnson et al., 1991
	<i>sec</i>	F: GACATAAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCC	257	53	30	Johnson et al., 1991
	<i>sed</i>	F: CTAGTTTGGTAATATCTCCT R: TAATGCTATATCTTATAGGG	317	54	30	Johnson et al., 1991
	<i>see</i>	F: AGGTTTTTTCACAGGTCATCC R: CTTTTTTTTCTTCGGTCAATC	209	54	30	Mehrotra et al., 2000
	<i>seg</i>	F: AAGTAGACATTTTTGGCGTTCC R: AGAACCATCAAACCTCGTATAGC	287	54	30	Omoe et al., 2002
	<i>seh</i>	F: GTCTATATGGAGGTACAACACT R: GACCTTACTTATTTTCGCTGTC	213	55	30	Omoe et al., 2002
	<i>sei</i>	F: GGTGATATTGGTGTAGGTAAC R: ATCCATATTCTTTGCCTTTACCAG	454	56	30	Omoe et al., 2002
	<i>sej</i>	F: CATCAGAACTGTTGTTCCGCTAG R: CTGAATTTTACCATCAAAGGTAC	142	64	30	Nashev et al., 2004
Biofilm formation	<i>icaA</i>	F: CCTAACTAACGAAAGGTAG R: AAGATATAGCGATAAGTGC	1315	49	32	Vasudevan et al., 2003
	<i>icaD</i>	F: AAACGTAAGAGAGGTGG R: GGCAATATGATCAAGATAC	381	50	32	Vasudevan et al., 2003
	<i>bap</i>	F: CCCTATATCGAAGGTGTAGAATTG R: GCTGTTGAAGTTAATACTGTACCTGC	971	59	40	Cucarella et al., 2001
MSCRAMMs	<i>clfA</i>	F: ATTGGCGTGGCTTCAGTGCT R: CGTTTCTTCCGTAGTTGCATTTG	288	56	25	Ghasemian et al., 2015
	<i>clfB</i>	F: ACATCAGTAATAGTAGGGGCAAC R: TTCGCACTGTTTGTGTTTGCAC	204	56	25	Ghasemian et al. 2015

	<i>fnbA</i>	F: CATAAATTGGGAGCAGCATCA R: ATCAGCAGCTGAATTCACCATT	128	56	25	Ghasemian et al. 2015
	<i>fnbB</i>	F: GTAACAGCTAATGGTCGAATTGATACT R: CAAGTTCGATAGGAGTACTATGTTC	524	56	25	Ghasemian et al. 2015
	<i>fib</i>	F: CTACAACACTACAATTGCGTCAACAG R: GCTCTTGTAAGACCATTTTCTTCAC	405	56	25	Ghasemian et al. 2015
	<i>cna</i>	F: AAAGCGTTGCCTAGTGGAGA R: AGTGCCTTCCCAAACCTTTT	192	56	25	Ghasemian et al. 2015
	<i>eno</i>	F: ACGTGCAGCAGCTGACT R: CAACAGCATCTTCAGTACCTTC	301	56	25	Ghasemian et al. 2015
	<i>ebps</i>	F: CATCCAGAACCAATCGAAGAC R: AGTTACATCATCATGTTTATCTTTTG	188	52	25	Ghasemian et al. 2015
	<i>bbp</i>	F: AACTACATCTAGTACTCAACAACAG R: ATGTGCTTGAATAACACCATCATCT	574	56	25	Ghasemian et al. 2015
Antiseptic-resistance	<i>qacA, qacB</i>	F: TCCTTTTAATGCTGGCTTATACC R: AGCCKTACCTGCTCCAACATA	220	56	30	Zmantar et al., 2011
	<i>qacC</i>	F: GGCTTTTCAAAATTTATACCATCCT R: ATGCGATGTTCCGAAAATGT	249	56	30	Zmantar et al., 2011
Antibiotic-resistance	<i>blaZ</i>	F: CAGTTCACATGCCAAAGAG R: TACTCTTGCGGTTTC	772	57	35	Gómez-Sanz et al., 2010
	<i>mecA</i>	F: GGGATCATAGCGTCATTATTC R: AACGATTGTGACACGATAGCC	527	57	33	Gómez-Sanz et al., 2010
	<i>mecC</i>	F: GCTCCTAATGCTAATGCA R: TAAGCAATAATGACTACC	304	51	30	Cuny et al., 2011
	<i>aac6'aph2'</i>	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	220	55	32	Gómez-Sanz et al., 2010
	<i>ant4</i>	F: GCAAGGACCGACAACATTTTC R: TGGCACAGATGGTCATAACC	165	51	30	Rossi, 2016
	<i>ermA</i>	F: TCTAAAAAGCATGTAAAAGAA R: CTTCGATAGTTTATTAATATTAG	645	51	30	Gómez-Sanz et al., 2010
	<i>ermB</i>	F: GAAAAGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTA	639	49	35	Gómez-Sanz et al., 2010
	<i>ermC</i>	F: TCAAAACATAATATAGATAAA R: GCTAATATTGTTTAAATCGTCAAT	642	52	30	Gómez-Sanz et al., 2010
	<i>ermF</i>	F: CGGGTCAGCACTTTACTATTG R: GGACCTACCTCATAGACA AG	466	51	30	Chung et al., 1999

	<i>ermT</i>	F: CCGCCATTGAAATAGATCCT R: TTCTGTAGCTGTGCTTTCAAAAA	200	52	30	Gómez-Sanz et al., 2010
	<i>msrA</i>	F: GGCACAATAAGAGTGTTTAAAGG R: AAGTTATATCATGAATAGATTGTCCTGTT	940	54	30	Gómez-Sanz et al., 2010
	<i>cfr</i>	F: TGAAGTATAAAGCAGGTTGGGAGTCA R: ACCATATAATTGACCACAAGCAGC	746	50	35	Kehrenberg & Schwarz, 2006
	<i>vgaC</i>	F: ACGAGGGGACAATCACGCCG R: TTGGTCGTCGGTTCGTCTGC	156	58	33	Gómez-Sanz et al., 2010
	<i>lnuA/A'</i>	F: GGTGGCTGGGGGGTAGATGTATTAAGTGG R: GCTTCTTTTGAATAACATGGTATTTTTTCGATC	323	57	34	Gómez-Sanz et al., 2010
	<i>lnuB</i>	F: CCTACCTATTGTTTGTGGAA R: ATAACGTTACTCTCTATTTC	944	54	34	Arana et al., 2014
	<i>lsaB</i>	F: TGCCGAAGCCATGTACCGTCC R: CGGTTAGACCAACCAGCCGAACG	396	56	30	Gómez-Sanz et al., 2010
	<i>lsaE</i>	F: CGGCTATAGAACCGTTTGT R: AGTTATTGTGGCAACTCAAATC	310	52	30	Wendlandt et al., 2013
	<i>tetK</i>	F: TTAGGTGAAGGGTTAGGTCC R: GCAAACCTCATTCCAGAAGCA	697	55	25	Gómez-Sanz et al., 2010
	<i>tetL</i>	F: CATTTGGTCTTATTGGATCG R: ATTACACTTCCGATTTCCGG	456	50	25	Gómez-Sanz et al., 2010
	<i>tetM</i>	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	657	52	25	Agersø & Sandvang, 2005
<i>agr</i> typing	<i>agrI</i>	F: GTCACAAGTACTATAAGCTGCGAT R: ATGCACATGGTGCACATGC	440	55	25	Shopsin et al., 2003
	pan- <i>agrII</i>	F: GTATTACTAATTGAAAAGTGCCATAGCC R: ATGCACATGGTGCACATGC	572	55	25	Shopshin et al., 2003.
	pan- <i>agrIII</i>	F: CTGTTGAAAAAGTCAACTAAAAGCTC R: ATGCACATGGTGCACATGC	406	55	25	Shopshin et al., 2003.
	pan- <i>agrIV</i>	F: CGATAATGCCGTAATACCCG R: ATGCACATGGTGCACATGC	588	55	25	Shopshin et al., 2003.
	pan- <i>agr</i>					
<i>spa</i> typing	<i>spa 1</i>	F: TAAAGACGATCCTTCGGTGAGC	Variable	53	35	www.ridom.com
	<i>spa 2</i>	R: CAGCAGTAGTGCCGTTTGCT				

<sup>1</sup>F= Forward; R= Reverse

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## **CHAPTER 4**

*Antimicrobial efficacy of selected essential oils against S. aureus from subclinical bovine mastitis*

**Antimicrobial activity of selected essential oils against *Staphylococcus aureus* from bovine mastitis**

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### Abstract

**Abstract:** *Staphylococcus aureus* is a major cause of subclinical mastitis in dairy cows, and its development of antibiotic resistance has limited effective treatment efficacy. Essential oils (EOs) are natural products with a wide range of antimicrobial properties that could be used to treat bovine mastitis. This study aims to investigate the antimicrobial activity of EOs against *S. aureus* isolated from subclinical bovine mastitis cases in the State of São Paulo – Brazil. A total of 14 *S. aureus* isolates were selected, based on the presence of biofilm-forming genes (*icaA*, *icaD*, and *bap*), and were cultured to a final concentration of  $10^3$  CFU.mL<sup>-1</sup> for Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) analysis of five EOs (*Citrus aurantium bergamia* - bergamot, *Copaifera reticulata* - copaiba, *Foeniculum vulgare* - fennel, *Zingiber officinale* -ginger, and *Ocimum basilicum* - basil). Chemical compositions of EOs were characterized using gas chromatography coupled to a mass selective detector (GC/MSD). Basil and bergamot EOs exhibited the highest antimicrobial activity against *S. aureus* strains, with mean MIC/MBC values of  $1.561 \pm 0.223/2.806 \pm 0.255$  mg.mL<sup>-1</sup> and  $2.782 \pm 0.228/4.396 \pm 0.198$  mg.mL<sup>-1</sup>, respectively. The primary compounds in basil EO were methyl-chavicol, linalool, and  $\alpha$ -humulene, while bergamot EO predominantly contained linalyl acetate, limonene, and linalool. This research highlights the potential of basil and bergamot EOs as natural antimicrobial agents for treating bovine mastitis caused by *S. aureus*, offering a potential alternative to traditional antibiotics and contributing to animal welfare and public health. In addition, it emphasizes the need for further studies to validate the long-term effects, optimal dosages, and application methods.

**Keywords:** Herbal medicine; antimicrobial effect; dairy cows; MIC; MBC; bovine mastitis; antibiotic resistance

## 1. Introduction

Bovine mastitis, one of the most prevalent and costly diseases of dairy cows, is primarily caused by *Staphylococcus aureus* (Zaatout et al., 2020). This pathogen is a Gram-positive coccus, characterized by its catalase-positive and facultative anaerobic nature, which can grow in a wide range of temperatures, pH, and water activity (Bhunias, 2018). It is notably prevalent in Brazil, where it is a leading cause of foodborne outbreaks (A. C. da Silva et al., 2020). It possesses a range of virulence factors, including enzymes, toxins, and survival strategies that facilitate the evasion of host defenses, such as the ability to adhere to surfaces and form biofilms (Bhunias, 2018; Jenul & Horswill, 2019; Zhu et al., 2018).

Mastitis can be classified into two forms: clinical mastitis, which shows evident signs, such as udder inflammation, redness, warmth, and swelling, representing the cardinal signs of inflammation. Additionally, it leads to visible changes in milk, such as clots and discoloration (Sharun et al., 2021). The second form is subclinical mastitis (SM), which lacks noticeable signs; however, due to the silent development of the disease, SM has the tendency to progress into chronic infections (Gonçalves et al., 2020; Tomanić et al., 2023). The effect of mastitis on dairy farms is significant, leading to reduced milk production, increased culling rates, and decreased milk quality (Gonçalves et al., 2018; Sharun et al., 2021). Conventional treatments for mastitis primarily depend on antibiotic usage (Ruegg, 2017). Unfortunately, the excessive and inappropriate use of antibiotics has contributed to antibiotic resistance in *S. aureus*, leading to significant hindrances in mastitis treatment. As a result, the efficacy of conventional treatment protocols have been compromised (da Silva Abreu et al., 2021; Sharun et al., 2021). Consequently, there is a

growing need to find effective alternatives for mastitis treatments and to reduce the antibiotic resistance problem (Cheng & Han, 2020; Tomanić et al., 2023).

Plant extracts have emerged as significant therapeutic alternatives, particularly due to the increasing antimicrobial resistance to conventional antibiotics (Ni et al., 2021). Throughout the years, common knowledge has proven indispensable for gaining insight into the healing effects of essential oils (EOs). EOs are composed of a complex mixture of volatile molecules, which are specific to each plant, including their range of bioactivities; these molecules include alkaloids, monoterpenes, carotenoids, flavonoids, isoflavones, phenolic acids, and oxygen-containing, non-oxygenated terpene hydrocarbons, and aldehydes (Ni et al., 2021; Tavares et al., 2022). The mechanisms of antimicrobial activity of EOs include degradation of the cell wall and cytoplasmic membrane, cytoplasm coagulation, inhibition of toxic bacterial metabolites, and inhibition of the bacterial efflux system. However, the efficacy of antimicrobial activity can vary depending on the pathogen and the composition of the EO (Álvarez-Martínez et al., 2021; Caneschi et al., 2023; Man et al., 2019). For example, these compounds have a hydrophobic characteristic, which confers greater effectiveness against Gram-positive bacteria. On the other hand, Gram-negative bacteria present lipopolysaccharides that cause rejection of EOs, hindering their absorption and antimicrobial activity (Álvarez-Martínez et al., 2021; Ni et al., 2021).

The use of EOs as an alternative to treat *S. aureus* bovine mastitis has not been extensively studied. There is limited research on the topic (Caneschi et al., 2023), and further studies are needed to confirm the safety and efficacy of EOs for use in dairy cows. In this context, studies regarding the antimicrobial activity of new compounds, including

EOs, especially to develop new treatments against mastitis, are increasingly important (Lopes et al., 2020).

Based on the abovementioned reasons, this study aims to evaluate the antimicrobial activity of five EOs that could be considered potential antimicrobial agents for treating subclinical mastitis caused by *S. aureus*. It seeks to identify natural compounds relevant to the dairy industry by offering effective and sustainable mastitis treatments.

## **2. Materials and Methods**

### *2.1. Preparation of bacterial strains*

Before initiating the study, ethical approval was secured from the Ethical Committee on Animal Use at the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil, with protocol number 3020/2013. A total of 14 *S. aureus* strains were initially sourced from raw milk. Samples were collected from cows with SM from commercial dairy herds in the Midwest of São Paulo State, Brazil. The selection criteria for cows were specifically designed to focus on those exhibiting signs of chronic SM, whilst excluding cows with a history of clinical mastitis. Identification of SM was based on consecutive biweekly somatic cell count (SCC) tests and culture-positive results. Specifically, cows were considered to have SM if they had at least two out of three SCC tests with counts exceeding 200,000 cells/mL. These *S. aureus* strains were sourced from a prior companion study conducted at the Milk Quality Research Laboratory of the University of São Paulo. A comprehensive description is available in previous studies (Gonçalves et al., 2018, 2020). Therefore, the strains were selected based on the presence of biofilm-forming genes (*icaA*, *icaD*, and *bap*). The cultures were stored on nutrient agar

slopes at 4°C until microbiological analysis. Then, bacterial suspensions were prepared by subculturing 100 µL of each strain in 9 mL of Brain Heart Infusion broth (BHI, Kasvi, Brazil) and incubated at 35 ± 1°C for 24 h. After this period, inoculum was standardized in sterile 0.85% saline solution to a turbidity of 0.5 on the McFarland scale (equivalent to 1.5 × 10<sup>8</sup> CFU/mL). For the purpose of this study, bacterial suspensions were adjusted to contain 10<sup>3</sup> colony-forming units (CFU)/mL.

## 2.2. Essential oils

A total of five commercial EOs were purchased from BioEssência® (São Paulo, Brazil). Table (1) describes the characteristics of the EOs evaluated in our study.

**Table (1).** Botanical and geographical characteristics of EOs.

Botanical Origins	Plant Family	Common names of EOs	Geographical Origin
<i>Citrus aurantium bergamia</i>	Rutaceae	Bergamot	Brazil
<i>Copaifera reticulata</i>	Fabaceae	Copaiba	Brazil
<i>Foeniculum vulgare</i>	Apiaceae	Fennel	Brazil
<i>Zingiber officinale</i>	Zingiberaceae	Ginger	Brazil
<i>Ocimum basilicum</i>	Lamiaceae	Basil	Brazil

## 2.3. GC/MSD analysis of EOs

The chemical composition of EOs was performed at the Multidisciplinary Center of Chemical, Biological and Agricultural Research (CPQBA) of UNICAMP using an HP-6890 gas chromatograph coupled with an HP-5975 mass selective detector (GC/MSD). The analyses were carried out using the following analytical conditions: HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm), injector temperature of 220 °C, column temperatures of 60 °C, 3 °C/min, 240 °C, and detector temperature of 250 °C. Injected



volume was 1  $\mu\text{l}$ , carrier gas was helium at a 1  $\text{mL}/\text{min}^{-1}$  flow rate and a 40:1 split ratio. Identification of the analytes was performed by comparing their retention indices (IR) using a coinjection of a mixture of hydrocarbon standards (C-8 to C-24) with the electronic equipment's database from the National Institute of Standards and Technology (NIST-11) (*National Institute of Standards and Technology. NIST Chemistry WebBook SRD 69.*, n.d.) and available literature data (Adams, 2007).

