YASMIN NEVES VIEIRA SABINO

INHIBITION OF *Staphylococcus aureus* VIRULENCE BY *Bacillus* spp. AS A POTENTIAL THERAPY TO CONTROL BOVINE MASTITIS

Thesis submitted to the Agricultural Microbiology's Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Hilário Cuquetto Mantovani

Co-adviser: Tiago Antônio de O. Mendes

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Author

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Hilário Cuquetto Mantovani
Adviser

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Focus, dedication, strength, courage, and faith.

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ABSTRACT

SABINO, Yasmin Neves Vieira, D.Sc., Universidade Federal de Viçosa, February, 2023. **Inhibition of *Staphylococcus aureus* virulence by *Bacillus* spp. as a potential therapy to control bovine mastitis.** Adviser: Hilario Cuquetto Mantovani. Co-adviser: Tiago Antônio de Oliveira Mendes.

S. aureus is a common pathogen of humans and animals that causes long-lasting infections. In the food production sector, bovine mastitis caused by *S. aureus* is a common disease that severely affects milk production and quality. The treatment of bovine mastitis is challenging due to the resistance of *S. aureus* to many available antibiotics, and the development of new therapeutic approaches to combat antibiotic resistant bacteria has been a research priority. The objective of this study was to investigate anti-virulence compounds that inhibit hemolytic activity and biofilm formation by *S. aureus*, which are virulence factors associated with the severity and persistence of bovine mastitis, respectively. We evaluated the capacity of *Bacillus* spp., a bacterium that produces an arsenal of biologically active metabolites, to produce anti-virulence compounds. The active compounds were identified and characterized, and their mechanisms of action were evaluated. The results showed that *Bacillus subtilis* and *Bacillus velenzesis* strains were able to secrete anti-hemolytic and anti-biofilm compounds in their supernatants. The anti-hemolytic activity of *Bacillus* spp. against *S. aureus* was attributed to the production of lipopeptides. Crude extracts containing lipopeptides were able to completely abolish the hemolytic activity of *S. aureus* at certain concentrations. After purification, iturin was the class of lipopeptides that showed the highest anti-hemolytic activity. Molecular docking analyses revealed the ability of iturin to bind to the heptameric structure of hemolysin, both outside and inside the hemolysin pore. In turn, exopolysaccharides produced by both *Bacillus* species showed a dose-dependent anti-biofilm effect. At a final concentration of 1 mg/mL, exopolysaccharides were able to reduce biofilm formation by up to 83% depending on the *S. aureus* strains tested. The characterization of the exopolysaccharides revealed that the compounds produced by *B. velezensis* 87 and *B. subtilis* TR47II have a similar structure of approximately 30 kDa, composed mainly of glucose and mannose. The results indicated that these exopolysaccharides possibly inhibit biofilm formation mainly due to a modification of the abiotic surface characteristics. In conclusion, the

study reveals for the first time the potential use of *Bacillus* spp. and its metabolites to control the virulence of *S. aureus* associated with bovine mastitis.

Keywords: *Staphylococcus aureus*. Bovine mastitis. *Bacillus* spp. Anti-virulence. Biofilm. Hemolysin.

RESUMO

SABINO, Yasmin Neves Vieira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2023. **Inibição da virulência de *Staphylococcus aureus* por *Bacillus* spp. como uma potencial terapia para o controle de mastite bovina.** Orientador: Hilario Cuquetto Mantovani. Coorientador: Tiago Antônio de Oliveira Mendes.

S. aureus é um patógeno que causa infecções duradouras em humanos e animais. No setor de produção de alimentos, a mastite bovina causada por *S. aureus* é um fenômeno comum que afeta severamente a produção e a qualidade do leite. O tratamento da mastite bovina é desafiador devido à resistência de *S. aureus* a muitos antibióticos disponíveis, e o desenvolvimento de novas abordagens terapêuticas para combater bactérias resistentes a antibióticos tem sido uma prioridade de pesquisa. O objetivo deste estudo foi investigar compostos anti-virulência que inibem a atividade hemolítica e a formação de biofilme por *S. aureus*, que são fatores de virulência associados à severidade e persistência da mastite bovina, respectivamente. Nós avaliamos a capacidade de *Bacillus* spp., uma bactéria que produz um rico arsenal de metabólitos biologicamente ativos, de produzir compostos anti-virulência. Os compostos ativos foram identificados e caracterizados e seus mecanismos de ação foram avaliados. Os resultados mostraram que estirpes de *Bacillus subtilis* e *Bacillus velenzesis* foram capazes de secretar compostos anti-hemolíticos e anti-biofilme em seus sobrenadantes. A atividade anti-hemolítica de *Bacillus* spp. contra *S. aureus* foi atribuída à produção de lipopeptídeos. Extratos brutos contendo lipopeptídeos foram capazes de abolir completamente a atividade hemolítica de *S. aureus* em certas concentrações. Após purificação, iturina foi a classe de lipopeptídeos que apresentou maior atividade anti-hemolítica. Analises de docking molecular revelaram a capacidade da iturina de se ligar à estrutura heptamérica da hemolisina, tanto fora quanto dentro do poro da hemolisina. Por sua vez, exopolissacarídeos produzidos por ambas as espécies de *Bacillus* mostraram um efeito anti-biofilme dose-dependente. Em uma concentração final de 1 mg/mL, os exopolissacarídeos foram capazes de reduzir a formação de biofilme em até 83%, dependendo das estirpes de *S. aureus* testadas. A caracterização dos exopolissacarídeos revelou que os compostos produzidos por *B. velezensis* 87 e *B. subtilis* TR47II tem uma estrutura semelhante de aproximadamente 30 kDa, composta principalmente por glicose e manose. Os resultados indicaram que esses exopolissacarídeos possivelmente inibem a formação

do biofilme principalmente devido a uma modificação das características abióticas da superfície. Em conclusão, o estudo revela pela primeira vez o potencial de *Bacillus* spp. e seus metabólitos no controle da virulência de *S. aureus* associado à mastite bovina .

Palavras-chave: *Staphylococcos aureus*. Mastite bovina. *Bacillus* spp. Anti-virulência. Biofilme. Hemolisina.

SUMMARY

GENERAL INTRODUCTION

Brazil is among the largest milk producers in the world, with approximately 35 million tons of milk being produced annually (Embrapa 2022). However, the Brazilian dairy herds show low milk productivity (about 2,192 liters/cow/year) compared to developed countries, such as the USA (about 11,000 liters/cow/year), the largest milk producer in the world (Embrapa 2022). Although many factors affect milk productivity of Brazilian dairy herds, the occurrence of mastitis in dairy farms is reported as the main cause of reduced milk quality and production. Mastitis is the most frequent inflammatory disease of the mammary gland in dairy cows, causing severe effects on animal health and significant economic losses to dairy production systems (Ruegg 2017). The total cost of mastitis in a herd of 159 lactating cows was estimated to be US$ 61,623.13 per year, with 54.9% representing the losses caused by the reduction of milk production (Guimarães *et al.* 2017).

Staphylococci, coliforms, enterococci, and streptococci are considered the main etiological agents of bovine mastitis (Smulski *et al.* 2011; Gomes *et al.* 2016a). Among these, *Staphylococcus* is the genera most commonly isolated from dairy cows with mastitis (Leitner *et al.* 2011), with *S. aureus* being frequently associated with subclinical symptoms of the disease (Oliveira *et al.* 2007). *S. aureus* is a Gram-positive bacterium frequently reported as a nosocomial pathogen and a common cause of skin and soft tissue infections (Tong *et al.* 2012). The capacity of *S. aureus* to colonize and persist in different hosts can be largely attributed to the outstanding variety of virulence factors that it can produce, including toxins, adhesins, and immune evasion molecules, in addition to biofilm formation (Monistero *et al.* 2018; Haag *et al.* 2019; Cheung *et al.* 2021).