#### 2.4. Determination of minimum inhibitory concentrations (MIC)

The determination of broth microdilution technique was performed using the microdilution technique. Initially, EO density was performed in triplicate to determine the volume of EO stock solution needed to achieve the desired concentration by the relationship between mass and volume. For the MIC procedures, 360  $\mu\text{l}$  of TSB broth (Tryptone Soy Broth) was dispensed into the first column of a 96-well acrylic plate, along with 0.5% Tween 80 and 40  $\mu\text{l}$  of essential oil, reaching a final concentration of 10% of essential oil in the first well. Then, 200  $\mu\text{l}$  of TSB broth with 0.5% Tween 80 were distributed into the wells of the following columns. After homogenization of the contents in the wells of the first column, a two-fold serial dilution was performed. An aliquot of 200  $\mu\text{l}$  of the mixture from the first well of the row was transferred into the second well, and so on consecutively. Subsequently, the strains were standardized on the McFarland scale, adjusted with sterile saline to a concentration of  $10^5$  CFU/mL, and 2  $\mu\text{l}$  of the microbial suspension obtained were inoculated, reaching a final concentration of  $10^3$  CFU/mL per well. The microplates were incubated at 37 °C for 24 h. Afterward, growth confirmation was carried out in the wells by applying 50  $\mu\text{l}$  of resazurin solution with a concentration of 0.01% and then waiting ~5 to 10 minutes to perform a visual reading of the occurrence of colorimetric reaction, and evaluation of the growth inhibition of

microorganisms (Da Silva et al., 2018; De Souza et al., 2020). All tests were performed in triplicate. The MIC was calculated according to the well with the lowest concentration of essential oil that presented a blue color, which indicates the absence of microbial growth.

#### *2.5. Determination of the Minimum Bactericidal Concentration (MBC)*

To determine the Minimum Bactericidal Concentration, 10  $\mu$ l were removed from the MIC and the three previous wells and subsequently deposited on nutrient agar plates by micro-drop technique. Later, the plates were incubated at 37 °C/24 h. At the end of incubation, a reading was performed, and the following criteria were considered: Sections with microbial growth indicated bacteriostatic activity of the essential oil in question at the analyzed concentration; and sections with no microbial growth showed bactericidal activity. The well with the lowest concentration that exhibited a bactericidal effect was recorded as MBC (Knezevic et al., 2016).

#### *2.6. Statistical Analysis*

Data were reported as the mean  $\pm$  standard deviation (SD) of three measurements. Statistical analysis to evaluate the difference between groups was performed using the analysis of variance (ANOVA), followed by Tukey's range test to analyze data with the significance level set at  $p < 0.05$ . All statistical analyses were performed using SPSS Statistics 21 software (IBM Corp., Armonk, NY, USA) (Xing et al., 2019).

### **3. Results**

#### *3.1. Chemical composition of the essential oils*

The composition of EOs obtained by GC/MSD analysis are presented in Table (2), and their chromatograms are shown in Figure (1). Among the several chemical components found in bergamot EO, linalyl acetate, limonene, and linalool were the most abundant compounds (accounting for 39.18%, 29.88%, and 16.91%, respectively). Sesquiterpenes were found as the major compound of ginger EO, containing mainly alpha-zingiberene (32.76%), ar-curcumene (14.03%), and  $\beta$ -sesquiphellandrene (12.95%). Basil EO presented phenylpropanoids, monoterpenes, and sesquiterpenes, especially methyl-chavicol (72.86%), linalool (18.76%), and  $\alpha$ -humulene (1.55%) as its major compounds. The copaiba oil is rich in sesquiterpenes, containing mainly trans-caryophyllene (47.43%),  $\alpha$ -humulene (7.95%),  $\alpha$ -trans-bergamotene (7.59%), and  $\alpha$ -copaene (6.81%). Fennel EO contained mainly a combination of monoterpenes and phenylpropanoids, with trans-anethole (81.36%), followed by fenchone (5.84%) and  $\alpha$ -pinene (4.56%) being the primary compounds.

**Table (2).** Chemical composition of EOs identified by GC/MSD.

Essential Oil	RI	RI (Lit.)	Compound	% rel.
Bergamot	932	932	$\alpha$ -pinene	0.56
	971	969	sabinene	0.59
	975	974	$\beta$ -pinene	3.49
	989	988	$\beta$ -myrcene	0.77
	1023	1020	p-cymene	0.69
	1029	1029	Limonene	29.88
	1057	1054	$\gamma$ -terpinene	3.83
	1102	1095	Linalool	16.91
	1227	1227	nerol (cis-geraniol)	1.06
	1239	1238	neral (cis-citral)	0.69
	1258	1257	Linalyl acetate	39.18
	1269	1267	geranial (trans-citral)	0.86
	1372	1423	linalyl butyrate	1.48
Copaiba	1335	1335	$\delta$ -elemen	0.67
	1347	1495	$\alpha$ -cubebene	1.36

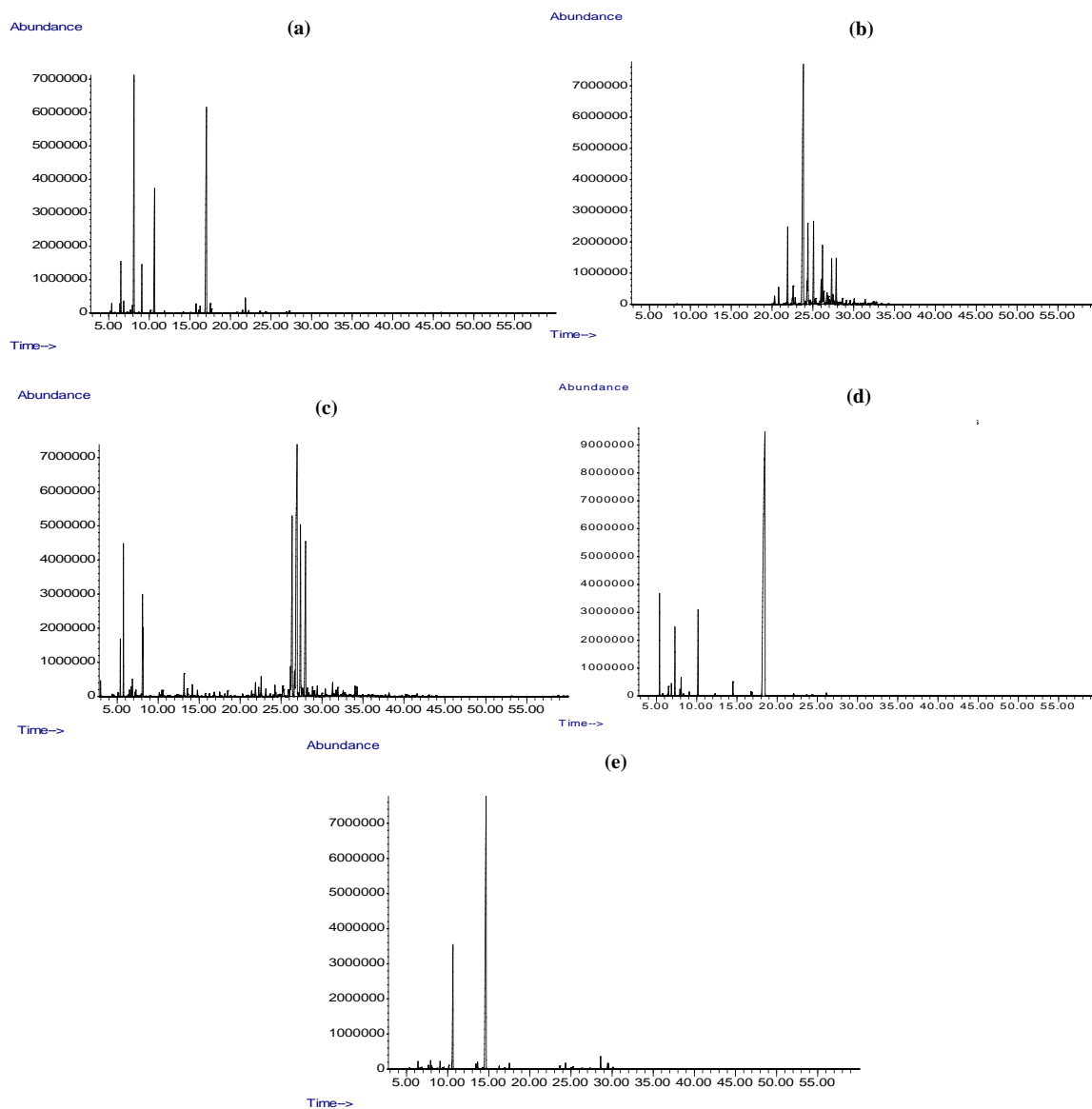
	1374	1374	$\alpha$ -copaene	6.81
	1388	1387	$\beta$ -cubebene	0.44
	1390	1389	$\beta$ -elemene	1.75
	1396	1398	cyperene	0.54
	1421	1419	trans-caryophyllene	47.43
	1432	1434	$\gamma$ -elemene	2.04
	1435	1432	$\alpha$ -trans-bergamotene	7.59
	1452	1452	$\alpha$ -humulene	7.95
	1456	1454	trans- $\beta$ -farnesene	0.36
	1458	1458	allo-aromadendrene	0.43
	1475	1478	$\gamma$ -muurolene	2.43
	1479	1484	germacrene D	5.69
	1483	-	M = 204	1.59
	1492	1493	epi-cubebol	1.04
	1493	1500	bicyclogermacrene	0.75
	1497	1500	$\alpha$ -muurolene	0.66
	1507	1505	$\beta$ -bisabolene	4.11
	1511	1513	$\gamma$ -cadinene	0.6
	1521	1522	$\delta$ -cadinene	4.11
	1541	-	M = 204	0.59
	1579	1582	caryophyllene oxide	0.59
	1614	1618	junenol	0.46
	932	932	$\alpha$ -pinene	4.56
	975	974	$\beta$ -pinene	0.55
	989	988	$\beta$ -myrcene	0.59
	1004	1002	$\alpha$ -phellandrene	3.55
	1023	1020	p-cymene	0.34
Fennel	1027	1029	limonene	1.2
	1056	1054	$\gamma$ -terpinene	0.21
	1088	1086	fenchone	5.84
	1197	1195	p-allylanisole (estragole)	0.97
	1251	1249	cis-anethole	0.63
	1291	1282	trans-anethole	81.36
	1478	1484	germacrene D	0.21
	800	801	hexanal	0.28
	932	932	$\alpha$ -pinene	1.66
Ginger	947	946	camphene	5.01
	984	981	6-methyl-5-hepten-2-one	0.31
	989	988	$\beta$ -myrcene	0.57
	1028	1029	limonene	4.88
	1030	1026	1,8-cineole (eucalyptol)	2.27

	1164	1165	endo-borneol	1.13
	1174	-	M = 166	0.24
	1189	1186	$\alpha$ -terpineol	0.54
	1373	1374	$\alpha$ -copaene	0.74
	1382	1379	geranyl-acetate	0.42
	1390	1389	$\beta$ -elemene	1.17
	1404	1405	sesquijene	0.37
	1431	1434	$\gamma$ -elemen	0.45
	1455	1454	trans- $\beta$ -farnesene	0.42
	1478	1478	$\gamma$ -muurolene	1.75
	1483	1479	ar-curcumene	14.03
	1490	1496	valenceno	1.58
	1498	1493	$\alpha$ -zingiberene	32.76
	1509	1505	$\beta$ -bisabolene	11.72
	1516	1520	7-epi- $\alpha$ -selenene	0.62
	1525	1521	$\beta$ -sesquiphellandrene	12.95
	1531	1529	trans- $\gamma$ -bisabolene	0.39
	1547	1548	elemol	0.51
	1562	1561	trans-nerolidol	0.56
	1587	-	M = 222	0.34
	1611	-	M = 222	0.77
	1628	-	M = 222	0.53
	1685	-	M = 222	0.56
	1692	-	M = 220	0.48
	971	969	sabinene	0.57
	1015	1014	$\alpha$ -terpinene	0.32
	1023	1020	p-cymene	0.71
	1056	1054	$\gamma$ -terpinene	0.69
	1087	-	n.i.	0.37
	1101	1095	linalool	18.76
	1171	1167	menthol	0.61
Basil	1176	1174	terpin-4-ol	0.82
	1202	1195	p-allylanisole (estragole)	72.86
	1269	1264	trans-citral (geranial)	0.64
	1433	1432	$\alpha$ -trans-bergamotene	0.7
	1541	1452	$\alpha$ -humulene	1.55
	1563		trans-	
		1562	methoxycinnamaldehyde	0.76
	1565	-	M = 164	0.64

RI: Retention index.

RI (Lit.): Retention index from literature data (Adams, 2007).

In order to provide a comprehensive overview of the chemical compositions of the essential oils examined in this study, we have grouped the identified compounds into their respective chemical classes based on the information delineated in Table (2). For Bergamot EO, monoterpenes emerged as the most abundant class, making up roughly 56.72% of the total composition. This was followed by esters, which contributed around 40.66%. Alcohols and aldehydes were present but less abundant, constituting approximately 1.06% and 1.55%, respectively. Copaiba EO was characterized by sesquiterpenes, accounting for 86.36% of the overall composition. Oxygenated sesquiterpenes made up a smaller fraction, approximately 0.59%. Compounds designated as 'M=204' comprised about 2.18% of the EO composition. In the case of Fennel EO, phenylpropanoids were overwhelmingly dominant, contributing to about 82.62% of the total composition. Monoterpenes followed, accounting for roughly 11.00%, while sesquiterpenes were minimally present, constituting about 0.21%. For Ginger EO, sesquiterpenes were the major class, making up approximately 73.09% of the total oil composition. Monoterpenes were also significant, contributing about 13.89%. Oxygenated monoterpenes and aldehydes were found in smaller amounts, representing 2.27% and 0.28%, respectively. Lastly, basil EO was primarily constituted of phenylpropanoids, which accounted for 73.62% of the total composition. Monoterpenes comprised the second largest category, contributing about 21.57%, while sesquiterpenes were found in lesser proportions, around 2.25% of the total composition.

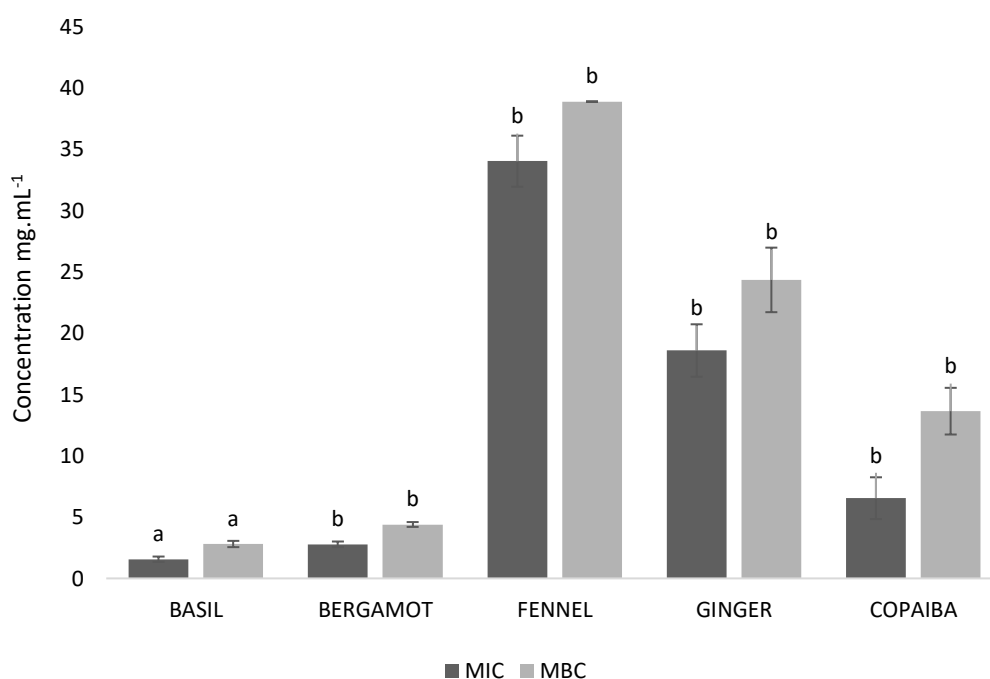


**Figure (1).** Chromatographic profiles of EOs tested by GC/MSD. The area represented by the peaks corresponds to each component's proportions in the mixture. **(a)** Bergamot EO; **(b)** Copaiba EO; **(c)** Ginger EO; **(d)** Fennel EO; **(e)** Basil EO.

### 3.2. Antimicrobial activity of EOs against *S. aureus*

The antimicrobial activity of the EOs was evaluated using MIC and MBC tests.

The results of the mean values are shown in Figure (2).



**Figure (2).** Minimum inhibitory concentration and Minimum bactericidal concentration results of EOs tested against *S. aureus* strains. Bars represent mean  $\pm$  SD. Means with different letters are significantly different ( $p < 0.05$ ).

Based on the antimicrobial activity results, the EOs displayed varying levels of effectiveness against *S. aureus* strains, with a clear decreasing order of activity: basil EO > bergamot EO > copaiba EO > ginger EO > fennel EO.

The two most effective EOs were basil and bergamot, with mean MIC/MBC values of  $1.561 \pm 0.223/2.806 \pm 0.255$  and  $2.782 \pm 0.228/4.396 \pm 0.198$  mg.mL<sup>-1</sup>, respectively. These EOs exhibited higher antimicrobial activity against *S. aureus* isolates than copaiba and ginger EO, which had mean MIC/MBC values of  $6.541 \pm 1.705/13.637 \pm 1.903$  and  $18.58 \pm 2.138/24.341 \pm 2.631$  mg.mL<sup>-1</sup>, respectively. Fennel EO had the highest MIC values with a mean of  $34.02 \pm 2.083$  mg.mL<sup>-1</sup> and did not exhibit a bactericidal effect, even at the highest concentration applied.



Concerning the results of the MIC/MBC tests for each *S. aureus* strain, they are displayed in Supplementary Materials Figure (S1) and (S2), respectively. The MIC/MBC values of basil and bergamot EOs ranged between 0.149-2.378/0.298-4.755 and 0.277-4.435/1.109-8.87 mg.mL<sup>-1</sup>, respectively. Copaiba and ginger EOs had MIC/MBC values ranging between 1.11-17.76/4.44-35.52 and 4.605-36.84/9.21-36.84 mg.mL<sup>-1</sup>, respectively. MIC values of Fennel EO ranged from 19.44 to 38.88 mg/mL<sup>-1</sup>.

Furthermore, when comparing the antimicrobial activity of the EOs, Basil emerged as the most effective EO, demonstrating significantly lower MIC and MBC against *Staphylococcus aureus*. Bergamot, Fennel, Ginger, and Copaiba showed no significant difference in their antimicrobial activities.

#### **4. Discussion**

Bovine mastitis, associated with *S. aureus* infection, demands preventive measures in dairy farming (Gonçalves et al., 2018; Ruegg, 2017; Sharun et al., 2021). Managing bovine mastitis presents a formidable challenge, mainly due to the biofilm-forming capabilities of *S. aureus*, facilitated by genes such as *icaA*, *icaD*, and *bap*. These genes enable the bacteria to firmly attach to the bovine mammary epithelium and form biofilms, which can enhance bacterial resistance to antimicrobial agents, often leading to persistent and recurrent infections (Campos et al., 2022; V. Silva et al., 2021). In light of this, our isolate selection was strategic, aiming to assess the antimicrobial potency of EOs as agents against *S. aureus*, even in the presence of genes capable of forming biofilms.

Overuse of antibiotics has led to resistance in pathogens (da Silva Abreu et al., 2021; Munive Nuñez et al., 2023), driving the search for alternative treatments without exacerbating antibiotic resistance (Lopes et al., 2020; Tomanić et al., 2023). Several

studies have shown that the chemical constituents of EOs are abundant in aromatic compounds, which can be classified into two structural groups: terpenoids and phenylpropanoids (Caneschi et al., 2023; Ni et al., 2021). These phenolic compounds of EOs can modify the permeability of the cell membrane by penetrating the phospholipid bilayer of the bacterial cell wall (Sakkas & Papadopoulou, 2017). In our study, it was observed that among the tested EOs, basil EO exhibited the highest antimicrobial activity, with low MIC/MBC; which indicates their ability to inhibit and kill *S. aureus* at relatively low concentrations. The phenolic compounds and monoterpenes found as the major components of these EOs must be associated with their antimicrobial properties (72.86%). Supporting our findings, Falowo et al. (2019) reported that basil EO contains bioactive chemicals such as estragole (41.40%), 1,6-octadien-3-ol, 3,7-dimethyl (29.49%), and bergamotene (5.32%). Based on these observations, we hypothesize that the presence of estragole and other bioactive compounds in basil EO plays a crucial role in its remarkable antimicrobial activity against *S. aureus* strains.

Terpenes and terpenoids represent the most active phytochemicals studied, with properties for treating or preventing diseases, followed by polyphenols (such as phenolic acids and flavonoids) (Álvarez-Martínez et al., 2021; Masyita et al., 2022). Our findings support these claims, as these compounds, constituting up to 90% of most EO compositions, exhibit diverse chemical and biological properties (Sakkas & Papadopoulou, 2017). Our study reported that bergamot EO was found to be the second most effective antimicrobial agent against *S. aureus* strains according to the MIC/MBC results. Among the several chemical components contained in this EO, monoterpenes such as linalyl acetate (39.18%), limonene (29.88%), and linalool (16.91%) were found to be the most abundant. A study performed to discover the biological activities of

bergamot EO suggested good antimicrobial activity against *S. aureus* and identified d-limonene (60.44%) and  $\gamma$ -terpinene (20.28%) as the major compounds contributing to this activity (Xing et al., 2019). The significant presence of these active compounds, particularly the monoterpenes, in bergamot EO may account for its notable antimicrobial properties.

It is important to note that the antimicrobial activity of EOs can vary depending on the specific pathogen and the composition of the EO. For instance, EOs of basil and bergamot were found to be active against Gram-positive bacteria (*S. aureus* and *B. subtilis*). On the other hand, perilla EO strongly inhibited yeast growth (Lv et al., 2011), adding complexity to the application of EOs as antimicrobial agents. While our results are promising, indicating that basil and bergamot EO could be helpful in treating *S. aureus* infections in subclinical bovine mastitis, more research is needed. Their low MIC and MBC values indicate that they could help control the growth of *S. aureus* in milk, thereby reducing the risk of mastitis in dairy cows. However, further research must corroborate these findings, and the safety and efficacy of using EOs in dairy cows must be thoroughly evaluated. Future studies should focus on assessing the long-term effects of EO treatment, including appropriate dosage, application methods, and potential interactions with other medications or treatments. It is essential to ensure these compounds' safe and effective management in bovine mastitis, without negatively impacting milk quality or human health.

Sesquiterpenes are well-known for their bioactive properties that display good inhibitory activity against *S. aureus* (Bagheri et al., 2020). Copaiba EO varies in its phytochemical composition depending on its species. The primary components include sesquiterpenes, diterpenes, and  $\beta$ -caryophyllene (de Oliveira et al., 2020). In line with

this, our results demonstrated a high concentration of sesquiterpenes in the tested copaiba EO, containing mainly trans-caryophyllene (47.43%),  $\alpha$ -humulene (7.95%),  $\alpha$ -trans-bergamotene (7.59%), and  $\alpha$ -copaene (6.81%). These findings support the research conducted by de Faria et al. (2017), who also identified sesquiterpenes as the major compound in Copaiba EO and showed its antimicrobial activity against coagulase-negative *Staphylococcus*. Overall, our results reinforce that sesquiterpenes are bioactive compounds responsible for the observed antimicrobial activity against the strains we tested.

Studies have shown that Ginger EO can help prevent the growth of various pathogens by targeting the bacterial cell membrane and genetic material (Ni et al., 2021; Wang et al., 2020). Although Ginger EO exhibits some antimicrobial activity against *S. aureus* strains, its efficacy appears to be comparatively lower when compared to the other EOs tested in our study. Sesquiterpenes were the primary compound of this EO, mainly containing  $\alpha$ -zingiberene, ar-curcumene, and  $\beta$ -sesquiphellandrene. In line with our findings, Dal Pozzo et al. (2011) evaluated the activity of EOs from various plants, including ginger, basil, rosemary, and sage EO, along with the major compound cineole, an reported no antimicrobial activity against *S. aureus*. While ginger EO exhibits some antimicrobial effects, our study confirms that it has lower efficacy compared to other tested EOs.

Based on the MIC results, the fennel EO, predominantly composed of monoterpenes and phenylpropanoids, exhibited minimal activity and showed no activity in the MBC test against most of the tested *S. aureus* strains. Interestingly, our results concerning fennel EO diverge from some of the existing literature. In comparison, some studies have reported its antimicrobial potential (Ghasemian et al., 2020; Moreira

Gonçalves et al., 2020). Likewise, Kwiatkowski et al. (2019) demonstrated that trans-anethole at a concentration of 4% displayed antistaphylococcal effects. However, our results are consistent with those of Rani et al. (2021), who determined that fennel EO has insignificant impact on *S. aureus*. Overall, these findings suggest that fennel EO may not be a potent solution for fighting against *S. aureus* that have biofilm-forming genes (e.g., *icaA*, *icaD*, and *bap*, such as the current study).

Above all, and based on previous studies that report EO compounds represent the main source of chemical diversity (Álvarez-Martínez et al., 2021; Bagheri et al., 2020; Ni et al., 2021), we support the relevance of the current finding that includes natural compounds' biological properties. The discovery of these antimicrobial agents against *S. aureus*, which causes bovine mastitis, may have several benefits not only in the dairy industry but also for the pharmaceutical and medical industries. In addition, this study offers practical benefits for dairy breeders. Using EOs as an antibiotic alternative could align with sustainable farming practices. This is especially valuable in the current context of increased antibiotic resistance, and consumer preference for natural treatments. These insights contribute to improved herd health, milk quality, and overall farm sustainability. Given the rich source of chemical diversity in EOs, future studies should focus on key bioactive elements in EOs with proven efficacy against *S. aureus*. Additionally, further studies are needed to confirm the safety and effectiveness of EOs for use in dairy cows with bovine mastitis; and evaluate the appropriate dosage and application methods. In this context, ensuring that EOs do not negatively impact milk quality or human health becomes essential.

## **5. Conclusions**

In conclusion, our study reported that basil and bergamot are the most efficient antimicrobial EOs among the five EOs tested against *S. aureus* causing bovine mastitis. The phenylpropanoid compounds and monoterpenes found to be the major components of these EOs must be associated with their antimicrobial properties. This study contributes to the development of potential alternatives to conventional antibiotics, as an approach to mitigate the challenges posed by antibiotic-resistant *S. aureus*.

**Supplementary Materials:** The following supporting information can be downloaded at: (The access link to the supplementary data will be provided in the final version of the article upon acceptance), Figure (S1): Minimum inhibitory concentration results of EOs tested against *S. aureus* strains, Figure (S2): Minimum bactericidal concentration results of EOs tested against *S. aureus* strains.

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#### **Abbreviations:**

The following abbreviations are used in this manuscript:

EO	Essential oil
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
GC/MSD	Gas chromatography coupled to mass selective detector
CFU/mL	Colony-forming units per milliliter
SM	Subclinical mastitis
SCC	Somatic cell count
BHI	Brain heart infusion broth
SD	Standard deviation
ANOVA	Analysis of variance

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## SUPPLEMENTARY MATERIAL DATA

## Antimicrobial activity of selected essential oils against *Staphylococcus aureus* isolated from bovine mastitis

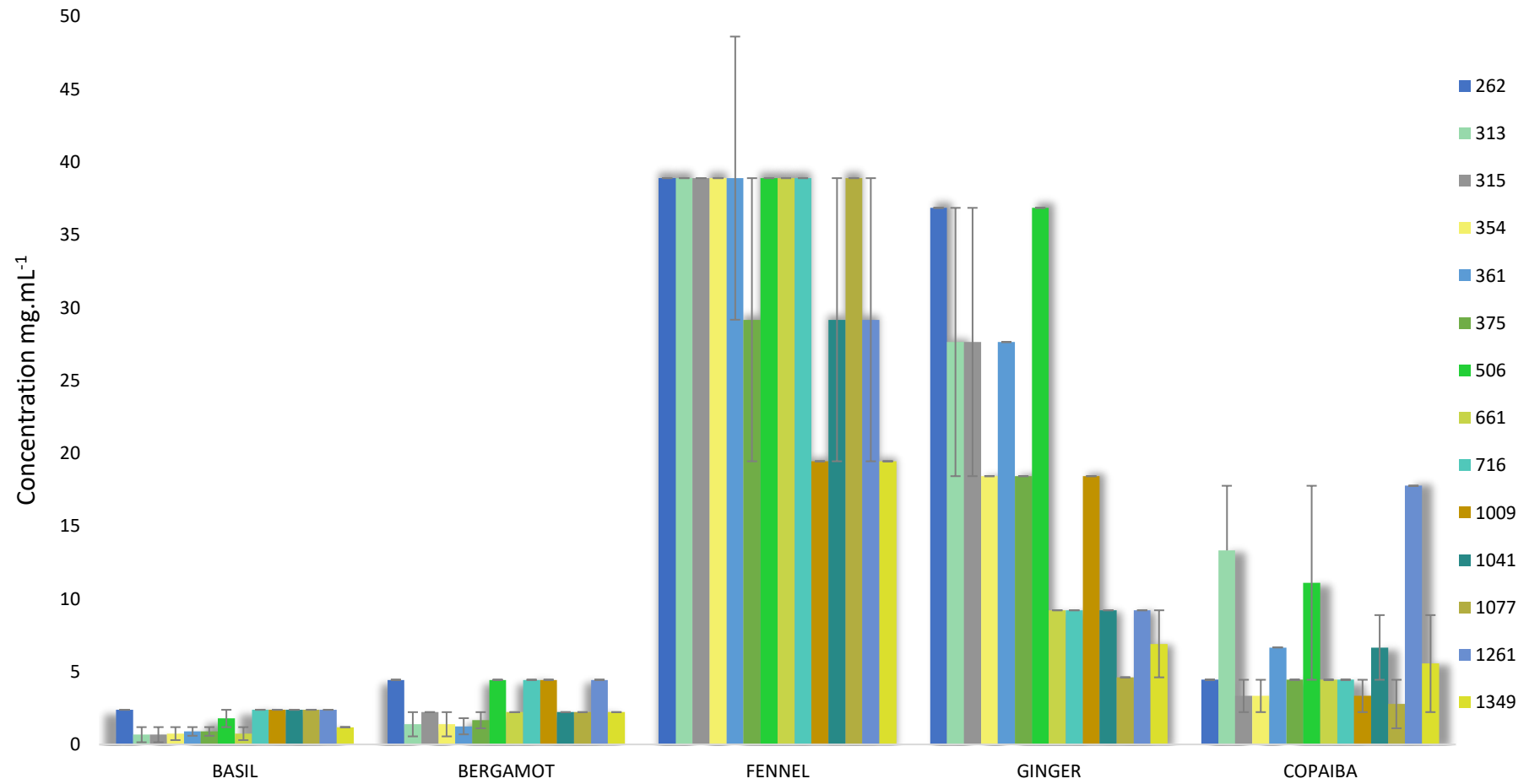
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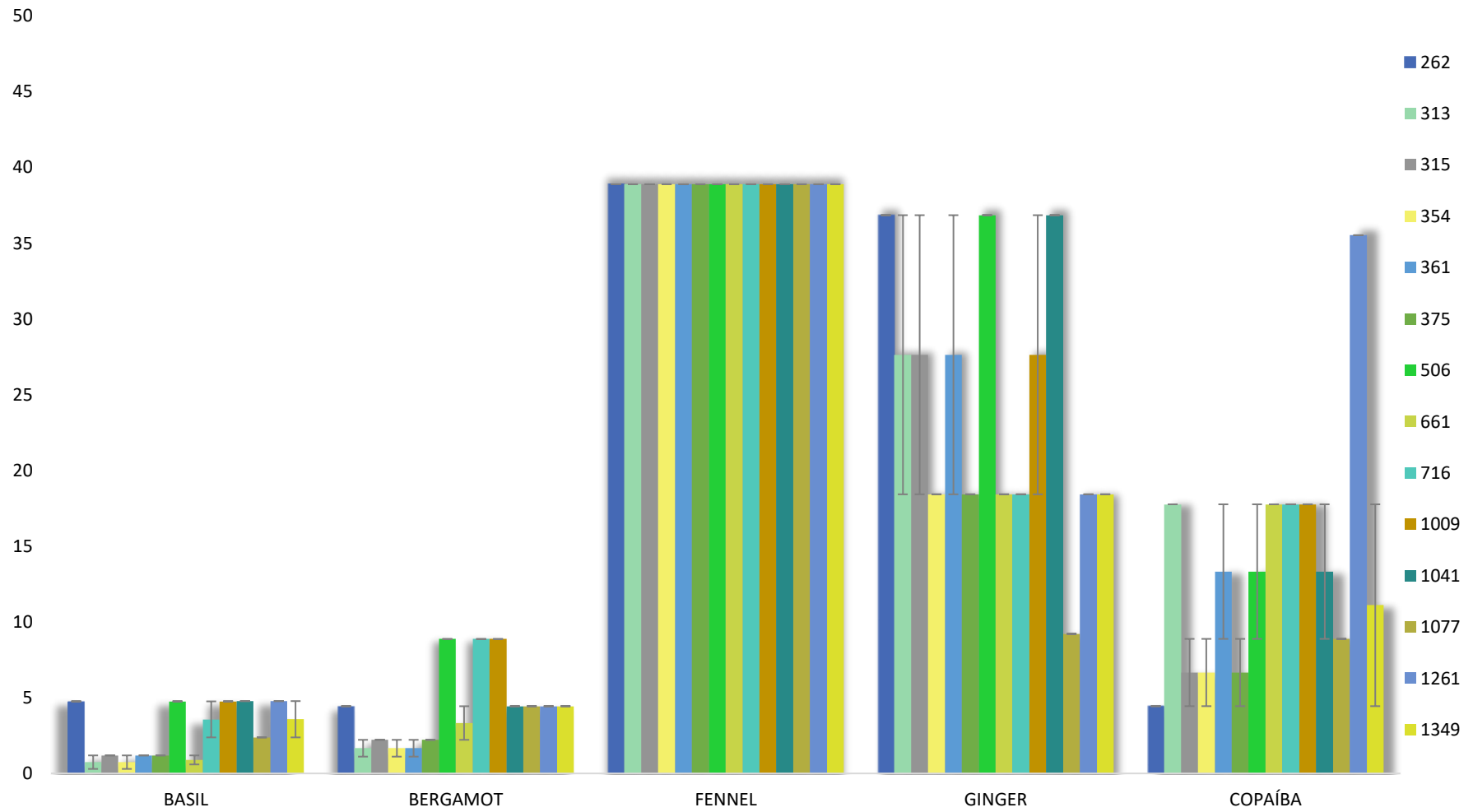
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**Figure S1.** Minimum inhibitory concentration results of EOs tested against *S. aureus* strains.



**Figure S2.** Minimum bactericidal concentration results of EOs tested against *S. aureus* strains.

## CHAPTER 5

*The potential of clove, arrayan, and spearmint essential oils as natural antibiotic alternatives against biofilm-forming Staphylococcus aureus*

**The potential of clove, arrayan, and spearmint essential oils as natural antibiotic alternatives against biofilm-forming *Staphylococcus aureus***

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## ABSTRACT

The rise of antibiotic-resistant *Staphylococcus aureus* in bovine mastitis has intensified the need for alternative therapeutic strategies. This study investigates the potential of selected essential oils (EOs) and their synergistic combinations as natural alternatives to antibiotics, specifically targeting biofilm-forming *S. aureus* isolates isolated from bovine mastitis obtained in the State of São Paulo – Brazil. In this experimental study, eight different isolates were selected, and the bacterial suspensions were inoculated in a final concentration of  $10^7$  CFU.mL<sup>-1</sup> to perform the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Luma chequeen* (Arrayan), *Mentha spicata* (Spearmint) and *Eugenia caryophyllus* (Clove) EOs. Chemical compositions of EOs were analyzed by gas chromatography coupled to a mass selective detector (GC/MSD). The fractional inhibitory concentration (FIC) index was investigated for the synergism of their possible combinations. Sub-minimum inhibitory concentrations (sub-MICs) were used to evaluate biofilm formation. In addition, the cytotoxicity of EOs on Vero cells was assessed using the MTT assay. Results were quantified using Optical Density (OD) measurements. GC/MSD analysis identified the main constituents of clove, arrayan, and spearmint EOs, which were eugenol (86.25%), limonene (32.64%), and carvone (63.21%), respectively. The MIC and MBC values ranged from 0.39 – 1.10 mg.mL<sup>-1</sup> and for 0.66 – 2,20 mg.mL<sup>-1</sup> for clove, 1.12- 4.48 mg.mL<sup>-1</sup> and 2.99 – 14.93 mg.mL<sup>-1</sup> for arrayan, and 1,35 – 18.56 mg.mL<sup>-1</sup> and 7.73 – 18.56 mg.mL<sup>-1</sup> for spearmint, respectively. Although cloves obtained better MIC values when analyzed alone, the best combination was spearmint + arrayan, which displayed a synergistic effect. For biofilm analyses, at 24 hours, the OD was 0.085, while at 48 hours, it reduced to 0.078. Further, OD decreased from 0.094 at 1/4 MIC to 0.069 at 1/16 MIC. Tested EOs showed cytotoxicity on Vero cells; clove EO had an IC<sub>50</sub> of 0.021, while arrayan EO had a higher IC<sub>50</sub> of 0.231. While clove EO exhibited the best antimicrobial and antibiofilm properties when tested alone, the combination of spearmint and arrayan showed synergistic effects. EOs tested demonstrated antimicrobial and antibiofilm activities against a variety of *S. aureus* isolates. However, the cytotoxicity observed in Vero cells raises concerns about the safety of these EOs for therapeutic application in bovine mastitis. Further studies are needed to optimize formulations and assess in vivo efficacy and safety.