In the context of bovine mastitis, biofilm formation and the synthesis of extracellular toxins such as alpha-hemolysin play an important role in *S. aureus* colonization and invasion (Pérez *et al.* 2020). Alpha-hemolysin is a well-studied toxin produced by *S. aureus* capable of damaging multiple cell types in the host, including red and white blood cells, as well as keratinocytes, epithelial, and endothelial cells (Divyakolu *et al.* 2019a; Ahmad-Mansour *et al.* 2021). A previous study showed that a mammary gland infection in mice induced by a mutant strain of *S. aureus* unable to produce alpha and beta-hemolysin resulted in greater host survival, lower recovery of bacteria, and better preservation of mammary cell structure compared to the infection

caused by the wild-type strain, showing the importance of hemolysins to the pathogenicity of *S. aureus* in mastitis (Bramley *et al.* 1989).

Biofilm formation, in turn, has been associated with chronic and recurrent mastitis infections in dairy cattle (Melchior *et al.* 2006). In *S. aureus*, the formation of a biofilm begins with the production of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), important for bacterial adhesion to the host cell, followed by the secretion of extracellular polymeric substances, especially polysaccharide intercellular adhesion (PIA) (Otto 2008). The protection of *S. aureus* cells by the biofilm matrix reduces the susceptibility of *S. aureus* to antimicrobials. Also, the decreased growth rate of sessile cells and the presence of a genetically heterogeneous bacterial population inside the biofilm contribute to this resistance (Prenafeta 2014).

Currently, the control of bovine mastitis in dairy cows depends heavily on antibiotics (Gomes and Henriques 2016). Although antibiotics are considered effective to treat bovine mastitis, the treatment efficacy varies between animals and bacterial strains. Antibiotic-resistant strains may also be selected with repeated application of intramammary antibiotics and antibiotic residues in the milk can interfere with fermentation processes in the dairy industry or cause allergic reactions in consumers (Kurjogi *et al.* 2019). Therefore, alternative treatments have been proposed for mastitis including the use of bacteriophages, nanoparticles, and natural compounds with antimicrobial activity (Gomes and Henriques 2016; Godoy-Santos *et al.* 2019). However, these strategies are mostly focused on killing the pathogens, thus imposing selective pressures in the intramammary ecosystem, which in turn can promote the emergence of resistant strains (Hughes 2014). Therefore, anti-virulence therapies could represent a potential alternative to downregulate the pathogenicity of pathogens while imposing a lower selective pressure (Dickey *et al.* 2017; Shoham and Greenberg 2017).

Bacteria are a rich source of compounds with biological applications (Abdelghani *et al.* 2021) with fast, low-cost, and eco-friendly production. *Bacillus* comprises a genus of spore-forming bacteria known for the synthesis of secondary metabolites with potential industrial applications (Lyngwi *et al.* 2014; Elshaghabee *et al.* 2017). Studies have shown that *Bacillus* spp. can produce compounds with anti-virulence activity. For example, lipopeptide produced by *Bacillus subtilis* (fengycin) inhibited the quorum sensing system of *S. aureus* from human origin (Piewngam *et al.*

2018). Different compounds produced by *Bacillus* spp. have also demonstrated activity against biofilm formation by *S. aureus* (Sayem *et al.* 2011; Kalpana *et al.* 2012; Giri *et al.* 2019). Nonetheless, little is known about the efficacy of anti-virulence compounds on the pathogenesis of bacteria associated with bovine mastitis. Therefore, this study aimed to investigate anti-virulence compounds produced by *Bacillus* spp. that could attenuate the pathogenicity of *S. aureus* isolated from bovine mastitis.

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CHAPTER 1

Anti-virulence compounds against *Staphylococcus aureus* associated with bovine mastitis: a new therapeutic option?

Yasmin Neves Vieira Sabino[1], Paul David Cotter[2], Hilário C. Mantovani[1,3]

[1]Department of Microbiology, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil

[2]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[3]Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Madison, WI, USA

Corresponding author: Hilário C. Mantovani, Present address: Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Madison, WI, USA, e-mail: hcmantovani@wisc.edu; hcm6@ufv.br

Abbreviated running headline: *S. aureus* anti-virulence therapy

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ABSTRACT

Bovine mastitis represents a major economic burden faced by the dairy industry. *S. aureus* is an important and prevalent bovine mastitis-associated pathogen in dairy farms worldwide. The pathogenicity and persistence of *S. aureus* in the bovine mammary gland are associated with the expression of a range of virulence factors that can lead to the production of several toxins and biofilm formation. The traditional therapeutic approach to treating bovine mastitis includes the use of antibiotics, but these antimicrobials can leave chemical residues in milk and contribute to the selection of antibiotic-resistant strains. Therapeutic approaches that target the expression of virulence factors in *S. aureus* rather than bacterial viability have several advantages to control udder infections, including lower selective pressure towards the development of resistance, and little impact on the host commensal microbiota. This review summarizes the potential of anti-virulence therapies to control bovine mastitis-associated *S. aureus* focusing on anti-toxin, anti-biofilm, and anti-quorum sensing compounds. It also covers potential sources of new anti-virulence inhibitors and presents screening strategies for identifying these compounds based on phenotypic and molecular assays as well as chemical analysis and computational modeling.

Keywords: biofilm, quorum sensing, screening, *Staphylococcus aureus*, toxins, virulence factors

1 INTRODUCTION

The global population is expected to reach 10.9 billion by 2100 (United Nations 2019), and the demand to feed the growing population sustainably is a major challenge for the agri-food sector. Therefore, to pace the projected increase in inhabitants, it is predicted that agricultural production will have to double or treble to meet the food demand in 2100 (Rohr *et al.* 2019). Together with the environmental crisis and the spread of infectious diseases, these challenges represent major public concerns in modern society (Rohr *et al.* 2019).

The increased demand for animal-based protein is being followed by an intensification of agricultural systems and expressive gains in production efficiency (Godfray and Garnett 2014). In this scenario, antibiotics will continue to play an important role in livestock production to promote animal growth and prevent and/or treat infectious diseases in farm animals (Hao *et al.* 2014; Muurinen *et al.* 2021). However, the intensive use of antibiotics in agriculture, even at subtherapeutic concentrations, can lead to antibiotic residues in animal-derived foods, as well as the emergence and spread of antibiotic-resistant bacteria across hosts and the environment, which represents a potential threat to human health (Serratosa *et al.* 2006).

In dairy cattle, the infection of the mammary gland referred to as mastitis, is the main cause of antibiotic use and economic loss in the dairy sector (Ashraf *et al.* 2018; Stevens *et al.* 2019). *Staphylococcus aureus* is an important etiological pathogen that causes contagious mastitis and chronic infections that are difficult to eradicate (Zaatout *et al.* 2020). In addition, *S. aureus* can cause disease in a range of animal hosts, including humans, and at distinct body sites, thereby making this bacterium even more significant. The widespread prevalence and importance of *S. aureus* as a pathogen can be greatly attributed to its arsenal of virulence factors and the genomic plasticity, which facilitates, for example, the acquisition of antibiotic resistance genes (Guerillot *et al.* 2019; Cheung *et al.* 2021). Notably, with respect to this review, the virulence factors of *S. aureus* will be highlighted due to their importance for bacterial pathogenicity in bovine mastitis (Pérez *et al.* 2020).