**Keywords:** cytotoxicity, antimicrobial effect, MIC, FIC Index

## ***Introduction***

*Staphylococcus aureus* is a highly adaptable pathogen renowned for its ability to develop antibiotic resistance, presenting significant challenges to diagnosing and treating subclinical bovine mastitis (Bonsaglia et al., 2018; Cheng and Han 2020; Tomanić, Samardžija, and Kovačević 2023). Its adaptability is complicated by several virulence factors, including enzymes and toxins, which are crucial in its disease-causing capability. Furthermore, this pathogen employs survival strategies to persist and defeat host defenses, such as the adhesion capacity and biofilm formation (Bhunja 2008; Jenul and Horswill 2019; Zhu et al. 2018). In addition, the tendency of these infections to become more chronic has limited treatment for this disease (Ruegg 2017; Zaatout, Ayachi, and Kecha 2020).

The pathogenicity of *S. aureus* in bovine mastitis is complicated by its ability to form biofilms. This significant virulence factor enhances their capacity to adhere to and colonize the mammary glands' epithelial surfaces, which serve as a protective barrier against antibiotics (Archer et al. 2011; Campos et al. 2022). Other virulence factors, such as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which are proteins that allow bacteria to adhere to surfaces in the mammary gland, also contribute to the persistence and severity of the infection (Aslantaş and Demir 2016; Zaatout et al. 2020; Zuniga et al. 2015).

The dairy industry faces a significant challenge in managing bovine mastitis, a prevalent disease that notably impacts milk quality and yield. Subclinical mastitis, a type of mastitis that shows no apparent signs or symptoms, is a particularly challenging condition, often caused by *S. aureus* (Cheng & Han, 2020; Dyson et al., 2022; Gonçalves et al., 2023). Historically,  $\beta$ -lactam antibiotics like penicillins have been the treatment of choice for bovine mastitis. However, their diminishing effectiveness, due to the

pathogen's adaptability and increasing antibiotic resistance, has led to an urgent call for alternative therapeutic strategies (Gonçalves et al. 2022; Lopes et al. 2020; da Silva Abreu et al. 2021; Zaatout et al. 2020).

In this context, essential oils (EOs) have emerged as a promising avenue for research. These complex mixtures of volatile compounds, including alkaloids, monoterpenes, and flavonoids (Ni et al. 2021; Tavares et al. 2022), have been used for medicinal purposes and as therapeutic agents since ancient times due to their pharmacological and psychological properties (Beeby et al. 2020). However, literature on their safety and efficacy in treating bovine mastitis is still growing, highlighting the need for further studies (Caneschi et al. 2023; Lopes et al. 2020; Tomanić et al. 2023).

EOs operate through multiple mechanisms to exert their antibacterial effects. Their lipophilic nature allows them to penetrate bacterial cell membranes easily, destabilizing cellular architecture (Sakkas and Papadopoulou 2017; Tariq et al. 2019). This disruption compromises the membrane's integrity, affecting various cellular functions such as membrane transport mechanisms, energy production, and other metabolic activities (Ni et al. 2021). Therefore, it is noteworthy to consider the complexity of bacterial cell walls. Gram-negative bacteria, for instance, have a complex lipopolysaccharide layer that can hinder the absorption of EOs, making them more resistant to these compounds (Álvarez-Martínez et al. 2021; Ni et al. 2021). On the other side, Gram-positive bacteria have simpler, peptidoglycan-based cell walls, making them more susceptible to EO treatments (Caneschi et al. 2023; Patterson, McElmeel, and Wiederhold 2019).

EOs display a range of antimicrobial activities, which can be influenced not only by their primary components but also by potential synergistic interactions with trace elements (Bagheri et al. 2020; Chouhan, Sharma, and Guleria 2017) thereby contributing

to combating microorganisms that may cause diseases (Álvarez-Martínez et al. 2021; Caneschi et al. 2023). Some studies suggest that specific blends of EOs can enhance their antimicrobial efficacy (Caneschi et al. 2023; Milagres de Almeida et al. 2023). However, the potential interactions between different compounds must be considered, as they can be synergistic, additive, or even antagonistic (Chouhan et al. 2017). Furthermore, EOs have shown promise in inhibiting biofilm formation by interfering with quorum sensing mechanisms, suppressing gene transcription related to bacterial motility, or directly affecting bacterial motility (Melo et al. 2019). Given the complexity of EOs and their potential for synergistic interactions, they offer a promising avenue for developing new antimicrobial agents (Bagheri et al. 2020; Chouhan et al. 2017; Tariq et al. 2019).

Cytotoxicity is another crucial factor to consider when evaluating the potential of EOs as alternative treatments. The safety profile of EOs can vary, depending on the type of EO, its concentration, and the specific cell type risks exposed (García-Salinas et al. 2018; Ribeiro et al. 2020). In vitro assays, such as the MTT test, offer a reliable method for assessing the cytotoxicity of these compounds (Brożyna et al. 2021; Surowiak et al. 2022)

Based on the reasons mentioned above and the increasing prevalence of antibiotic-resistant *S. aureus* in bovine mastitis cases highlights the importance of exploring alternative treatments, this study aims to evaluate the antimicrobial and antibiofilm efficacy of clove, arrayan, and spearmint EOs against *S. aureus* isolated from bovine mastitis, in vitro techniques, including cytotoxicity tests, were used to identify the most effective and safe EOs for potential therapeutic applications. Given the increasing failure rates of conventional antimicrobial treatments, this research offers valuable insights into alternative treatments for bovine mastitis, thereby contributing to more sustainable and effective disease management in the dairy industry.

## ***Material and methods***

### ***Preparation of bacterial isolates***

A total of 8 *S. aureus* isolates were used in this study and came from raw milk from cows diagnosed with SM in the State of São Paulo, Brazil. These isolates, detailed in Table 1, were provided from a previous study carried out at the Milk Quality Research Laboratory, University of São Paulo, as described by Gonçalves et al. (2018), and were previously characterized with the presence of biofilm-forming and MSCRAMMs genes (Munive Nuñez et al. 2023). The bacterial cultures were obtained from frozen stocks, and then 100 µL of each isolate was subcultured into 9 mL of Brain Heart Infusion broth (BHI, Kasvi, Brazil) and incubated at  $35 \pm 1^\circ\text{C}$  for 24 h. Following incubation, the inoculums were standardized in sterile 0.85% saline solution to achieve a turbidity equivalent to the 0.5 McFarland standard, corresponding to approximately  $1.5 \times 10^8$  colony-forming units (CFU).mL<sup>-1</sup>. For the purpose of this investigation, the bacterial culture concentrations were adjusted to  $10^7$  CFU.mL<sup>-1</sup>.

**Table 1.** Genotypic characteristics of selected *S. aureus* isolates for biofilm formation and MCRAMMs Genes.

Isolate	Biofilm formation and MCRAMMs genes
262	<i>icaD, clfA, clfB, cna, fnbA, eno, fib, ebps</i>
313	<i>icaA, icaD, bap, clfA, clfB, fnbA, eno, fib</i>
506	<i>icaD, clfA, clfB, cna, fnbA, fib, ebps</i>
661	<i>icaA, icaD, bap, clfA, clfB, fnbA, eno, fib</i>
1009	<i>fib</i>
1077	<i>icaA, icaD, bap, clfA, clfB, fnbA, eno, fib</i>
1261	<i>icaA, icaD, bap, clfA, clfB, fnbA, eno, fib</i>
1349	<i>icaA, bap, fib</i>

### *Essential oils*

Three commercial EOs were purchased from BioEssência® (São Paulo, Brazil) for clove, Nua® (Lima, Perú) for spearmint, and Eop® (Lima, Perú) for arrayan. Table 2 details the botanical origins, plant families, common names, and geographical origins of the EOs evaluated in our study.

**Table 2.** Botanical and geographic origins of evaluated EOs.

Botanical Origins	Plant Family	Common Names of EOs	Geographical Origin
<i>Eugenia caryophyllus</i>	Myrtaceae	Clove	Brazil
<i>Luma chequen</i>	Myrtaceae	Arrayan	Peru
<i>Mentha spicata</i>	Lamiaceae	Spearmint	Peru

### *Gas Chromatography Coupled with a Mass Selective detector (GC/MSD) analysis*

The chemical composition of the EOs was analyzed at the Multidisciplinary Center of Chemical, Biological, and Agricultural Research (CPQBA) of UNICAMP. This analysis utilized an HP-6890 gas chromatograph coupled to an HP-5975 mass selective detector (GC/MSD). Analyses were carried out under the following analytical conditions: HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm), with an injector temperature set at 220°C, column temperatures of 60°C, 3°C/min, 240°C, and detector temperature of 250°C. The injected volume was 1.0 mL, the carrier gas was helium at a 1 mL/min<sup>-1</sup> flow rate, and a 40:1 split ratio. Identification of the analytes was performed by comparing their retention indices (RI) using a coinjection of a mixture of hydrocarbon standards (C-8 to C-24) with the electronic equipment's database (NIST-11) and data available in the literature (Adams 2007).

### ***Determination of minimum inhibitory concentrations (MIC)***

Previously, the EOs density was measured in triplicate to ascertain the volume of stock solution of EOs that was needed to achieve the desired concentration by the relationship between mass and volume. The broth microdilution technique was used, where initially 360  $\mu\text{l}$  of TSB broth (Tryptone Soy Broth) were distributed in the first column of a 96-well acrylic plate, followed by the addition of 0.5% Tween 80 and 40  $\mu\text{l}$  of EO, reaching a final concentration of 10% of EO in the first well. Then, 200  $\mu\text{l}$  of TSB broth, containing 0.5% Tween 80, were distributed across the wells of the following columns. A two-fold serial dilution was carried out after homogenizing the contents of the wells of the first column. From the first well of the row, 200  $\mu\text{l}$  of the homogenized mixture was transferred to the second well, and this step was repeated sequentially for subsequent wells. Subsequently, the isolates were standardized on the McFarland scale, adjusted with sterile saline, and 2  $\mu\text{l}$  of the microbial suspension obtained were inoculated, reaching a final concentration of  $10^7$  CFU. $\text{ml}^{-1}$  per well. The microplates were incubated at  $37^\circ\text{C}$  for 24 h to promote bacterial growth. Afterward, the growth confirmation was carried out in the wells by adding 50  $\mu\text{l}$  of resazurin solution with a concentration of 0.01% and allowing a reaction time of 5 to 10 minutes in order to perform a visual reading of the occurrence of colorimetric reaction and evaluation of the growth inhibition of microorganisms (Perazzo et al. 2012). All tests were performed in triplicate. The MIC was calculated according to the well with the lowest concentration of EO that presented a blue color, which indicates the absence of microbial growth.

### ***Determination of the Minimum Bactericidal Concentration (MBC)***

To determine the Minimum Bactericidal Concentration, 10  $\mu\text{l}$  were removed from the MIC and three previous wells and deposited on nutrient agar plates by microdrop technique. Later, they were incubated at  $37^\circ\text{C}/24\text{h}$ . At the end of incubation, the reading

was performed, and the following criteria were considered: Sections with microbial growth indicated bacteriostatic activity of the EO in question at the analyzed concentration, and sections with no microbial growth showed bactericidal activity. The well with the lowest concentration that exhibited a bactericidal effect was recorded as MBC (Knezevic et al. 2016).

### ***Synergistic interaction between EOs***

The antibacterial effects of double (clove/arrayan, spearmint/arrayan, and clove/spearmint) and triple EOs combinations (clove/spearmint/arrayan) against bacterial isolates were evaluated using the microdilution technique for dilution and evaluation of growth and/or inhibition using resazurin and the determination of MBC. The concentration in the first well was 1/2 of the MIC of each EO to be combined and tested against the bacterial isolates, which were added at a concentration of  $10^7$  CFU.mL<sup>-1</sup>. Negative control and growth control (medium with inoculum but without EOs) were included in each microplate.

The fractional inhibitory concentration (FIC), defined as the concentration of an antimicrobial agent combined with a defined concentration of another agent was calculated. Finally, the fractional inhibitory concentration index (FICI) of each combination was calculated to determine the type of interaction by the method reported by Fratini et al. (2017) using the following formula for double combinations  $\sum FICI = FIC (A) + FIC (B)$ :

$$\text{where } FIC (A) = \frac{MIC(A) \text{ in combination}}{MIC (A) \text{ alone}} \text{ and } FIC (B) = \frac{MIC(B) \text{ in combination}}{MIC (B) \text{ alone}},$$

$$\text{and for triple combinations } \sum FICI = FIC (A) + FIC (B) + FIC (C)$$

$$\text{where } FIC (A) = \frac{MIC(A) \text{ in combination}}{MIC (A) \text{ alone}}, FIC (B) = \frac{MIC(B) \text{ in combination}}{MIC (B) \text{ alone}}$$



$$\text{and } FIC(C) = \frac{MIC(C) \text{ in combination}}{MIC(C) \text{ alone}}$$

The  $\sum$  FICI were interpreted as follows: synergistic effect ( $FICI \leq 1$ ), commutative (or additivity) effect ( $FICI = 1$ ), indifferent effect ( $1 < FICI \leq 2$ ), and antagonistic effect ( $FICI > 2$ ).

### ***Biofilm adhesion assessment***

To analyze the *biofilm adhesion ability of the S. aureus isolates*, the bacterial cells were exposed to sub-inhibitory concentrations (sub-MIC) of EOs using microtiter-based crystal violet staining. EOs tested were spearmint + arrayan, spearmint, arrayan, and clove. The method was performed based on the described processes with modifications to optimize the assay (Budri et al. 2015; Zhu et al. 2018) using sub-MIC concentrations with 1/4 and 1/6 MIC, corresponding to 0.25 and 0.0625 MIC values. *S. aureus* samples were diluted to  $10^7$  CFU.mL<sup>-1</sup> in TSB and incubated at 37°C for 24 h. Subsequently, 100  $\mu$ l of each prepared suspension and 100  $\mu$ l of sub-MIC concentrations of EOs were transferred into a 96-well microtiter plate and incubated at 35 °C for 24 to 48 h. After incubation, the broth was carefully withdrawn, and the wells were washed three times with 1X phosphate-buffered saline (PBS) at pH 7.4, then dried and stained with 150  $\mu$ l of 1% crystal violet for 30 min and washed additionally three times. The biofilm-adhered cells were resuspended in 150  $\mu$ l of 95% ethanol, and the plate was left at room temperature for 30 min. The absorbance was measured at 570 nm using a multimode plate reader (EnSpire Multimode Plate Reader, Perkin Elmer). A known biofilm producer, *S. aureus* ATCC6538, was selected as the positive control. The experiment was repeated three times separately for each isolate, and the data were expressed as the mean absorbance value  $\pm$  standard error of the mean (SEM) (Djordjevic, Wiedmann, and McLandsborough 2002).

### ***Preparation of stock solution of EO for cytotoxicity study***

A stock solution was prepared by dissolving the three EOs separately (Clove - *Eugenia caryophyllus*, spearmint - *Mentha spicata*, and Arrayan - *Luma chequen*) in dimethyl sulfoxide (DMSO) to a final concentration of 10% for detection of cytotoxicity.

### ***Vero Cell Preparation***

Isolated and cryopreserved Vero cells were used at the Cell Culture and Immunology Laboratory of the Scientific University of the South, Lima, Peru, maintained in 75-cm<sup>2</sup> cell culture in flasks of Dulbecco's modified Eagle's medium (DMEM) supplemented with serum. 10% fetal bovine (FBS) and 100 µg gentamicin/mL were used as a standard medium. The flasks were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and visualized daily with the help of an inverted microscope to obtain monolayers with a cell confluence of 90 to 95%.

### ***MTT-based cytotoxicity assay***

The cytotoxicity of the EOs on the Vero cells was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This method was adapted from Federman et al. (2016). Aliquots of 200 µL of  $2 \times 10^5$  Vero cells suspension were added to each well for a final cell density of  $\sim 4 \times 10^4$  cells per well dispensed in a round-bottom 96-well tissue culture plate and incubated at 37°C with 5% CO<sub>2</sub> for 24 h. The media was removed, and the cells were washed three times with DMEM. The media was replaced with 200 µL of DMEM containing 10% FBS, 100 µg gentamicin / mL, and different EO concentrations (0, 0.025, 0.05, or 0.1%) were used. The plates were incubated for either 1 or 24 h at 37°C with 5% CO<sub>2</sub>. The media was removed again, and the cells were vigorously washed three times with DMEM before 200 µL of DMEM supplemented with 10% FBS, and gentamicin (100 µg/mL) was added to each well with

20  $\mu\text{L}$  of 5  $\text{mg}\cdot\text{mL}^{-1}$  of MTT, reconstituted in  $1\times$  PBS for 2 h. The cells were incubated for 2 h to reduce MTT at  $37^{\circ}\text{C}$ , and 5%  $\text{CO}_2$ . Lysed cells did not react with MTT. Afterward, the medium was removed, and 200  $\mu\text{L}$  of DMSO was added to each well. The 96-well plate was shaken for 10 to 15 min. The absorbance was read at 570 nm with a blank of 5% DMSO.