The resistance of *S. aureus* strains to many commercially available antibiotics has caused the failure of some therapeutic approaches, including the treatment of mastitis. The bacteriological cure rate of bovine mastitis caused by *S. aureus* using

traditional treatments such as antibiotics varies from 20 to 50% (Roy and Keefe 2012; Côté-Gravel and Malouin 2019). Moreover, as noted above, the presence of antibiotic residues in animal-derived food can be harmful to human health and, therefore, milk from animals treated with antibiotics cannot be used, leading to further economic losses (Priyanka *et al.* 2017). Given this, the development of new therapies to treat bovine mastitis caused by *S. aureus* is urgently needed.

Recently, the potential of anti-virulence therapy has gained attention in clinical and veterinary settings (Dickey *et al.* 2017; Shoham and Greenberg 2017). This approach targets the expression of virulence factors, aiming to decrease the capacity of pathogens to cause disease without affecting their growth (Fleitas Martinez *et al.* 2019). This review aims to summarize the role of virulence factors associated with mammary gland colonization by *S. aureus* and highlight the advances and challenges of anti-virulence therapies as an alternative to traditional therapeutic approaches. We also present current methods for screening anti-virulence inhibitors as well as potential hotspots for the discovery of anti-virulence molecules that could be directed to treat bovine mastitis.

2 MAIN VIRULENCE FACTORS OF *S. aureus* INVOLVED IN MASTITIS INFECTIONS

S. aureus infections in bovine mastitis can be described as a biphasic process where the synthesis of bacterial adhesins is followed by the production of several pore-forming exotoxins and extracellular enzymes (Pérez *et al.* 2020). Adhesins allow *S. aureus* to recognize host structures and initiate colonization. Toxins such as hemolysins, leukotoxins, and enterotoxins, in addition to exoenzymes, including several proteases and lipases, facilitate bacterial invasion and the escape from the host immune system (Pérez *et al.* 2020) (Figure 1).

Many virulence factors are involved in the adhesion step, including fibronectin (FnBP), fibrinogen (FgBP), collagen binding (Cna) proteins as well as clumping factors (ClfA and ClfB) (Pérez *et al.* 2020; Campos *et al.* 2022). These matrix-associated proteins are known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Among the MSCRAMMs, fibronectin-binding protein A (FnBPA) and fibronectin-binding protein B (FnBPB) are primarily responsible for *S. aureus* adherence (Lammers *et al.* 1999; Campos *et al.* 2022). A greater invasion of bovine mammary epithelial cells (bMEC) was observed when *fnb* genes were

overexpressed (Pereyra *et al.* 2016) while a strain lacking FnBPs had a reduced ability to colonize the mammary gland of mice (Brouillette *et al.* 2003). These observations indicate that FnBPBs are essential for the colonization of the bovine mammary gland by *S. aureus in vivo*.

Biofilm formation is also important for bacterial colonization of the bovine mammary gland (Pérez *et al.* 2020; Seethalakshmi *et al.* 2020). Biofilms are defined as well-structured and dynamic microbial communities that remain physically attached to a surface. Sessile cells are typically embedded in an extracellular matrix composed primarily of exopolysaccharides, proteins, nucleic acids, and lipids (Yin *et al.* 2019). The first step of *S. aureus* biofilm formation is the adhesion to epithelial cells of the mammary gland, a process in which MSCRAMMs play an important role (Zaatout *et al.* 2020). The production of extracellular polymeric substances is important in the second step of biofilm formation, which includes biofilm proliferation and maturation. The main component of the extracellular matrix is the polysaccharide intercellular adhesion (PIA), a homopolymer of β-1,6-N-acetylglucosamine (NAG) residues encoded by the *ica* operon (Arciola *et al.* 2015). The final step in biofilm formation is the bacterial detachment by the action of proteases (Boles and Horswill 2008), nucleases (Beenken *et al.* 2012), and phenol soluble modulins (PSMs) that break noncovalent interactions between cells and the matrix (Le *et al.* 2014). Biofilms are considered a common phenotype among *S. aureus* strains isolated from bovine mastitis (Pedersen *et al.* 2021) and this phenotype is associated with therapeutic failure, chronic infections, and mastitis recurrence (Melchior *et al.* 2006). The low efficacy of antimicrobials against biofilms is due to the difficulties to reach the cells embedded in the extracellular matrix at a bactericidal concentration. The stress-tolerant state and decreased growth rate of sessile cells, and the genetically heterogeneous bacterial population forming the biofilm also contribute to reducing the susceptibility of *S. aureus* cells to antimicrobials (Prenafeta 2014).

After colonization, *S. aureus* secretes virulence factors that allow the invasion and destruction of the mammary gland tissue (Côté-Gravel and Malouin 2019). *S. aureus* produces several membrane-damaging exotoxins, such as alpha- and beta-hemolysins (Vandenesch *et al.* 2012). Alpha-hemolysin (Hla), encoded by the gene *hla*, is the most characterized *S. aureus* hemolysin (Vandenesch *et al.* 2012). It causes damage to different cell types including erythrocytes, monocytes, and keratinocytes, in addition to epithelial and endothelial cells (Divyakolu *et al.* 2019; Ahmad-Mansour *et*

al. 2021). The beta-hemolysin (Hlb) is considered less cytotoxic but its sphingomyelinase activity makes cells more susceptible to alpha-hemolysin activity (Cifrian *et al.* 1996). The importance of Hla and Hlb in bacterial virulence during intramammary infections was confirmed using double mutants of *S. aureus* (Δ*hla* and Δ*hlb*) that failed to express these proteins. Cows infected with the mutants showed a lower inflammatory response compared to animals infected with the wild-type toxigenic strain (Kenny *et al.* 1992). Analysis of virulence of *S. aureus* double mutants (*Δhla* and *Δhlb*) using a murine model of mammary gland infection showed greater survival of mice and lower recovery of bacteria from the mammary gland 48 h post inoculation. The histopathological analysis indicated that glands infected with the *S. aureus* mutant (Δ*hla*/Δ*hlb*) had less necrosis and better preservation of the cell structure (Bramley *et al.* 1989).

S. aureus also secretes superantigens (SAs), including staphylococcal enterotoxins (SE), staphylococcal enterotoxins like (SEl), and toxic shock syndrome toxin (TSST-1), which have potent immunostimulatory activity (Campos *et al.* 2022). Therefore, these toxins play a role in the inflammatory response due to their mitogenic activity on T-cells (Tuffs *et al.* 2018). In cattle, *S. aureus* RF122 lacking the gene encoding TSST-1 toxin, was unable to induce clinical mastitis (Wilson *et al.* 2018). In addition to their importance in mastitis development, the presence of these toxins in milk poses a risk of food poisoning in humans (Ahmad-Mansour *et al.* 2021).

In addition, *S. aureus* produces leukotoxins to destroy host immune cells. Leukotoxins target cells involved in host defense, such as macrophages, neutrophils, and monocytes by interacting with membrane receptors and forming a β-barrel pore (Tromp and van Strijp 2020). Among other leukotoxins synthesized by *S. aureus* of bovine origin, LukMF` is a potent toxin against bovine neutrophils that reduces host defense leading to faster colonization of the bovine udder (Vrieling *et al.* 2015; Pérez *et al.* 2020). A comparison of cows infected with *S. aureus* strains that produced a high and intermediate amount of LukMF` revealed that only the first group developed severe clinical symptoms, demonstrating the importance of leukotoxins in the *S. aureus* pathogenesis in bovine mastitis (Vrieling *et al.* 2016).

The pathogenicity of *S. aureus* is coordinated by a complex regulatory system that controls the synthesis of virulence factors. The regulatory network includes two-component systems such as the accessory gene regulator (*agr*), *S. aureus* exoprotein expression (SaeRS), staphylococcal respiratory regulator (SrrAB), and autolysis

regulator locus (ArlTS). These systems play an important role in sensing environmental signals and coordinating the activity of alternative sigma factors and cytoplasmic regulators (Jenul and Horswill 2019). Among these, the *agr* system is the best characterized and it is known to coordinate the transition from the colonization to the invasive phase of *S. aureus* infections (Novick and Geisinger 2008).

The *agr* locus is 3.5 kb in size and contains the genes *agrA*, *agrB*, *agrC,* and *agrD*, which control the expression of two divergent transcripts from promoters P2 and P3, referred to as RNAII and RNAIII, respectively (Le and Otto 2015). The *agrD* transcript encodes a precursor peptide called the autoinducing peptide (AIP), which is processed and exported by AgrB, a transmembrane endopeptidase. AgrC and AgrA constitute a two-component regulatory system. When AIP accumulates at high concentrations in the extracellular medium, the sensory protein AgrC recognizes the AIP and is activated by autophosphorylation. This process induces phosphorylation of the response regulator AgrA, which binds to promoters P2 and P3. The expression of transcript RNAIII is induced from promoter P3, and RNAIII promotes the translation of several virulence factors, such as the toxin Hla, serine proteases (SspA, SspB, etc), and the lipase Geh. In contrast, RNAIII blocks the translation of adhesins, protein A and coagulase. AgrA also positively controls the expression of phenol-soluble modulins (psm), which are involved in the biofilm dispersal phenotype (Le and Otto 2015) (Figure 1).

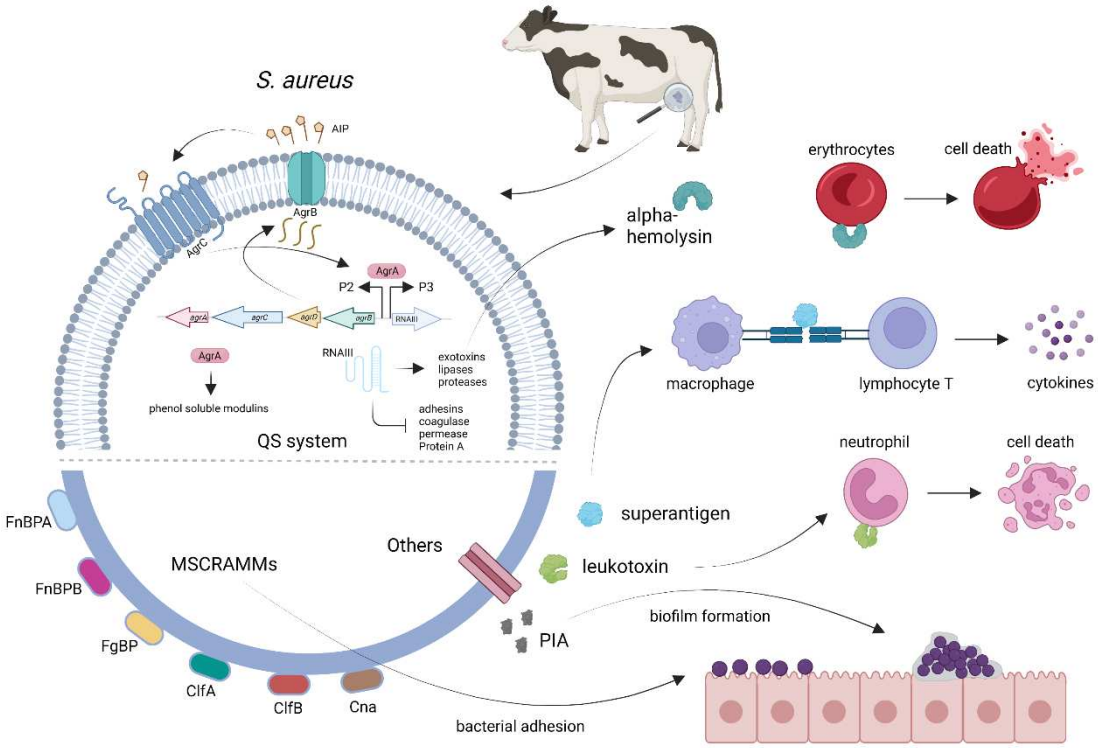

Figure 1. Virulence traits of *S. aureus* in bovine mastitis. The production of surface proteins, also known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), allows bacterial adhesion and host colonization. The production of polysaccharide intercellular adhesion (PIA) contributes to biofilm formation. *S. aureus* produces leukotoxins promoting the death of immune defense cells and superantigens inducing the release of pro-inflammatory cytokines. The production of several proteins involved in *S. aureus* virulence is coordinated by the accessory gene regulator (*agr*) system. The autoinducer peptide (AIP) is produced by *agrD* and matured and exported by AgrB. AgrC is a sensor protein that recognizes AIP and activates AgrA, a response regulator. AgrA promotes the transcription of phenol-soluble modulins and RNAIII. RNAIII, in turn, inhibits the translation of proteins involved in bacterial colonization and promotes the translation of proteins involved in bacterial invasions, such as alpha-hemolysin. Alpha-hemolysin destroys erythrocytes by forming pores. The figure was constructed using BioRender.

3 ANTI-VIRULENCE THERAPY AGAINST *S. aureus*

The increase in infections caused by multidrug-resistant bacteria in clinical and veterinary settings and the scarce therapeutic options to treat antibiotic-resistant

pathogens is a matter of public concern. Therefore, the development of efficacious therapies to combat microbial pathogens that can circulate across species is pivotal. Therapeutic strategies based on an anti-virulence approach have emerged as an alternative to depriving bacteria of their virulence factors, therefore decreasing pathogenicity (Fleitas Martinez *et al.* 2019). Since the impact on pathogen growth is expected to be minimal, the evolutionary pressure toward resistance is likely to be lower compared to antibiotics (Sully *et al.* 2014; Quave *et al.* 2015; Vale *et al.* 2016). Moreover, the use of anti-virulence therapies ensures that only pathogens are targeted, with no collateral effects on the commensal microbiota (Dickey *et al.* 2017). In view of the previously described virulence factors of *S. aureus* and their role in bovine mastitis, here we describe the potential of several approaches that target virulence traits of *S. aureus* to prevent disease (Figure 2).

3.1 Hemolysin

Several approaches have been used to design anti-toxin treatments against *S. aureus*, including antibodies developed to impair the activity of hemolysins, such as 7B8, 1A9, 2A3.1, LTM-14, MEDI4893, and AR-301 (Kong *et al.* 2016; Ahmad-Mansour *et al.* 2021). Hemolysin-neutralizing antibodies were successful in protecting mice against lethal pneumonia caused by *S. aureus* (Ragle and Bubeck Wardenburg 2009; Hua *et al.* 2014) and also reducing abscess formation in a dermonecrosis model (Tkaczyk *et al.* 2012; Foletti *et al.* 2013). Moreover, some antibodies seem to act synergistically with antibiotics (Foletti *et al.* 2013; Hua *et al.* 2014) potentially increasing the efficacy of antibacterial treatments.

The development of vaccines using inactivated or modified proteins that share similarities with *S. aureus* toxins has also been evaluated as an anti-virulence approach. The active immunization of mice with a mutant form of alpha-hemolysin (Hla_{H35L}) lacking its pore-forming properties prevented staphylococcal pneumonia in mice. This protective effect was associated with the production of antigen-specific immunoglobulin G against the staphylococcal cytotoxins in the immunized mice (Bubeck Wardenburg and Schneewind 2008). The multi-component vaccine IBT-V02 is under development for the prevention of skin infections caused by *S. aureus* including methicillin-resistant *S. aureus* (MRSA) (Karauzum *et al.* 2021) and is

expected to enter phase I safety and immunogenicity trials (Ahmad-Mansour *et al.* 2021).