### ***Statistical Analysis***

Data are reported as the mean  $\pm$  SD of three measurements. To evaluate the significance of the difference between groups, analysis of variance (ANOVA) was performed, followed by Tukey's range test, which was applied for analysis of data with the level of significance set at  $P < 0.05$ . data were tabulated using. Another group comparison was made using the Kruskal-Wallis test, followed by Dunn's pos hoc test.  $\text{IC}_{50}$  (50% inhibitory concentration of cells) was also determined by non-linear regression analysis. All statistical analyses were performed using the scientific statistic software GraphPad Prism 7.0 (GraphPad for Windows, San Diego, CA -USA).

## ***Results***

### ***Chemical composition of EOs***

The qualitative and quantitative composition of EOs obtained by GC/MSD analysis are listed in Table 3. Their respective chromatograms are shown in Fig. 1. The analysis confirmed that clove EO showed the smallest number of components among all tested EOs. Their major constituents were eugenol (86.25%), trans-caryophyllene (10.39%), and alpha-humulene (2.79%). Regarding arrayan EO, there was a prevalence of monoterpenes, comprising limonene (32,64%), terpin-4-ol (8.86%), 1,8-cineole (7.99%), and linalool (7.52%). Finally, the most abundant compounds of the Spearmint

EO were the monoterpenes, such as carvone (63,21%), limonene (19,97%), and menthol (2.71%).

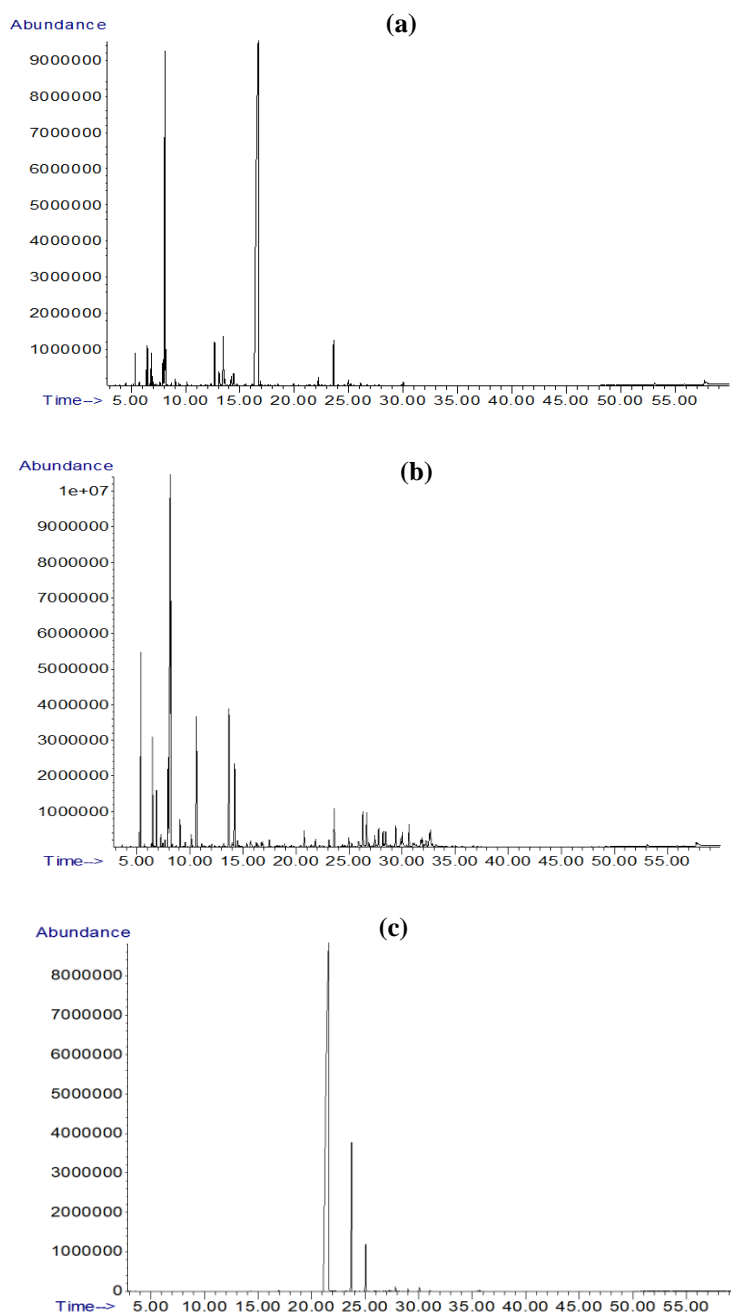
**Table 3.** Chemical composition of EOs identified by GC/MSD

EO	Rt (min)	RI	Compound	% rel.
Clove	21.55	1365	eugenol	86.25
	23.73	1418	trans-caryophyllene	10.39
	25.05	1451	alpha-humulene	2.79
	27.83	1521	delta-cadinene	0.29
	30.07	1579	caryophyllene oxide	0.28
Spearmint	16,66	1249	carvone	63,21
	8,09	1029	limonene	19,97
	13,46	1172	menthol	2,71
	23,61	1416	trans-caryophyllene	2,37
	12,66	1152	mint	1,93
	6,48	976	beta-pinene	1,27
	7,92	1024	para-cymene	1,17
	6,84	990	beta-myrcene	1,02
	13,08	1163	isomenthone	0,92
	5,36	932	alpha-pinene	0,87
	14,42	1196	cis-dihydro carvone	0,77
	14,20	1190	alpha-terpineol	0,60
	6,38	972	sabinene	0,50
	22,20	1381	beta-bourbonene	0,44
	13,62	1176	terpin-4-ol	0,36
	6,96	994	3-octanol	0,34
	24,97	1449	alpha-humulene	0,30
	9,06	1057	gamma-terpinene	0,25
	30,01	1578	caryophyllene oxide	0,25
	16,88	1254	carvotanacetone	0,23

	10,13	1087	terpinolene	0,18
	5,75	947	camphene	0,13
	7,65	1016	alpha-terpinene	0,13
	8,15	1030	limonene	32,64
	13,68	1178	terpin-4-ol	8,86
	8,20	1032	1,8-cineole	7,99
	10,62	1102	linalool	7,52
	5,37	932	alpha-pinene	6,33
	7,94	1024	para-cymene	5,82
	14,22	1191	alpha-terpineol	4,77
	6,49	976	beta-pinene	3,95
	26,29	1482	beta-selinene	2,38
	23,60	1416	trans-caryophyllene	2,24
	26,65	1491	alpha-selinene	2,03
	6,85	990	beta-myrcene	2,02
Arrayan	32,66	1649	beta-eudesmol	1,36
	30,64	1595	guaiol	1,31
	27,78	1520	delta-cadinene	1,20
	29,38	1562	trans-nerolidol	1,19
	9,07	1057	gamma-terpinene	1,13
	28,44	1537	selina-3,7(11)-diene	1,13
	30,03	1579	caryophyllene oxide	1,05
	28,19	1531	cadin-1,4-diene	0,98
	20,77	1347	alpha-terpinyl acetate	0,89
	32,55	1646	trans-dihydro Ocidentalol	0,87
	10,13	1088	terpinolene	0,71
	27,05	1501	alpha-murolene	0,63
	7,26	1005	alpha-phellandrene	0,57
	5,18	925	tricyclene	0,45

Rt: Retention time.

RI: Retention index.

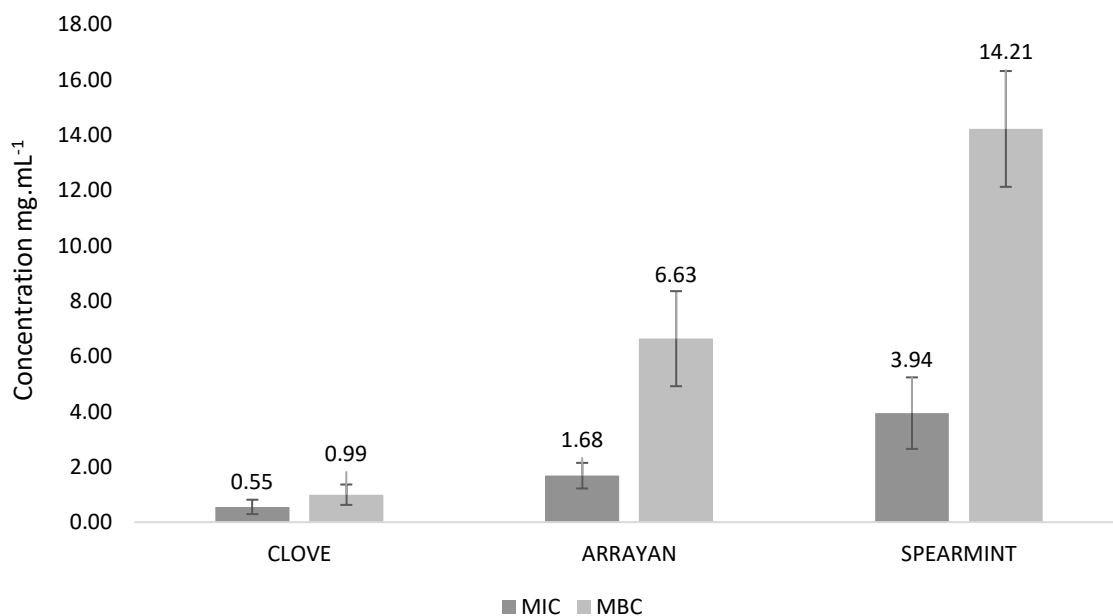


**Figure 1.** Chromatographic profiles of the EOs tested by GC/MSD. The area represented by the peaks corresponds to each component's proportions in the mixture. **(a)** Spearmint EO; **(b)** Arrayan EO; **(c)** Clove EO.

### *Antimicrobial activity of EOs against S. aureus*

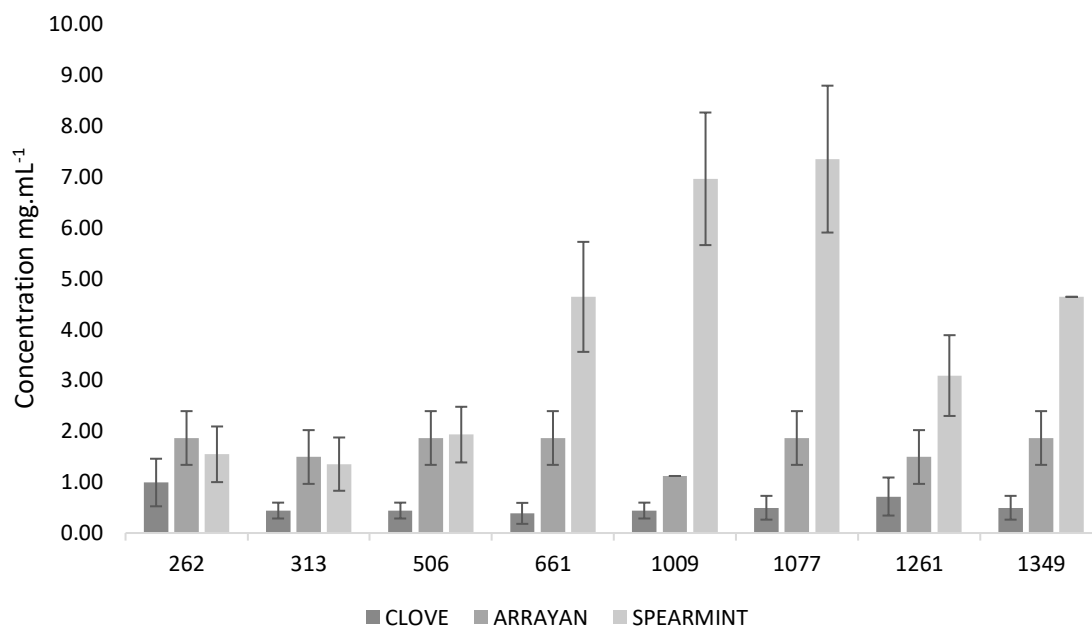
Comparing the mean of the antimicrobial activity results, the EOs displayed diverse levels of effectiveness against *S. aureus* isolates with the following decreasing order of activity: clove EO > arrayan EO > spearmint EO.

Mean MIC/MBC values ranged between 0.39 – 0.99 mg.mL<sup>-1</sup> and 0.66 – 2,20 mg.mL<sup>-1</sup> for clove EO, 1.12- 1.87 mg.mL<sup>-1</sup> and 2.99 – 12.69 mg.mL<sup>-1</sup> for arrayan EO, and 1,35 – 7.35 mg.mL<sup>-1</sup> and 7.73 – 18.56 mg.mL<sup>-1</sup> for spearmint EO, respectively (Fig. 2).

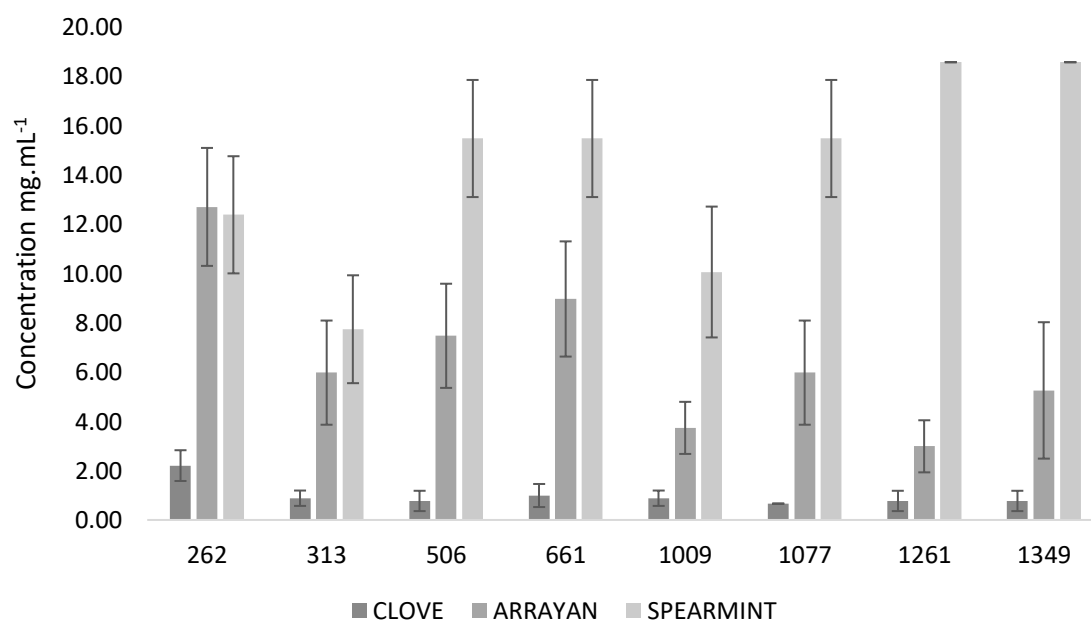


**Figure 2.** Minimum inhibitory concentration and Minimum bactericidal concentration results of EOs tested against *S. aureus*.

In addition, the results of the MIC and MBC of clove, arrayan, and spearmint EO against each *S. aureus* isolate are displayed in Fig. 3 and Fig. 4. For clove, isolates 262, 313, 506, 661, 1009, 1077, 1261, and 1349 had MIC values of 0.99, 0.44, 0.44, 0.39, 0.44, 0.50, 0.72, and 0.50 mg.mL<sup>-1</sup>, and MBC values of 2.20, 0.88, 0.77, 0.99, 0.88, 0.66, 0.77, and 0.77 mg.mL<sup>-1</sup>, respectively. For arrayan, the corresponding MIC values were 1.87, 1.49, 1.87, 1.87, 1.12, 1.87, 1.49, and 1.87 mg.mL<sup>-1</sup>, and MBC values were 12.69, 5.97, 7.47, 8.96, 3.73, 5.97, 2.99, and 5.25 mg.mL<sup>-1</sup>. For spearmint, the MIC values were 1.55, 1.35, 1.93, 4.64, 6.96, 7.35, 3.09, and 4.64 mg.mL<sup>-1</sup>, and the MBC values were 12.37, 7.73, 15.47, 15.47, 10.05, 15.47, 18.56, and 18.56 mg.mL<sup>-1</sup>.



**Figure 3.** Minimum inhibitory concentration results of EOs tested against *S. aureus*.



**Figure 4.** Minimum bactericidal concentration results of EOs tested against *S. aureus*.

### *Interactions of EOs double combinations*

Employing the microdilution technique, we calculated the MIC, FIC, and FICI to evaluate the interactions between the EOs. Four distinct EO combinations were examined for each bacterial isolate: clove and arrayan; spearmint and arrayan; clove and spearmint;



and a triple combination of clove, spearmint, and arrayan. In our study, a notable 21 combinations were categorized as "Synergistic" according to their FICI values, falling under the threshold of  $FICI \leq 1$ . From the synergism outcomes between EOs exhibited in Table 3, we can observe the qualitative classification by calculating the FIC Index of the MIC results.