Some types of nanoparticles, such as liposomes, can mimic the properties of the cell membrane and could be used to sequester bacterial toxins (Fang *et al.* 2015). Cholesterol-containing sphingomyelin liposomes sequestered cytolytic enzymes, such as alpha-hemolysins, and reduced tissue dermonecrosis in a murine cutaneous abscess model (Wolfmeier *et al.* 2018). Moreover, biomimetic toxin nanosponges that were formulated by coating the membranes of human red blood cells with polymeric nanoparticles could absorb and neutralize several hemolytic toxins (Chen *et al.* 2018).

Natural products are also a valuable source of anti-virulence compounds. Oligomeric compounds such as cyclodextrin derivatives produced from starch were tested as inhibitors of pore-forming proteins produced by *Bacillus anthracis* and *S. aureus* (Karginov *et al.* 2007). These molecules prevented the assembly of heptameric transmembrane pores formed by the protective antigen (PA) subunit of anthrax toxin and inhibited the activity of the alpha-hemolysin (Hla) of *S. aureus* (Karginov *et al.* 2007). Similarly, natural compounds present in morin hydrate from common guava, osage orange, and old fustic disrupted the assembly of the alpha-hemolysin pore, decreasing its hemolytic activity (Wang *et al.* 2015). Oroxin A, Oroxin B, and oroxylin A7-O-glucuronide, which are flavonoids present in fruits like strawberries, apples, and grapes, also prevent the transition of the hemolysin monomer to oligomer (Dong *et al.* 2013; Qiu *et al.* 2013).

Other frequent targets to reduce hemolysis are the regulatory mechanisms that control hemolysin expression, such as the quorum sensing (QS) system. The quorum sensing system, encoded by accessory gene regulator (*agr*) in *S. aureus*, positively regulates the expression of RNAIII. RNAIII is a small (514-nt) RNA that activates the translation of *hla* and other virulence factors via an antisense mechanism (Jenul and Horswill 2019). A study based on virtual screening and *in vitro* testing demonstrated that some small molecules, including diflunisal, an FDA-approved nonsteroidal anti-inflammatory drug, could inhibit the activation of the transcriptional factor AgrA (Khodaverdian *et al.* 2013). As a consequence, virulence genes that are involved in the hemolytic phenotype of *S. aureus* (e.g., *hla* and *psmα*) were down-regulated (Khodaverdian *et al.* 2013). Resveratrol, a polyphenol found in grapes, decreased the hemolytic activity of *S. aureus* by down-regulating the expression of the genes encoding RNAIII and alpha-hemolysin (Tang *et al.* 2019). A similar mechanism of

action has been demonstrated for chalcone and isorhamnetin, two anti-virulence compounds that inhibit the hemolytic activity of *S. aureus* by reducing the expression of relevant genes, such as *hla*, *agrA*, and RNAIII (Jiang *et al.* 2016; Zhang *et al.* 2017).

3.2 Biofilm

In clinical settings, biofilms are considered to play an important role in microbial infections, and attempts have been made to discover natural compounds with anti-biofilm activity. For example, chitosan is a linear polysaccharide highly soluble in an acid solution that has biocompatibility, low toxicity, and antimicrobial activity. Previous studies demonstrated that a 2.6 kDa chitosan had anti-biofilm activity against mastitis-associated *S. aureus* cells (Asli *et al.* 2017; Felipe *et al.* 2019). This low molecular weight chitosan prevented the persistence of internalized methicillin-resistant *S. aureus* (MRSA) in MAC-T cells, killed bacteria inside biofilms in a dose-dependent manner, and showed synergistic effects in combination with tilmicosin, a macrolide analog of erythromycin. Moreover, intramammary administration of the 2.6 kDa chitosan did not cause adverse effects (e.g. inflammation, cell toxicity) in mice or cows (Asli *et al.* 2017).

Efforts have also been made to identify compounds that interfere with biofilm formation by downregulating the genes involved in biofilm development (Ma *et al.* 2012; Baldry *et al.* 2016; Kong *et al.* 2018). A synthetic compound that inhibited the streptokinase enzyme of group A *Streptococcus*, named CCG-203592, was able to disrupt the biofilm structure of *S. aureus* without affecting microbial growth. This compound also inhibited the expression of several virulence genes in *S. aureus*, including *dltD* (D-alanine incorporation into teichoic acids), *atlA* (bifunctional autolysin); *psmα* (phenol soluble modulin α), SPA gene (immunoglobulin G-binding protein A), *lrgA* (murein hydrolase regulator), *sdrD* (SD-repeat-containing protein D), *sspB* (cysteine protease), *sigB* (Sigma B), RNAIII (gene expression regulator), *codY* (gene repressor), and *hla* (alpha-toxin) (Ma *et al.* 2012). Cytotoxicity analysis determined by the colorimetric MTT assay indicated that CCG-203592 was not cytotoxic to mammalian cells up to a concentration of 50 μM (Ma *et al.* 2012). Moreover, the benzimidazole derivative UM-C162 prevented biofilm formation in a dose-dependent manner without interfering with cell growth. More specifically, the expression of genes involved in biofilm formation, mainly those related to bacterial attachment such as *clfA*,

clfB, and *eno*, was reduced when *S. aureus* was treated with UM-C162 (Kong *et al.* 2018).

Several microbial products have shown potential as anti-biofilm agents. Polysaccharides, fatty acids, and peptides are examples of microbial-derived compounds that have demonstrated anti-biofilm effects against *S. aureus*. For example, exopolysaccharides produced by *Lactobacillus casei* NA-2, dispersed the biofilm of *S. aureus* by 31.8% and also significantly decreased biofilm formation at 500 ug/mL (Xu *et al.* 2020). Norlichexanthone, a small polyketide produced by fungi and lichens, reduced biofilm formation by *S. aureus* and repressed the SaeRS two-component system, considered a major regulator of *S. aureus* virulence (Baldry *et al.* 2016). Lipopeptides produced by *Bacillus* spp., *Lactobacillus* spp., *Pediococcus acidilactici,* and *Acinetobacter junii* can also act as anti-biofilm agents against *S. aureus* (Yan *et al.* 2019; Seethalakshmi *et al.* 2020; Algburi *et al.* 2021). Some of the lipopeptides studied showed quorum quenching activity (Algburi *et al.* 2017; Yan *et al.* 2019) and suppressed the expression of biofilm-related genes (Yan *et al.* 2019). Moreover, sophorolipids and mannosylerythritol lipids (MEL) produced by yeasts decreased the metabolic activity of sessile cells and disrupted the biofilm of *S. aureus* ATCC 6538 (Ceresa *et al.* 2020). Nonetheless, some of these compounds also show antimicrobial activity, and reductions in biofilm formation were probably due to a lower bacterial cell density.

3.3 Superantigens (SAgs)

Due to its importance in staphylococcal pathogenesis, some anti-virulence strategies have been designed to target the biosynthesis of superantigens or their activity. Antibodies can be used for this purpose as this allows prompt treatment based on passive immunization. Studies focusing on the development of antibodies against enterotoxin B (SEB), one of the most studied toxins from *S. aureus*, are limited. A human monoclonal antibody named HuMAbs-14 specific for SEB showed therapeutic properties and prevented the activity of toxins in a mouse model of SEB-induced mortality (Drozdowski *et al.* 2010). The monoclonal antibody 20B1, another SEB-neutralizing antibody, demonstrated a protective effect against a SEB-producing MRSA strain in mice, reducing skin tissue invasion and deep-abscess formation (Varshney *et al.* 2013).