**Table 3.** Results of MIC for combinations of EOs and FIC index.

Bacterial isolate	EO	MIC Combinations	FIC	FICI	Outcome
262	Clove	0.496	0.818	1.31	Indifferent
	Arrayan	0.933	0.489		
	Spearmint	0.773	0.122	0.61	Synergistic
	Arrayan	0.933	0.489		
	Clove	0.496	0.818	0.94	Synergistic
	Spearmint	0.773	0.122		
	Clove	0.496	0.818	1.43	Indifferent
	Spearmint	0.773	0.122		
	Arrayan	0.933	0.489		
	313	Clove	0.220	0.364	0.85
Arrayan		0.747	0.391		
Spearmint		0.677	0.122	0.66	Synergistic
Arrayan		0.747	0.391		
Clove		0.220	0.364	0.54	Synergistic
Spearmint		0.677	0.122		
Clove		0.220	0.122	1.03	Indifferent
Spearmint		0.677	0.364		
Arrayan		0.747	0.391		
506		Clove	0.220	0.364	0.85
	Arrayan	0.933	0.489		

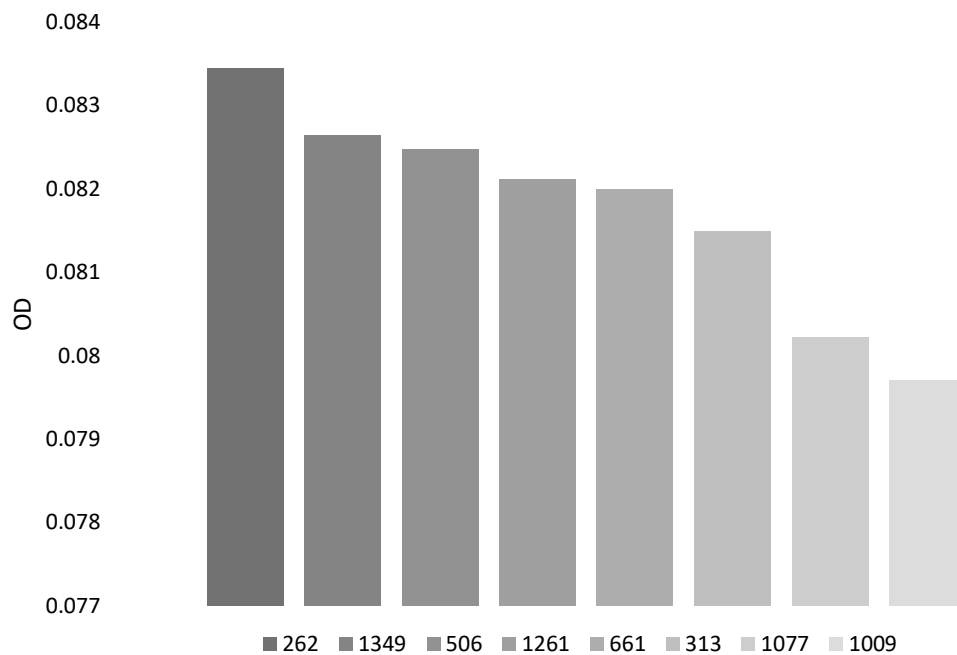
	Spearmint	0.967	0.174		
	Arrayan	0.933	0.489	0.66	Synergistic
	Clove	0.220	0.364		
	Spearmint	0.967	0.174	0.54	Synergistic
	Clove	0.220	0.364		
	Spearmint	0.967	0.174	1.03	Indifferent
	Arrayan	0.933	0.489		
	Clove	0.193	0.318		
	Arrayan	0.933	1.216	1.53	Indifferent
	Spearmint	2.320	0.347		
	Arrayan	0.933	1.010	1.36	Indifferent
661	Clove	0.193	0.318		
	Spearmint	2.320	0.417	0.74	Synergistic
	Clove	0.193	0.265		
	Spearmint	2.320	0.347	1.63	Indifferent
	Arrayan	0.933	1.013		
	Clove	0.220	0.364		
	Arrayan	0.560	0.293	0.66	Synergistic
	Spearmint	3.673	0.550		
	Arrayan	0.560	0.733	0.79	Synergistic
1077	Clove	0.220	0.364		
	Spearmint	3.673	0.660	1.02	Indifferent
	Clove	0.220	0.364		
	Spearmint	3.673	0.660	1.32	Indifferent
	Arrayan	0.560	0.293		
	Clove	0.358	0.188		
	Arrayan	0.747	0.489	0.68	Synergistic
1261	Spearmint	1.547	0.232		
	Arrayan	0.747	0.408	0.64	Synergistic
	Clove	0.358	0.188	0.47	Synergistic

	Spearmint	1.547	0.278		
	Clove	0.358	0.188		
	Spearmint	1.547	0.278	0.95	Synergistic
	Arrayan	0.747	0.489		
	Clove	0.220	0.364	0.66	Synergistic
	Arrayan	0.560	0.293		
	Spearmint	3.480	0.521	0.77	Synergistic
	Arrayan	0.560	0.244		
1009	Clove	0.220	0.364	0.99	Synergistic
	Spearmint	3.480	0.625		
	Clove	0.220	0.303		
	Spearmint	3.480	0.521	1.07	Indifferent
	Arrayan	0.560	0.244		
	Clove	0.248	0.409	0.90	Synergistic
	Arrayan	0.933	0.489		
	Spearmint	2.320	0.417	0.91	Synergistic
	Arrayan	0.933	0.489		
1349	Clove	0.248	0.409	0.83	Synergistic
	Spearmint	2.320	0.417		
	Clove	0.248	0.409		
	Spearmint	2.320	0.417	1.32	Indifferent
	Arrayan	0.933	0.489		

### ***Biofilm formation***

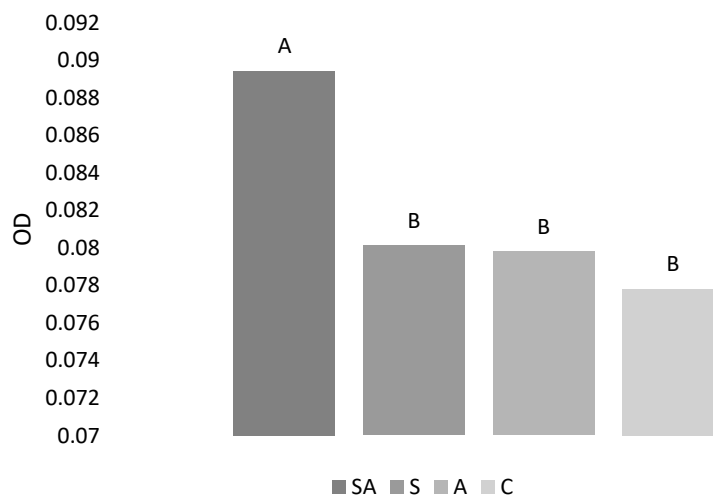
In our study of biofilm-forming capabilities among different *S. aureus* isolates OD was quantified. Notable variations were observed in the OD values across the isolates, as shown in Fig. 5. Specifically, isolates 262 and 1349 had the highest OD (0.083). Isolates 506, 1261, and 661 had similar OD values (0.082), while isolates 313, 1077, and 1009

had the lowest OD values (0.081, 0.080, and 0.080, respectively), despite these variations, no statistically significant differences were found.



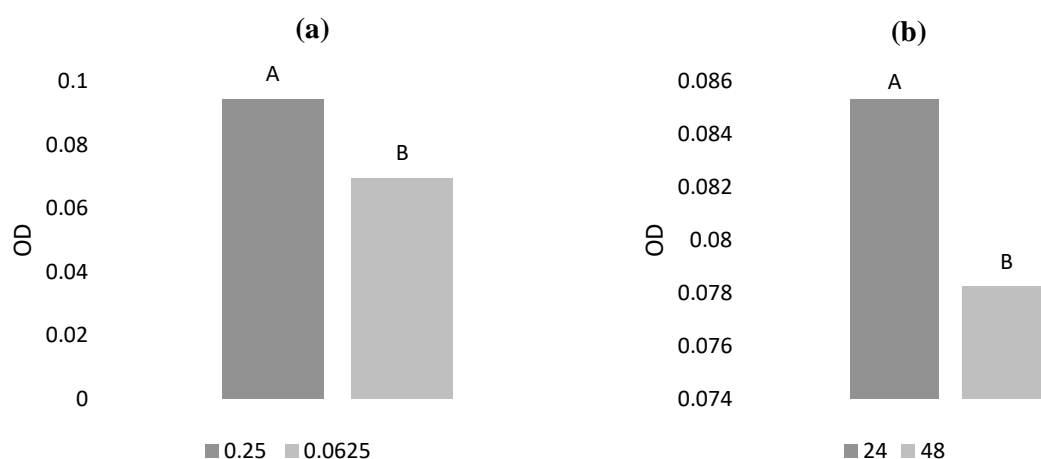
**Figure 5.** Biofilm formation across the different *S. aureus* isolates.

In addition, EOs tested exhibited distinct effects on biofilm formation as indicated by their respective OD values: SA (spearmint + arrayan): 0.089, S (spearmint): 0.080, A (arrayan): 0.080, C (clove): 0.078. Notably, the combination of SA was statistically different. These differential effects are visually represented in Fig. 6.



**Figure 6.** Effect of SA (spearmint + arrayan), S (Spearmint), A (arrayan), and C (clove) EOs, on the inhibition of biofilm formation by different *S. aureus* isolates.

We evaluate the impact of sub-MIC concentrations on biofilm formation in *S. aureus* isolates, as illustrated in Fig. 7a. Statistically significant differences were observed between two concentrations tested. Specifically, at a concentration of 1/4 MIC (0.25), the average OD was 0.094. This value significantly decreased to 0.069 at a concentration of 1/16 MIC (0.0625). Our study further explored the impact of time on biofilm formation in *S. aureus* isolates, as shown in Fig. 7b. OD measurements were taken at two distinct time points: 24 hours and 48 hours. A statistically significant decrease in biofilm formation was observed over this period. Specifically, the average OD reduced from 0.085 at 24 hours to 0.078 at 48 hours.



**Figure 7.** (a) Effect of Sub-MIC concentrations of EOs on biofilm formation of *S. aureus*. (b) Effect of time (24-28h) on biofilm formation of *S. aureus*.

### *Cytotoxicity effect of EOs*

All tested EOs showed varying degrees of cytotoxicity on Vero cells, and these results are shown in Table 4. Notably, clove EO exhibited significant cytotoxicity effects, aligning with the lowest IC<sub>50</sub> value (0.021). Arrayan EO, with an IC<sub>50</sub> value of 0.231, was less potent but exhibited significant cytotoxicity. The combination of clove and arrayan EO showed an IC<sub>50</sub> value of 0.077, which is lower than arrayan alone but higher than clove alone. Further, the spearmint and arrayan EO combination presented a higher IC<sub>50</sub>

value (0.367). Control compounds, Ethanol 30% and DMSO 5% showed much higher IC<sub>50</sub> values (8.97 and 15.1, respectively), confirming that the observed cytotoxic effects were specific to the EOs and not due to the solvent used.

**Table 4.** IC<sub>50</sub> values obtained for the selected EOs.

EO	IC <sub>50</sub> value <sup>1</sup>
Arrayan	0.231
Clove	0.021
Clove + Arrayan	0.077
Spearmint + Arrayan	0.367

<sup>1</sup> The IC<sub>50</sub> value represents the concentration of each compound that inhibits activity by 50%

### ***Discussion***

Bovine mastitis poses a significant challenge to developing sustainable dairy farming systems. This condition is caused by various pathogens, including *S. aureus*, which can have adverse consequences on dairy farms. In this context, prevention and treatment of bovine mastitis are essential (Zaatout et al. 2020). EOs are abundant in aromatic compounds that belong to phenolic compounds and can be classified into two structural groups: terpenoids, such as monoterpenes, sesquiterpenes, and diterpenes, while the other is phenylpropanoids (Aguirre, Borneo, and Leon 2013). EOs have garnered significant research interest focused on investigating natural products with bioactive compounds that may be applied as safe alternatives to current therapeutic options. Numerous works reveal that these compounds represent the main source of chemical diversity during pharmaceutical discoveries (Álvarez-Martínez et al. 2021; Bagheri et al. 2020; Ni et al. 2021).

Determining MIC and MBC values is fundamental for developing alternative antimicrobial agents, particularly in light of increasing antibiotic resistance. MIC values indicate the lowest concentration of an EO required to inhibit bacterial growth, while the MBC values identify the concentration of EOs that results in bacterial death (Balouiri, Sadiki, and Ibsouda 2016). Our findings revealed that MIC values for clove EO against all *S. aureus* isolates were lower than the MIC values for arrayan and spearmint, requiring lower concentrations for both inhibition and killing bacteria. This is consistent with the findings of the latter study, which reported MIC values ranging from 0.78 to 6.25  $\mu\text{L}/\text{mL}$  for clove EO against *S. aureus*, *S. agalactiae*, and *E. coli* (Aiemsaard et al. 2023). Existing literature demonstrates significant antibacterial properties of this EO against both Gram-positive and Gram-negative bacteria (Milagres de Almeida et al. 2023; Ni et al. 2021). Clove EO showed the smallest number of components among all tested EOs, and their major constituents were eugenol (86.25%), trans-caryophyllene (10.39%), and alpha-humulene (2.79%). Notably, their bioactive substances attracted the attention of researchers due to their relevance as antimicrobial agents.

Eugenol, a phenolic compound known for its antimicrobial activity against *S. aureus* and other gram-positive bacteria (da Silva et al., 2021; Milagres de Almeida et al., 2023), is the main component of clove EO, and its presence is related to its broad antimicrobial potential. Eugenol destroys bacteria through several modes of action that can modify the composition of the bacterial cytoplasm membrane, inducing cell wall destruction and inhibiting virulence factors, which lead to *S. aureus* cell lysis (Bagheri et al. 2020; Das et al. 2016; Marchese et al. 2017). The extremely reactive hydroxyl groups of eugenol can form hydrogen bonds with a particular bacterial enzyme, thereby interfering with its function and leading to cell membrane damage or failure (Guimarães et al. 2019). It is possible that the active ingredients in clove EO are more effective at

disrupting the cell membrane of *S. aureus*. Comparing our results to the literature, similar activity due to the major compound on clove EO was observed (Das et al. 2016; Marchese et al. 2017). It can thus be suggested that their antimicrobial properties against *S. aureus* make it an attractive option for treating bovine mastitis.

Our results found a high prevalence of sesquiterpenes in the EOs tested. *Trans*-caryophyllene, is a sesquiterpene commonly found in various EOs, including those derived from clove, thyme, oregano, and black pepper (Milagres de Almeida et al. 2023). Sesquiterpenes are bioactive compounds with good inhibitory activity against *S. aureus* (Bagheri et al. 2020). Its presence and unique properties make it worthy of discussion, particularly in the context of antimicrobial activities against gram-positive and gram-negative bacteria (Ghavam et al. 2020; Sengun et al. 2021). The combination of *trans*-caryophyllene and linalool, a monoterpene found as one of the primary compounds in arrayan EO tested in this study, was found to be the most effective in inhibiting biofilm formation in *S. aureus*. It was suggested that their combination decreased the expression levels of genes associated with initial cell adhesion and quorum sensing. Notably, *trans*-caryophyllene has been approved by the FDA and the European Food Safety Authority (EFSA) for use in cosmetics and as a flavor enhancer. Its low toxicity makes it an excellent candidate for further applications (Salinas et al. 2022).

Another essential chemical compound found in clove EO was  $\alpha$ -humulene, which exhibits a variety of biological activities, including antimicrobial properties. Particularly, it is known for its anti-inflammatory effects. Studies have shown its effectiveness against bacterial and fungal pathogens such as *S. aureus* and *Candida albicans* (Leite et al., 2021). Additional research on *Cannabis sativa* EO, which contains  $\alpha$ -humulene, also demonstrated moderate antibacterial and antibiofilm activities (Zengin et al. 2018).



The mechanisms through which terpenes present in EOs act against pathogens can vary. Arrayan EO contained as major constituents limonene, terpin-4-ol, 1,8-cineole, and linalool. Research has confirmed that limonene, also found as a major compound in the spearmint EO from our study, can effectively inhibit the growth of pathogenic bacteria such as *S. aureus* and *L. monocytogenes* (Han, Sun, and Chen 2019). Their antimicrobial activity is likely attributed to compromised cell membranes, enhanced membrane permeability, and reduced metabolic function in the respiratory system, along with the disruption of cell morphology and bacterial cell wall integrity (Han, Chen, and Sun 2021). It's noteworthy that limonene has been found to act synergistically with gentamicin. This combination amplifies the inhibitory effects on *S. aureus* and *E. coli*, including strains that are resistant to antibiotics (Costa et al. 2019). Moreover, limonene has been identified in EOs from bergamot, spearmint, arrayan, black pepper, and oregano (Gonçalves et al. 2006; Milagres de Almeida et al. 2023; Pedonese et al. 2017; Shahbazi 2015).

Another major monoterpene present in arrayan EO, Terpinen-4-ol, was found in a previous study in *Melaleuca alternifolia* EO. This compound has been the subject of comprehensive studies focusing on its antimicrobial attributes, especially its efficacy against *S. aureus* (Corona-Gómez et al. 2022). Additional studies have reinforced its potent bactericidal properties against *S. aureus*, likely by impeding the bacterial cell wall synthesis. Moreover, it has displayed significant antibiofilm activity, inhibiting biofilm formation even at sub-MIC concentrations (Cordeiro et al. 2020).

Carvone was the dominant monoterpene presented in spearmint EO, which agrees with the study by Shahbazi (2015), which demonstrates notable biological activities of this compound in vitro and in vivo. It possesses potent antimicrobial properties against a variety of microorganisms, including MRSA. Moreover, it has shown efficacy in inhibiting biofilm formation by *S. aureus*. Its mechanism of action involves penetrating

the bacterial cell membrane, thereby increasing cell permeability. Other pharmacological effects include antifungal, antiparasitic, anti-neuraminidase, antioxidant, anti-inflammatory, and anticancer activities (Bouyahya et al. 2021).

The MIC results revealed were critical in determining the starting concentrations for the subsequent tests. A clear understanding of the individual antimicrobial efficacy of each EO is critical for interpreting the outcomes of combined treatments. The study began with concentrations that were half of the MIC values, setting the stage for evaluating synergistic or antagonistic interactions. In fact, synergism is a strategy that brings advantages concerning microbial control (Tariq et al. 2019). These interactions may potentiate the effect and may be beneficial in diseases or interfere with their activity and, therefore, should be avoided. Based on our results, the lower MIC and MBC values, particularly for clove EO, highlight its potential as an effective antimicrobial agent, either alone or in combination.

Our study sheds light on the complex interactions between EOs. While individual MIC values provide an initial insight into the antimicrobial potential of each EO, our FICI results reveal a more complex scenario. When tested alone, clove was the most potent EO-inhibiting *S. aureus* growth, exhibiting the lowest MIC value of 0.193 mg.mL<sup>-1</sup>. Spearmint, despite a higher MIC value of 3.673 mg.mL<sup>-1</sup>, exhibited strong synergistic potential when combined with other EOs, as evidenced by low FIC and FICI values. Notably, blends of clove and spearmint revealed even more potent effects at inhibiting specific *S. aureus* isolates, such as 1261, 313, 506, and 262.