S. aureus secretes a range of toxins and the development of vaccines to neutralize these proteins is challenging. A multivalent vaccine (IBT-V02) containing toxoids of enterotoxins A and B, TSS1, alpha-hemolysin, Panton-Valentine leukocidin (PVL), LukS, LukF, and LukAB, is currently under study (Karauzum *et al.* 2021). It has been reported that IBT-V02 confers protection to mice and rabbits against skin infections caused by *S. aureus*, and will enter a Phase I clinical study (Karauzum *et al.* 2021). A fusion toxoid vaccine composed of the TSST-1, SEA, and SEB toxins was shown to increase antibody titers that broadly neutralized these SAgs demonstrating protective efficacy in a mouse model of toxic shock (Venkatasubramaniam *et al.* 2019).

Vaccines targeting the superantigens separately have also been studied. An inactive form of SEB expressed in a *Lactococcus lactis* strain was tested as an oral vaccine in an infected mouse model and generated a significant antibody titer that led to improved survival of the animals (Asensi *et al.* 2013). An in-human phase 1 study was conducted with the vaccine STEBVax, which contains a recombinant isoform of the SEB toxin with site-directed mutations incorporated in the histocompatibility complex class II (MHC-II) binding site (Chen *et al.* 2016). STEBVax enhanced the elicitation of functional antibodies against SEB and its dose escalation was considered safe up to 20 µg. Furthermore, a recombinant TSST-1 variant and a modified TSST-1 antigen vaccine were also explored to prevent *S. aureus* infections in humans (Schwameis *et al.* 2016) and mice (Hu *et al.* 2003), respectively.

The screening of natural compounds that suppress superantigens is another target for the discovery of new anti-virulence inhibitors. The olive chemical 4-hydroxytyrosol in its pure and commercial form was able to reduce the effects of the superantigen enterotoxin A (SEA) *in vitro* (Friedman *et al.* 2011). Perilla oil obtained from *Perilla frutescecens* (L.) Briton decreased the production of ETA, ETB, and TSS1 by *S. aureus* by downregulating gene expression in a dose-dependent manner (Qiu *et al.* 2011). Moreover, a soluble T-cell receptor variable domain was created using a yeast display technology to neutralize superantigen enterotoxin C (SEC) and SEB (Mattis *et al.* 2013).

3.4 Leukotoxins

Vaccines and antibodies have been developed and tested against *S. aureus* leukocidins. A polyclonal immunoglobulin preparation containing antibodies specific to

the Panton-Valentine leukocidin (PVL) is available commercially for intravenous use in humans. This preparation was able to neutralize pore formation and the cytopathic effect of *S. aureus* supernatants and purified PVL on polymorphonuclear neutrophils (Gauduchon *et al.* 2004). Moreover, humanized heavy chain-only antibodies were generated against PVL and neutralized toxin damage *in vivo* (Nguyen *et al.* 2003). Monoclonal antibodies were also constructed against LukAB resulting in a significantly lower bacterial count in a murine model of sepsis (Thomsen *et al.* 2017).

A rationally designed vaccine containing an attenuated subunit of LukS-PV and LukF-PV from *S. aureus* conferred protection in a mouse bacteremia model (Karauzum *et al.* 2013). The vaccine IBT-V02, as mentioned earlier, also confers protection against PVL (Karauzum *et al.* 2021). The vaccine PentaStaph is under development using PVL and other staphylococcal antigens (Ahmad-Mansour *et al.* 2021). However, it should be noted that some of these products have failed in protecting immunized individuals in clinical trials, which, for example, was the case of the antibody preparation ASN100 (Magyarics *et al.* 2019) and the vaccine StaphVax (Landrum *et al.* 2017).

Some molecules can also interfere with the activity of *S. aureus* leukocidins by blocking their activity. A human neutrophil peptide 3 (HNP3), a defensin that interacts with LukS-PV and LukF-PV, prevented pore formation and decreased the cytotoxic effects of PVL (Cardot-Martin *et al.* 2015). In addition, an *in vitro* study demonstrated that the *n*- zwitterionic surfactant tetradecyl phosphocholine ($C_{14}PC$) protects human primary immune cells against the activity of PVL and alpha-hemolysin (Liu *et al.* 2020).

3.5 Quorum sensing system

Various approaches have been evaluated in an attempt to inhibit the *S. aureus* quorum sensing system (Fleitas Martinez *et al.* 2019). Natural and synthetic compounds have shown quorum-quenching activity and inhibit quorum sensing in *S. aureus* by targeting different components of the system. Some molecules inhibit the synthesis of RNAIII (Gov *et al.* 2001; Ma *et al.* 2015), while others are autoinducing peptide (AIP) derivatives that prevent induction of the *S. aureus* quorum sensing system by AIPs (Otto *et al.* 2001; Tal-Gan *et al.* 2016). Secondary metabolites also inhibit proteins of the signal transduction pathway, including the AgrA inhibitors, hydroxyemodin, and phloretin (Daly *et al.* 2015; Zhou *et al.* 2015) and the AgrC

inhibitors, solonamide, cochinmicin, and avellanin (Mansson *et al.* 2011; Desouky *et al.* 2015; Igarashi *et al.* 2015; Wang and Muir 2016).

Notably, other species of *Staphylococcus* produce AIPs that can inhibit the quorum sensing system of *S. aureus* (Canovas *et al.* 2016; Mahmmod *et al.* 2018; Peng *et al.* 2019). A study that used reporter gene fusions to monitor the transcriptional activity of important virulence factors and regulators in *S. aureus*, reported that cell-free supernatants from 77% of the non-*aureus* staphylococci (NAS) isolated from milk and the teat surface of dairy cows reduced the expression of the *hla* gene (Mahmmod *et al.* 2018). Additionally, 70% of the NAS reduced the expression of RNAIII and 61% reduced the expression of *spa* in *S. aureus* (Mahmmod *et al.* 2018). Among the species analyzed, *Staphylococcus chromogenes* isolated from milk and *Staphylococcus xylosus* isolated from both milk and the teat surface showed consistent downregulation of the *S. aureus* virulence genes (Mahmmod *et al.* 2018). In another study, approximately 71% of the staphylococci isolated from animals, corresponding to 17 distinct species, inhibited the quorum sensing system of *S. aureus* via a similar mechanism (Canovas *et al.* 2016).