Moreover, the enhanced synergistic effect observed in clove and spearmint and arrayan and spearmint EO combinations was surprising, given their higher individual MIC and MBC values. The synergistic interaction between these EOs can be associated with terpenes as the main components that improve the external membrane porosity of

bacteria, resulting in easier compound access into the cytoplasm. Terpenes and terpenoids are found in abundance in EOs and are the most active phytochemicals studied, having properties for treating or preventing diseases, followed by polyphenols (Álvarez-Martínez et al. 2021; Masyita et al. 2022). Taken together, our study reinforces the idea that certain EOs may become significantly more effective when combined, even if they are less potent individually. The complex interactions between EOs and bacteria, including the phytochemical composition of the EO and the inherent resistance mechanisms of *S. aureus* isolates, may influence the response found.

While our study found notable synergistic interactions in certain double combinations, the triple combinations of clove, arrayan, and spearmint EO displayed predominantly indifferent outcomes, as evidenced by their FICI values. This is unexpected, given the individual and dual synergistic properties. However, the chemical constituents of each EO can interact in complex ways, and the lack of synergistic activity could be attributed to the active compounds in one EO that could compete with those in another, neutralizing their individual antimicrobial effects when used in a triple combination. The proportions in which the EOs were combined could also play a role (Burt 2004). Altogether, adding a third EO might not offer additional benefits and could even dilute the overall efficacy. Our findings caution that 'more' is not always 'better' in EO combinations, possibly indicating a saturation effect or competitive inhibition. Similarly, Vieira-Brock, Vaughan, and Vollmer (2017) found that blending different EOs (specifically orange, patchouli, peppermint, and clary sage) was equally effective as individual EOs in inhibiting *S. aureus*, indicating no additive effect.

Most of the toxicological information has focused on finding lethal or inhibitory concentrations and does not assess the effect with other concentrations. Studies on the effects of antimicrobials at sub-MIC concentrations against microorganisms are mainly

related to biofilm formation. An important aspect is that sub-MIC concentrations can alter the biofilm-forming phenotype of diverse bacterial species. It is important to highlight that the analyses for detecting anti-biofilm compounds usually use an inoculum at a concentration of  $10^7$  CFU.mL<sup>-1</sup>.

*S. aureus*, originating from bovine sources, is commonly characterized as a producer of biofilms (Campos et al. 2022; Felipe et al. 2017; Silva et al. 2021). A biofilm represents a microbial community of cells embedded in a polymer extracellular matrix, which in *S. aureus* consists mainly of the PIA. In addition, biofilm also content several MSCRAMMs proteins (Campos et al. 2022; Otto 2018). In addition, biofilms exhibit phenotypic and genetic traits distinct from planktonic cells, making them more resistant to antimicrobial treatments (Silva et al. 2022).

Research suggests that the efficacy of EOs to combat *S. aureus* biofilms is determined by diverse factors, such as hydrophobicity, reactivity, and rate of diffusion, as well as the composition and structural attributes of the biofilm itself. EOs can act in various ways to inhibit biofilm formation: they can block quorum sensing mechanisms, suppress the transcription of genes responsible for flagellar movement, or interfere with bacterial motility (Melo et al. 2019). The interaction with host matrix proteins and immune defenses adds another layer of complexity to the biofilm formation. For instance, biofilms can evade the host immune system by forming a physical barrier, making it difficult for immune cells to penetrate and clear the infection (Otto 2018; Scherr et al. 2014).

Knowing which strains form more robust biofilms is relevant for targeted antibacterial treatments, as biofilms are notoriously difficult to eradicate. For all isolates, the OD values range from 0.08 to 0.083, which suggests that these have some ability to biofilm formation. Isolate 262 appears to have the highest biofilm-forming ability with

an OD of 0.083, while isolate 1009 has the lowest with an OD of 0.080. The values are close to each other and are not significantly different. Notably, isolate 1009 exclusively contained the *fib* gene (encoding fibrinogen-binding protein) related to biofilm adhesion (Peng et al. 2023) and lacked other biofilm and MSCRAMMs genes common in the other isolates tested. The profile in this isolate may underline its distinct biofilm-forming abilities and a more susceptible response to the EOs tested.

In our study, we also investigated the time-dependent effects of EOs on biofilm formation among *S. aureus* isolates. Our findings showed a statistically significant reduction in biofilm formation from 24 to 48 h. Specifically, it decreases from 0.085 at 24 h to 0.078 at 48 h. One biological explanation for this result is nutrient depletion. As bacteria grow and the biofilm matures, they consume essential nutrients, which may weaken biofilms and restrict cell multiplication. This nutrient depletion can cause cells in the biofilm to die, reduce the capacity to form new biofilms, and activate a cascade of effects that signal cellular detachment. This detachment initiated by nutrient depletion and waste accumulation can also be affected by factors such as quorum sensing (Boles and Horswill 2011; François, Schrenzel, and Götz 2023; Hunt et al. 2004). This quorum sensing is based on producing chemical signals called autoinducers, which accumulate in the medium as *S. aureus* grows. A noteworthy justification is that phytochemical compounds in the EOs tested may influence gene expression related to biofilm formation, leading to reduced biofilm mass over time. Once autoinducers reach a threshold concentration corresponding to a specific population density, they alter the expression of genes (Salinas et al. 2022; Tariq et al. 2019).

Furthermore, we assessed the potential of EOs to inhibit biofilm formation at sub-MIC concentrations. We observed an intriguing dose-dependent effect characterized by varying OD values: 0.094 at 0.25 MIC (1/4 MIC) and 0.069 at 0.0625 MIC (1/16 MIC).

The higher OD value at 1/4 MIC suggests that there may be a concentration threshold below which the efficacy of EOs is significantly reduced. The lower OD at 1/16 MIC indicates that lowering the concentration of EOs results in more effective inhibition of biofilm formation while minimizing potential side effects. The observed data may reflect a hormetic response. Low doses of a substance can have a beneficial impact in hormesis, but the opposite happens at high doses. This is particularly relevant in biofilm formation, where sub-MIC concentrations can induce a protective or stimulatory effect on bacteria (Kaplan 2011). It could explain the enhanced biofilm inhibition at lower concentrations. Our intriguing observations in the dose-response have crucial implications for antimicrobial therapy, suggesting that lower doses of these EOs could be more effective, thus reducing potential side effects and costs.

It's noteworthy that sub-MIC concentrations can induce biofilm formation in various bacteria, including *S. aureus* and other Gram-positive and Gram-negative species. A study revealed that cinnamon EO and trans-cinnamaldehyde had a hormetic effect on biofilm formation in the multidrug-resistant bacteria *Acinetobacter baumannii* at medium concentrations. Interestingly, higher concentrations were either ineffective or promoted biofilm formation, suggesting that optimal concentrations for biofilm inhibition may vary depending on the bacterial strain (Ganić et al. 2022). This is a concern because it can make treating infections caused by bacteria forming biofilms difficult. There are some theories for how these low doses induce biofilm formation. One suggests that sub-MIC levels create sufficient stress to induce genes related to biofilm production without killing bacteria, while another theory is that extracellular DNA released by bacteria in the population most susceptible to the antibiotic stimulates the remaining bacteria to form biofilms (Ranieri, Whitchurch, and Burrows 2018).

Synergism between SA resulted in slightly higher OD values compared to individual EOs. This is an interesting aspect of the differential biofilm inhibition observed in combination. They presented OD values of 0.089 compared to the individual EOs (spearmint and arrayan with 0.080). This suggests that the combination between spearmint and arrayan may not be the most effective approach for biofilm inhibition. Also, combining EOs may have a saturation effect on the bacterial response (Vieira-Brock et al. 2017), reducing biofilm inhibition efficacy. From our findings, we infer that combining antimicrobial agents will necessarily result in enhanced efficacy, and combinations can be less effective in some cases than individual treatments. Our findings differ from research that revealed that synergistic combinations appear overall more effective than treating with either compound alone (Hawas, Verderosa, and Totsika 2022; Salinas et al. 2022).

Research by Caballero Gómez et al. (2022) provides valuable insights into inhibiting biofilm formation in *S. aureus*. The study employed a range of EO compounds, such as thymol, carvacrol, limonene, eugenol, geraniol, cinnamaldehyde, and disinfectant solutions, such as EDTA and HLE. These agents were tested at MIC and sub-MIC (0.5 or 0.25 MIC) concentrations against preformed biofilms of *Staphylococcus* sp. isolates. The study found significant synergistic effects in inhibiting biofilm formation when these agents were used. Another research showed that combined *Ocimum gratissimum* EO with ciprofloxacin and oxacillin has been shown to delay the growth curve of multi-resistant strains of *S. aureus* and *E. coli*, as well as a significant reduction in biofilm formation and cell viability (Melo et al. 2019).

Among the EOs tested, clove exhibited the most promising results. This finding suggests that clove EO was the most effective at inhibiting biofilm formation among the *S. aureus* isolates studied. As mentioned before, clove EO is rich in bioactive compounds

like eugenol, which may be responsible for the antibiofilm property. Eugenol and other bioactive components may disrupt bacterial cell membranes, inhibiting biofilm formation's initial attachment phase. Given its enhanced efficacy, clove EO could be a candidate for future antimicrobial therapies to control biofilm-associated infections, especially in *S. aureus* causing bovine mastitis. Previous research has demonstrated that eugenol exhibits antibacterial activity against MSSA and MRSA strains. Moreover, at sub-MIC concentrations, eugenol can inhibit biofilm formation. This is achieved by effectively eradicating pre-established biofilms, decreasing the expression of biofilms and enterotoxin-related genes, such as *icaD*, *sea*, and *sarA*, damaging the bacterial cell membrane, and leading to the leakage of cellular contents (Yadav et al. 2015) In addition, research has assessed the effect of eugenol on biofilm formation specifically against the Gram-negative *E. coli* O157:H7 suggesting that eugenol acts by destroying the biofilm barrier and diminishing the metabolic activity of bacteria (Cui et al. 2020; Kim et al. 2016).

Our research indicates that all the EOs evaluated in cytotoxicity assays demonstrated notable cytotoxic effects on Vero cells. The IC<sub>50</sub> values of the compounds were determined as the lowest concentration, reducing cell growth by ~50% in treated compared to untreated Vero cells. Specifically, clove EO emerged as the most cytotoxic, with the lowest IC<sub>50</sub> value of 0.021, aligning with prior studies that underscore its antimicrobial and cytotoxic capabilities. However, its specific efficacy against *S. aureus* in bovine mastitis remains to be investigated. This high cytotoxicity in clove EO may be due to its various bioactive compounds. Interestingly, one of these compounds,  $\delta$ -cadinene, a sesquiterpene, is not well-studied. However, this abundant metabolite has been shown to have therapeutic benefits, such as the one obtained from *Kadsura longipedunculata* (21.8%). It has also been shown to have some degree of cytotoxic



activity against some human cell lines (Mulyaningsih et al. 2010). A study showed that when examining individual compounds of Myrtaceae EOs on Vero and RC-37 cells,  $\alpha$ -pinene, eugenol, and leptospermone had low-risk concentrations at dilutions of 0.001%, 0.003%, and 0.001%, respectively. Interestingly, 1,8-cineole demonstrated minimal toxicity, with a harmless concentration of 0.1% (Schnitzler et al., 2008). This compound was present in Arrayan EO, which also displayed significant cytotoxicity, evidenced by its  $IC_{50}$  value of 0.231. Arrayan EO also contains limonene, a compound with demonstrated anticancer potential. Previous studies have shown that encapsulating limonene in nano-niosomes enhances its solubility and therapeutic efficacy. While these studies suggest that limonene's cytotoxicity could be helpful in a cancer therapy context (Hajizadeh et al. 2019), our findings indicate that this same cytotoxicity could be a limitation when the purpose is not to harm cells. It opens up avenues for further study. For instance, modifying the EO formulation may mitigate the cytotoxic effects.

Intriguingly, the clove and arrayan EO blend presented an  $IC_{50}$  value of 0.077, suggesting a potential synergistic effect. In contrast, the spearmint and arrayan EO combination did not exhibit such synergy, as indicated by its higher  $IC_{50}$  value of 0.367. Control substances, ethanol at 30% and DMSO at 5%, showed significantly higher  $IC_{50}$  values, confirming that the observed cytotoxic effects are specific to the EOs tested.

The use of EOs to treat bovine mastitis is a complex issue that requires careful consideration of several factors, including efficacy, safety, and regulatory approval. Our study was conducted *in vitro* using Vero cells, which may not fully simulate the *in vivo* conditions within bovine mammary glands; however, the cytotoxic effects observed could also be harmful to bovine mammary cells or other tissues. Safety studies, including *in vivo* tests, would be necessary to determine appropriate dosing and assess safety. Advanced formulation techniques, such as emulsions, gels, or encapsulation, could offer

more targeted and safer delivery methods. The potential for combining EOs with other antimicrobial agents should also be attractive since it could enhance their efficacy (Abd El-Hamid et al. 2023).

Therefore, further research is needed to optimize the formulation of EOs to minimize cytotoxic effects while preserving its potent antibacterial and antibiofilm activities. This could lead to more effective therapies against *S. aureus* causing bovine mastitis infections, addressing the growing concern of antimicrobial resistance of this pathogen.

### ***Conclusion***

Clove, arrayan, spearmint, and their synergistic combinations show promising antimicrobial and antibiofilm properties but present cytotoxicity in cells and require a nuanced approach for application, especially in sensitive applications like bovine mastitis treatment. While our findings could have significant implications for alternative treatment strategies in the dairy industry, they are preliminary and necessitate further research for validation.

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# CHAPTER 6

*General discussion*

## General discussion

The exploration of EOs as alternative antimicrobial agents has gained considerable attention, especially against antibiotic-resistant pathogens such as *S. aureus* (Caneschi et al., 2023; Lopes et al., 2020; Tomanić et al., 2023). Our research has delved into various aspects of EOs potential as a natural antibiotic alternative against *S. aureus*, a common pathogen known for its biofilm-forming and antibiotic-resistant capabilities. Overall, we established the potential of EOs tested, such as *Citrus aurantium bergamia*, *Copaifera reticulata*, *Foeniculum vulgare*, *Zingiber officinale*, *Ocimum basilicum*, *Luma chequen*, *Mentha spicata*, and *Eugenia caryophyllus* as viable antimicrobial agents against *S. aureus* resistant to antibiotics.

The role of *S. aureus* in causing bovine mastitis has been an area of ongoing research. This pathogen is responsible for subclinical bovine mastitis, a prevalent and economically significant disease in dairy herds. The disease is characterized by mammary gland inflammation that can decrease milk quality and yield (Cheng & Han, 2020; Dyson et al., 2022). Cows with subclinical mastitis produce less quantity of milk, and this milk has a higher somatic cell count (SCC). SCC measures the number of white blood cells in milk and is a good indicator of inflammation (Gonçalves et al., 2018). One key attribute that enables *S. aureus* to establish and maintain these infections is its array of virulence factors (Zaatout et al., 2020). Ongoing research is focused on developing new and improved methods for preventing, diagnosing, and treating bovine mastitis caused by *S. aureus* (Cheng & Han, 2020).

Screening *S. aureus* isolates for virulence and antimicrobial resistance genes was a critical step in our study to evaluate EOs as potential treatments for bovine mastitis infections caused by this pathogen, especially given the growing problem of antimicrobial resistance, which makes it more challenging to treat *S. aureus* infections. It's worth noting

that bovine mastitis is the most common reason for antibiotic use in dairy production, and the emergence of antimicrobial resistance in *S. aureus* continues as a significant challenge in treating this disease (Bonsaglia et al., 2018; Cheng & Han, 2020; Tomanić et al., 2023). By understanding the virulence and antimicrobial resistance profiles of our isolates, we can develop more targeted and effective treatments for this important pathogen. Our study found that the *S. aureus* isolates we screened presented a diverse virulence and antimicrobial resistance profile by phenotypic and genotypic testing, highlighting the importance of this step. This information allowed us to identify the EOs that were effective against our isolates, including those that were characterized as resistant to traditional antibiotics.

The findings about the virulence profile of our isolates highlight the importance of continued research on *S. aureus* virulence and antimicrobial resistance. Our study successfully screened 57 *S. aureus* isolates for various virulence genes, identifying several prevalent such as *icaD*, *bap*, *fnbA*, *clfA*, *icaA*, *see*, and *clfA*, among others, and antimicrobial resistance genes such as *blaZ* and *aac6'aph2'*. These genes encode various proteins and factors that contribute to *S. aureus* to adhere to host tissues, evade the immune system, and establish infections. This suggests that *S. aureus* isolates tested can cause infections that are more challenging to treat, especially in the context of bovine mastitis.

Enterotoxins, biofilm formation, and MSCRAMM genes are important virulence factors in bovine mastitis, as evidenced by our study. For enterotoxin genes, the frequencies were *see* (64.9%), *sed* (61.4%), and *seh* (3.5%). Forty-seven isolates (82.5%) presented at least one enterotoxin-encoding gene, and only ten isolates (17.5%) did not have any enterotoxin gene in their profile. Enterotoxins have been shown to play an essential role in the pathogenesis of bovine mastitis, and it is known that most *S. aureus*

strains isolated from this disease harbor one or more enterotoxin genes (Bonsaglia et al., 2018; Rall et al., 2014; Ren et al., 2020). Moreover, these may remain, increasing the possibility of foodborne poisoning (Campos et al., 2022; Grispoldi et al., 2021).

Notably, EOs efficacy against *S. aureus* at a concentration of  $10^3$  CFU.mL<sup>-1</sup> (Chapter 4) sets a baseline for their antimicrobial potential. However, the results from Chapter 5, conducted at  $10^7$  CFU.mL<sup>-1</sup>, provide a more realistic assessment of their efficacy, given that higher bacterial concentrations often simulate more severe infections. *S. aureus* originating from bovine sources is commonly characterized as a producer of biofilms (Campos et al., 2022; Felipe et al., 2017; V. Silva et al., 2021). Our research has revealed the efficacy of EOs like clove, arrayan, and spearmint against biofilm-forming isolates of *S. aureus*. Biofilms protect bacteria from antibiotics and the host immune system. When bacteria form biofilms, it becomes difficult for antibiotics to penetrate the matrix. This physical barrier can make it difficult for immune cells to reach and clear the infection, thereby making treating these infections particularly challenging (Otto, 2018; Scherr et al., 2014; Zaatout et al., 2020). The *bap*, *icaA*, and *icaD* genes are associated with the genes that encode for MSCRAMMs, which are specific staphylococcal surface proteins that play a crucial role in initial attachment, colonization, intracellular internalization, and biofilm initiation (Klein et al., 2012). They provide a great capacity for adhesion and biofilm production in *S. aureus* isolates, which is essential for the colonization and persistence of the pathogen (Castilho et al., 2017). Our results showed that 98.2% of isolates contained at least one biofilm-forming gene. The most frequent biofilm formation gene detected was *icaD*, with 94.7% (n = 54), followed by *bap* with 78.9% (n=45), and *icaA* with 66.7% (n = 38). The *icaA* and *icaD* genes are important factors in promoting intercellular adhesion and forming multilayered biofilms (Miao et al., 2019; V. Silva et al., 2021). Our findings are similar to the results of

Vasudevan *et al.* (2003), who found *icaA* and *icaD* genes in 35 (100%) *S. aureus* isolates from bovine mastitis cases. Moreover, the high prevalence of the *bap* gene is consistent with the results reported by Salimena *et al.* (2016), who determined that this gene was present in 95.6% of the isolates from the milk of dairy herds in Brazil. In addition, the most frequent MSCRAMMS genes detected were *fib* (93%), *fnbA* (82.5%), *clfA* (80.7%), *clfB* (73.7%), and *eno* (63.2%). On the other hand, the less prevalent were *cna* (7%) and *ebps* (7%). Altogether, these virulence factors play an important role in the persistence of *S. aureus*, causing bovine mastitis infection (Aslantaş & Demir, 2016; Zaatout *et al.*, 2020; Zuniga *et al.*, 2015). The EOs' ability to inhibit growth and disrupt biofilm-forming *S. aureus*, as examined in Chapter 5, suggests that they may represent a promising way to disrupt these robust bacterial communities, especially given the high prevalence of biofilm-forming genes in the isolates explored.

The biofilm-associated and MSCRAMMs genes identified in this study, such as *icaD*, *bap*, and *icaA*, play critical roles in enhancing the bacteria's ability to survive antibiotic treatment. Notably, the antibiotic resistance profiles of our isolates were a concern, underscoring the need for alternative treatment strategies. Our findings contribute to the growing evidence of antibiotic resistance. We found an alarming prevalence of the *blaZ* gene (94.7%), indicative of penicillin resistance, in the *S. aureus* isolates tested. This is particularly concerning given that  $\beta$ -lactams, including penicillin, are commonly used antibiotics to treat mastitis diseases (Aslantaş & Demir, 2016). This finding underlines the urgent need for more responsible antibiotic use, as many producers do not perform the necessary diagnosis and treat the animals inappropriately, which can lead to the development of resistance to antimicrobials (Adkins & Middleton, 2018; Tomanić *et al.*, 2023).



*Spa* typing, a molecular typing technique for *S. aureus*, has shown that *spa* type t605 exists in multiple countries, including Brazil, Canada, and Italy (Campos et al., 2022). All our isolates were characterized as *spa* type t605. It suggests that this *spa* type is widespread in the Brazilian dairy herds and may be descendants of a common ancestor. The study conducted by Bonsaglia *et al.* (2018) agrees with our results by finding a high frequency of *spa* type t605 (70.5%) in *S. aureus* isolates from cows with SM in São Paulo State, Brazil. Likewise, Silva *et al.* (2013) observed a predominance of *spa* type t605 (37.5%) in milk from cows subclinically infected. The high prevalence of our findings suggests that these isolates are well adapted to bovine herds in Brazil, indicating the presence of an endemic *S. aureus* widely disseminated that may be descendants of a common ancestor, which can invade and remain in the bovine mammary cells, causing chronic SM. These findings corroborate what other studies have reported by showing the persistence of *spa* type t605 (Veh *et al.*, 2015; Cândido *et al.*, 2020).

The study also investigated the genetic diversity of *S. aureus* isolates, finding that *agr* type II was the most prevalent. None of the isolates was *agr* type I or IV, and 22 tested negative for all known types. These findings are consistent with results from other studies in the region, where *agr* type II was frequently identified in *S. aureus* isolates from dairies and dairy cows with mastitis (Bonsaglia et al., 2018; da Silva Cândido et al., 2020; Schmidt et al., 2017; N. C. C. Silva et al., 2013; Soares et al., 2017). The high prevalence of *agr* type II *S. aureus* in São Paulo State, Brazil, is concerning from an epidemiological point of view, as it suggests that this strain may be well adapted to dairy environments in the region and represent a significant cause of subclinical bovine mastitis, thereby, this underscores the importance of developing and implementing effective control strategies in order to reduce the prevalence of this pathogen.

EOs are abundant in aromatic compounds. These belong to phenolic compounds and can be classified into two structural groups: terpenoids, such as monoterpenes, sesquiterpenes, and diterpenes, while the other is phenylpropanoids (Aguirre et al., 2013). EOs have garnered significant research interest focused on investigating natural products with bioactive compounds that may be applied as safe alternatives to current therapeutic options. Numerous works reveal that these compounds represent the main source of chemical diversity during discoveries in the pharmaceutical and medical area (Álvarez-Martínez et al., 2021; Bagheri et al., 2020; Ni et al., 2021). Variations in the EO components can be related to the nature and content of secondary metabolites depending on dissimilarities in the geographical sources, differences in growth, temperature, plant age, harvest season, soil characteristics, and the EO distillation process. These factors could affect the yield and chemical composition of EOs (Bagheri et al., 2020; Ni et al., 2021).

Understanding the MIC and MBC values is vital for developing alternative antimicrobial agents, particularly in light of increasing antibiotic resistance. MIC values indicate the lowest concentration of an EO required to inhibit bacterial growth, while the MBC values identify the concentration of EOs that results in bacterial death (Balouiri et al., 2016). Assessing MIC and MBC assays on *S. aureus* strains becomes vital since these parameters provide insights into the potency of EOs (Ghasemian et al., 2020). For this reason, this study is an essential tool because it may provide new strategies to control and successfully combat this important mastitis pathogen.

Among the EOs tested against *S. aureus* at a concentration of  $10^3$  CFU.mL<sup>-1</sup>, basil EO exhibited the highest antimicrobial activity, with MIC/MBC values ranging between 0.149-2.378/0.298-4.755 mg.mL<sup>-1</sup>. Our study infers that the presence of estragole (72.86%) and other bioactive compounds in basil EO plays a crucial role in its remarkable

antimicrobial activity against this pathogen. Supporting our findings, Falowo et al. (2019) reported that basil EO contains bioactive chemicals such as estragole. This organic compound naturally occurs in various plants and has garnered attention for its antimicrobial properties. A 2022 study by Galgano et al. revealed that the *Ocimum basilicum* EO, which contains about 73% estragole, exhibited bactericidal effects against *S. aureus* strains. Other studies have also corroborated the antimicrobial potential of EOs containing estragole against this pathogen, reinforcing the importance of the compound for this activity (Bezerra et al., 2021; Mota et al., 2015). In addition, Bergamot EO was the second most effective antimicrobial agent, with MIC/MBC values for bergamot EO between 0.277-4.435/1.109-8.87 mg.mL<sup>-1</sup>. The most abundant compounds in this EO were linalyl acetate (39.18%), limonene (29.88%), and linalool (16.91%). We also suggest that bergamot EO may be effective for treating *S. aureus*.

The use of varying concentrations, as well as sub-MIC levels, allowed us to gain a comprehensive understanding of how EOs can potentiate the effects of antibiotics. EOs tested against *S. aureus* at a concentration of 10<sup>7</sup> CFU.mL<sup>-1</sup> exhibit antimicrobial properties even at sub-MIC concentrations. Understanding the effects of EOs at sub-MIC concentrations is crucial in the context of antibiotic resistance, making them promising candidates for alternative or adjunctive therapies. Our studies have demonstrated that EOs such as clove, arrayan, and spearmint exhibited antimicrobial properties even at sub-MIC levels.

While clove displayed the lowest MIC value, the combination with spearmint revealed potent inhibitory effects against *S. aureus* isolates tested. Interestingly, despite its higher MIC value, spearmint showed strong synergistic potential when combined with other EOs. This suggests that the efficacy of EOs is not solely dependent on their individual MICs but is significantly influenced by their combinations. In addition, the

observed synergistic effect is not only due to the antimicrobial properties of the EOs but also their phytochemical compositions. Terpenes and terpenoids, abundant in EOs, improve bacterial membrane porosity, thus enhancing antimicrobial action (Álvarez-Martínez et al., 2021; Masyita et al., 2022). These complex interactions between EOs and bacterial strains, including their inherent resistance mechanisms, seem to play a critical role in the observed effects.

Our study found strong synergistic interactions in double EO combinations, notably between clove and spearmint, and arrayan and spearmint. However, the triple combinations displayed predominantly indifferent outcomes, possibly due to competitive inhibition or a saturation effect where adding more components does not necessarily improve efficacy (Burt, 2004). This aligns with findings by Vieira-Brock et al. (2017), who noted that blending different EOs did not result in additive effects, cautioning that 'more' is not always 'better' in EO combinations.

These EOs tested were effective against antibiotic-resistant *S. aureus*, even at low concentrations. Further, they can also interrupt bacterial survival strategies, making them a promising new approach to managing antibiotic resistance. Additionally, the significant decrease in MIC values from EO combinations strongly indicates their synergistic capabilities.

Overall, the results of this study are promising, as they confirm the antibacterial potential of EOs against antibiotic-resistant isolates in both  $10^3$  CFU.mL<sup>-1</sup> and  $10^7$  CFU.mL<sup>-1</sup>. The EOs tested not only inhibited bacterial growth but also appeared to disrupt biofilm formation, a critical factor in the persistence of infections. These findings align with earlier studies that have identified the antimicrobial and antibiofilm properties of EOs (Salinas et al., 2022; Yadav et al., 2015; Zhu et al., 2018).

The cytotoxicity assays conducted on Vero cells revealed important insights into the biological impact of EOs beyond their antimicrobial capabilities. The  $IC_{50}$  values, which indicate the concentration of EOs reducing cell growth by ~50%, were determined for each EO. It was found that clove EO had the lowest  $IC_{50}$  value of 0.021, making it the most cytotoxic among the tested EOs. This high cytotoxicity could be attributed to its bioactive compounds, one of which is  $\delta$ -cadinene. This sesquiterpene has not been studied extensively but can potentially provide therapeutic benefits (Mulyaningsih et al., 2010) (Mulyaningsih et al. 2010). Arrayan EO also displayed significant cytotoxicity with an  $IC_{50}$  value of 0.231. Interestingly, it contains 1,8-cineole, which demonstrated minimal toxicity in previous studies, and limonene, a compound with demonstrated anticancer potential (Schnitzler et al., 2008; Hajizadeh et al., 2019). The cytotoxic effects observed in our in vitro study using Vero cells raise essential questions about the safety of these EOs when applied to bovine mastitis or other tissues. Advanced formulation techniques such as emulsions, gels, or encapsulation could offer more targeted and safer delivery methods. Further, in vivo studies are essential to determine appropriate dosing and assess safety. Given the cytotoxic effects observed, optimizing the EO formulations to mitigate these cytotoxic effects while preserving their antibacterial properties is a critical future direction.

In addition, considering the limitations of current antibiotics and the rapid emergence of resistance (Tomanić et al., 2023), these findings have important implications, particularly in the treatment of bovine mastitis. EOs exhibited a better antimicrobial effect when in combination than those tested independently. For this reason, we corroborate that interactions between EO compounds can potentiate the antimicrobial effect at lower concentrations. The observed synergistic effects offer a promising avenue

for antimicrobial treatments, reducing the dosages required thereby minimizing potential toxic effects (Tariq et al., 2019).

The combined findings highlight the potential and complexities of using EOs as antimicrobial agents. They offer new strategies to combat antibiotic-resistant bacteria, particularly *S. aureus*, which causes bovine mastitis. However, it is essential to consider factors such as efficacy, cytotoxicity, and safety when evaluating clinical applications. Optimizing EO formulations to balance antimicrobial efficacy with minimal cytotoxic effects will be a crucial next step. Additionally, in vivo studies are necessary for validating these promising in vitro results.

# CHAPTER 7

*General conclusions*

## **General conclusions**

This comprehensive study makes significant advances in exploring the potential of EOs as potential antimicrobial agents. Our findings also shed light on the virulence factors and antibiotic-resistant profiles of these strains. With detailed investigations into antibacterial efficacy, synergistic interactions, and cytotoxicity, this research offers a comprehensive understanding of the complexities of EOs. While promising in their antimicrobial properties, the study also underscores the need for caution due to the cytotoxic effects observed, particularly in treating bovine mastitis caused by the pathogen.

The research's findings offer hope and direction for future studies and is particularly significant given the increasing prevalence of antibiotic resistance *S. aureus* strains. The following steps involve optimizing EO formulations to balance efficacy and safety and conducting in vivo tests to validate promising in vitro results. Furthermore, our research provides a foundation for exploring alternative antimicrobial therapies, leading to more targeted and safer treatment options against *S. aureus* strains.



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# Appendix 1

## Proof of publication

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## Virulence and antimicrobial resistance genes profiles of *spa* type t605 methicillin-susceptible *Staphylococcus aureus* isolated from subclinical bovine mastitis

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### Abstract

**Aims:** *Staphylococcus aureus* is one of the most common pathogens associated with mastitis in dairy herds worldwide. This study evaluated the profile of virulence and antimicrobial resistance genes of *spa* type t605 methicillin-susceptible *Staphylococcus aureus* isolated from subclinical bovine mastitis in São Paulo, Brazil.

**Methods and results:** A total of 57 *S. aureus* strains were screened by conventional PCR (Polymerase Chain Reaction) for 49 virulence genes. The most prevalent virulence genes detected were *icaD* (94.7%), *fib* (93%), *fnbA* (82.5%), *clfA* (80.7%), *bap* (78.9%), *clfB* (73.7%), *icaA* (66.7%), *see* (64.9%), and *sed* (61.4%). The *blaZ* (94.7%), *aac6'aph2'* (15.8%), and *ant4* (12.3%) genes were the most common antimicrobial resistance genes; however, *mecA* and *mecC* genes were not found. All methicillin-susceptible *S. aureus* (MSSA) strains were characterized through *spa* and *agr* typing. The *spa* type t605 was found in all isolates. By *agr* typing, the most prevalent were type II (56.1%). Antimicrobial resistance was determined by the disk diffusion method, and 93% showed resistance to at least one antibiotic. Penicillin resistance was the most prevalent (87.7%), followed by tetracycline (12.3%), oxacillin (10.5%), and gentamicin (10.5%) resistance.

**Conclusion:** Our study confirmed the *spa* type t605 as endemic, carrying a wide variety of virulence factors and high-level penicillin resistance. The profile seems to be associated with the colonization of MSSA and its persistence in subclinical mastitis.

### Significance and impact of the study

The methicillin-susceptible *Staphylococcus aureus* characteristics found here evidence its potential risk to human and bovine health and emphasize the need for continuous surveillance to control its presence. These findings improve the comprehension of its pathogenesis, genomic variations, and antibiotic resistance, which can help to enhance therapeutic recommendations and optimize bovine mastitis treatment.

**Keywords:** MSSA, MSCRAMMs, antibiotic resistance, *spa* typing, mastitis

### Introduction

Subclinical mastitis (SM) represents the most prevalent type of bovine mastitis, and *Staphylococcus aureus* is one of the main etiologic agents causing contagious intramammary infections (Ruegg 2017, Côté-Gravel and Malouin 2019, Dyson et al. 2022). The risk of *S. aureus* infection is increased when unhygienic practices are performed during milking; these include contaminated equipment, unsanitary environments, and the lack of trained personnel in dairy operations (Zaatout et al. 2019). Cows with SM caused by *S. aureus* present high SCC (>200 × 10<sup>3</sup> cells mL<sup>-1</sup>), but no visual changes in milk are observed (Gonçalves et al. 2018). However, it can cause human intoxication since bacteria can be eliminated through a thermal process, but enterotoxins may remain, increasing the possibility of foodborne poisoning (Grispoldi et al. 2021, Campos et al. 2022). In this regard, *S. aureus* has been associated with 9.4% and 7.8% of food-poisoning cases concerning milk and dairy products, respectively, with 120 584 cases re-

ported by the Brazilian Ministry of Health between 2009 and 2018 (Brasil 2019).

Molecular studies are important to evaluate the genetic diversity of pathogens and contribute to understanding sources, routes of transmission, prognosis, and adaptation mechanisms (McMillan et al. 2016). *Staphylococcus aureus* represents a significant issue for bovine udder health. Its adaptation to the tissue has been associated with persistence and specific genetic and phenotypic traits (Schmidt et al. 2017, Santos et al. 2020). In addition, this pathogen has various virulence factors and survival strategies to persist and defeat host defences, such as adhesion capacity and biofilm formation (Bhunia 2008, Zhu et al. 2018). The expression of these virulence factors is rigorously controlled by a series of regulatory genes influenced by the accessory gene regulator (*agr*). Their presence or absence plays an important role in the persistence of the pathogen causing mastitis (Rossi et al. 2021). Overall, mastitis can be reduced in the dairy industry by identifying the

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## Appendix 2

### SISGEN registration declaration



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

Número do cadastro:	<b>ACBF62E</b>
Usuário:	<b>Juliano Leonel Gonçalves</b>
CPF/CNPJ:	<b>056.313.399-60</b>
Objeto do Acesso:	<b>Patrimônio Genético</b>
Finalidade do Acesso:	<b>Pesquisa</b>

#### **Espécie**

**Staphylococcus aureus**

Título da Atividade: **Impacto da mastite bovina sobre a produção e a qualidade do leite**

#### **Equipe**

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#### **Parceiras Nacionais**

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