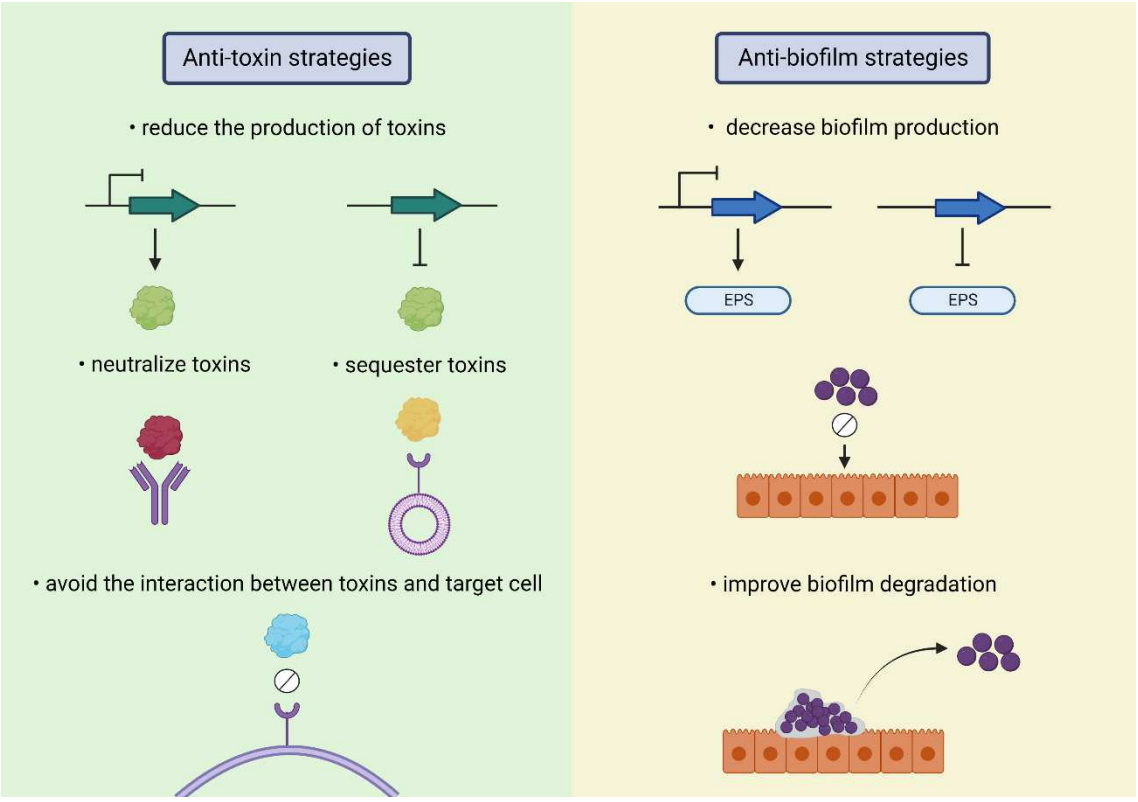

Figure 2. Anti-virulence strategies to decrease *S. aureus* pathogenicity. Strategies based on anti-toxins can decrease the transcription or translation of genes encoding

toxins, neutralize toxins using antibodies, sequester toxins using structures that mimic a cell surface, and block the interaction between toxin and target cell. Anti-biofilm activity can be achieved by decreasing the transcription and/or translation of biofilm-associated genes, preventing bacterial adhesion, and improving bacterial detachment. The graphical representation was constructed using BioRender.

4 SCREENING APPROACHES FOR THE IDENTIFICATION OF ANTI-VIRULENCE COMPOUNDS

Anti-virulence compounds have been identified mainly through phenotypic and genetic assays, as well as chemical and computer-assisted approaches. Each approach has advantages and disadvantages for achieving high-throughput screening (HTS) and varies in equipment requirements and costs (Table 1). In addition, the efficacy of each approach to the discovery of new bioactive agents depends on the virulence factor targeted.

4.1 Phenotypic assays

4.1.1 Agar plate-based assays

Activity-based bioassays have been developed for the detection of delta-hemolysin production. The method is based on the evaluation of synergistic hemolysis when the bacteria producing the hemolytic peptide are streaked adjacent to a beta-hemolysin disk in blood agar (Schweizer *et al.* 2011). This assay is useful to evaluate if the strain treated with anti-virulence compounds loses the capacity to produce delta-hemolysin within the beta-hemolysin zone, which indicates interference with the *agr* function and consequent expression of secreted and non-secreted virulence factors (Schweizer *et al.* 2011).

Genetically modified bacterial strains have been used to phenotypically monitor the stimulation or inhibition of the *S. aureus* quorum sensing system (Nielsen *et al.* 2010; Mansson *et al.* 2011). A *S. aureus* sensor strain was constructed by fusing the *hla* and *spa* genes with *lacZ* (Nielsen *et al.* 2010). Since the *spa* gene is negatively regulated and the *hla* gene is positively regulated by the quorum sensing system, under conditions that stimulate the quorum sensing system, *S. aureus* PC203 (*spa*::*lacZ*)

colonies grown in a medium with X-Gal turn light blue while the *S. aureus* PC322 (*hla*::*lacZ*) colonies become intensely blue in the same media. If the quorum sensing is repressed, the opposite phenotypes are observed. This methodology has been applied previously for the discovery of novel quorum-quenching compounds from marine bacteria (Mansson *et al.* 2011).

4.1.2 Multi-well plate assays

Multi-well assays are excellent tools for scaling up the experimental design for screening purposes. Natural and synthetic molecules with potential anti-hemolytic activity can be screened against *S. aureus* by growing the cultures with the extracts or molecules under evaluation until the bacteria reaches stationary phase. Upon centrifugation of the *S. aureus* cultures, the cell-free supernatants can be tested to evaluate spectrophotometrically (OD_{540}) the release of hemoglobin from washed and diluted erythrocytes (Tang *et al.* 2019). A compound or extract is considered to have anti-hemolytic activity when the release of hemoglobin promoted by the cell-free supernatants collected from treated *S. aureus* cultures is significantly lower in comparison to the controls.

In addition, biofilm inhibitors can be screened in a multi-well plate format using crystal violet colorimetric assays (Stepanović *et al.* 2000). For this, the biofilm producer (e.g., *S. aureus*) is inoculated in the presence or absence of the compound under evaluation in a multi-well plate. The cultures are incubated and the extent of biofilm adherent to the wells is quantified spectrophotometrically (OD_{570}) using the dye crystal violet. Compounds with anti-biofilm activity are expected to prevent biofilm formation and should, therefore, decrease the absorbance (OD_{570}) when compared to untreated controls (Isaac *et al.* 2017).

4.1.3 Fluorescence techniques

Screenings based on the induction of bacterial fluorescence through reporter genes have also been conducted to identify quorum-quenching molecules (Sully *et al.* 2014). A mutant reporter strain containing *agr::P3*, where P3 controls the expression of the green fluorescent protein (GFP), and also an AgrA-dependent lux reporter strain (luminescence induction) were constructed to screen a large library of chemical

compounds for selective activity against the *agr* quorum sensing system in *S. aureus* (Sully *et al.* 2014). This led to the identification of savarin, a compound that inhibited the activation of the P3 and/or *agrA* reporter genes, decreased the expression of several virulence genes (e.g., RNAIII, *hla*, *psm α*, and PVL), and promoted *agr*-dependent host defense both *in vivo* and *in vitro* without affecting the exponential growth phase of *S. aureus*.

Fluorescence techniques can also be used to assess the susceptibility of *S. aureus* cells to the host's immune system (Quave and Horswill 2014). The activity of leukotoxins was analyzed by adding *S. aureus* expressing green fluorescent protein (sGFP) to neutrophils and measuring fluorescence using flow cytometry to monitor cell viability (Schwartz *et al.* 2009; White *et al.* 2014). Anti-virulence compounds that block the production or activity of leukotoxins increase the susceptibility of *S. aureus* cells to neutrophil attack resulting in a decreased sGFP signal.

4.2 Chemical and molecular methods

Analytical techniques are useful for detecting and quantifying bacterial metabolites and therefore can be applied for the identification of new anti-virulence compounds (Quave *et al.* 2011; Quave and Horswill 2014). The production of hemolysins has been analyzed using chromatography and/or spectrometric methods. For example, liquid chromatography MS (LC-MS) (Somerville *et al.* 2003), and HPLC-MS (Hodille *et al.* 2016) have been used to detect delta-hemolysin in bacterial supernatants. Moreover, Whole-Cell Matrix-Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight MS (MALDI-TOF/TOFMS) was successfully applied to detect delta-hemolysin in a routine identification procedure of bacteria using an isolated colony (Gagnaire *et al.* 2012).

The production of autoinducing peptides (AIP) by *S. aureus* can also be detected by using ultra-high-performance liquid chromatography (UHPLC) coupled with electrospray ionization MS (Junio *et al.* 2013). Reductions in the production of AIP by *S. aureus* can be monitored and the production of different groups of AIP that cross-inhibit the quorum sensing system of *S. aureus* can be assessed using this approach.

Analysis of cell transcripts also allows the screening of compounds that can interfere with bacterial virulence (Quave and Horswill 2014). Northern blot analysis has been used to investigate the production of specific transcripts and could be a useful

approach to evaluate the activity of anti-virulence compounds (Mansson *et al.* 2011; Nielsen *et al.* 2014). Additionally, quantitative reverse transcription PCR (qRT-PCR) can be used to analyze the expression of several genes correlated to *S. aureus* virulence, such as genes involved in biofilm formation/degradation (i.e., *icaA*, *icaD*, *fnbA*, *fnbB*, *nuc*, *aur*), hemolysin production (i.e., *hla*, *hld*) and quorum sensing system (i.e., *agrA*, *agrC*, RNAIII) (Lee *et al.* 2013; Lakshmi *et al.* 2020). Typically, a comparison between gene expression in treatments and controls is performed to identify compounds that reduce the pathogenicity of *S. aureus* by downregulating the expression of specific virulence genes.

4.3 Virtual screening and chemical design

Three main strategies are used to identify or design structure-based compounds (Neville and Jia 2019) with potential anti-virulence activity. The first is based on the modification of known substrates, cofactors, or inhibitors to generate analogs that act as protein inhibitors (Persson *et al.* 2005; Larzabal *et al.* 2010). The second approach includes a computer-assisted virtual screening of molecules, in which molecular docking is performed *in silico* often using large libraries of chemical compounds and the protein of interest (Swietnicki *et al.* 2011). These analyses can provide information on predicted ligand-protein interactions as well as opportunities for optimization of such interactions. Lastly, it is possible to design small-molecule fragments that are positioned in the target site and scored *in silico* (Arya *et al.* 2015).

Virtual screening is a rapid and low-cost approach to identifying possible anti-virulence compounds against *S. aureus*. This method has been applied to screen for new compounds with potential anti-virulence activity and also in an attempt to repurpose FDA-approved drugs (Rashidieh *et al.* 2015; Mellini *et al.* 2019). For molecular docking, protein structures of some *S. aureus* virulence factors are already available in public databases such as the Protein Database (PDB) (https://www.rcsb.org/). This is the case for the well-characterized alpha-hemolysin and some domains of the quorum sensing proteins AgrA and AgrC. Another approach is the purification and determination of the crystal structure of proteins or domains of interest to search *in silico* for anti-virulence compounds that show chemical interactions with the virulence factor of *S. aureus* (Leonard *et al.* 2012; Khodaverdian *et al.* 2013).

Chemical synthesis based on a known backbone has also been used to discover new anti-virulence compounds against *S. aureus* (Kong *et al.* 2018). Benzimidazole, for example, possesses a wide range of biological activities (Keri *et al.* 2015), increasing the chances that its derivatives will also possess a variety of useful activities. Benzimidazole derivatives were generated by chemically replacing radical groups of the corresponding carboxylic acid intermediates by *de novo* synthesis. The products were tested *in vivo* and showed an anti-virulence activity against *S. aureus* in a *Caenorhabditis elegans* infection model (Kong *et al.* 2018).

Table 1. Advantages and disadvantages of assays used for screening new anti-virulence compounds

Assay	Advantages	Disadvantages	References
Agar-based plate assays	Low-cost Simple technique	Not amenable to large scale screening Qualitative results	(Nielsen, Nielsen et al. 2010, Schweizer, Furuno et al. 2011)
Well-plate assays	Medium-throughput screening Quantitative analysis	Medium cost/ equipment required	(Isaac, Bohl et al. 2017, Tang, Li et al. 2019)
Fluorescence assays	Medium-throughput screening Quantitative analysis High sensitivity	High cost/ equipment required	(Sully, Malachowa et al. 2014, White, Boyd et al. 2014)
Analytical techniques	High specificity and sensitivity	Not amenable to large scale screening High cost/ equipment required	(Junio, Todd et al. 2013, Hodille, Cuerq et al. 2016)
Molecular techniques	Mechanism of action explored	Not amenable to large scale screening High cost/ equipment required	(Nielsen, Mansson et al. 2014, Lakshmi, Bhaskar et al. 2020)
Virtual screening	Fast and High-throughput screening Low-cost	*In vitro* validation required	(Leonard, Bezar et al. 2012, Khodaverdian, Pesho et al. 2013)
Chemical synthesis	High specificity	High cost *In vitro* validation required	(Kong, Chee et al. 2018)

5 POTENTIAL HOTSPOTS FOR THE DISCOVERY OF ANTI-VIRULENCE COMPOUNDS AGAINST *S. AUREUS* CAUSING BOVINE MASTITIS

It is well-known that the commensal microbiota colonizing an ecological niche produces compounds that can inhibit or impair the growth of other bacteria as part of a competitive exclusion strategy (Hibbing *et al.* 2010), which can contribute to protecting the host against pathogens. Therefore, prospecting anti-virulence compounds in microbiomes of healthy individuals where bacterial pathogens can potentially colonize is a promising approach to identifying these molecules.

For example, when comparing animals with or without a history of mastitis infections, a higher abundance of members of the phylum *Bacteroidetes*, or the class *Clostridia*, and the genus *Bifidobacterium* was observed in the bovine teat microbiome of quarters with no history of mastitis (Falentin *et al.* 2016). These results indicated that the microbiota of the teat apex and the teat canal can influence the microbial composition and overall health of the mammary gland (Derakhshani *et al.* 2018), with healthy quarters showing greater microbiota diversity than mastitis quarters affected by mastitis (Falentin *et al.* 2016).

The microbiota of the teat apex and the teat canal of dairy cows vary in composition but share a high prevalence of members of the phylum *Firmicutes*, including *Staphylococcus*, *Lachnospiraceae*, *Ruminococcaceae,* and *Clostridiales* (Derakhshani *et al.* 2018). Although some coagulase-negative staphylococci (CoNS) can cause bovine mastitis, they are commonly non-pathogenic in animals and humans and studies have shown their potential to interfere with the quorum sensing system and reduce *S. aureus* colonization in pigs (Verstappen *et al.* 2017; Peng *et al.* 2019). Some reports indicate that *Staphylococcus epidermidis* from humans and *Staphylococcus sciuri*, *Staphylococcus cohnii*, *Staphylococcus saprophyticus* from pigs prevent *S. aureus* colonization (Iwase *et al.* 2010; Verstappen *et al.* 2017). Moreover, CoNS are also able to produce AIPs that inhibit the quorum sensing system of *S. aureus*, potentially playing a role in microbial competition and niche colonization (Peng *et al.* 2019).

The teat apex microbiota also contains a high proportion of *Actinobacteria*, represented by *Corynebacterium* and *Propionibacterium* species (Derakhshani *et al.* 2018). In the presence of *Corynebacterium* species, the expression of virulence factors from *S. aureus* was decreased, the quorum sensing system was strongly inhibited and

the bacteria demonstrated an increased adherence capacity, indicating a shift from pathogenic towards a commensal state (Ramsey *et al.* 2016). These observations demonstrate that some genera of the *Actinobacteria* phylum could be a valuable source of anti-virulence compounds against *S. aureus* in bovine mastitis.

Moreover, a study in humans demonstrated that the abolishment of nasal and intestinal *S. aureus* colonization was associated with the consumption of a probiotic *Bacillus* bacterium by a rural population (Piewngam *et al.* 2018). Although the production of antimicrobial peptides is considered a common mechanism of pathogen exclusion by probiotic lactic acid bacteria, the *Bacillus*-mediated *S. aureus* exclusion was attributed to the production of fengycin, a lipopeptide that showed inhibitory activity against the Agr quorum sensing system of *S. aureus* which is considered essential for successful intestinal colonization (Piewngam *et al.* 2018). Therefore, the screening and selection of *Bacillus* isolates with probiotic traits from healthy bovine udder could represent a potential source of anti-virulence compounds against *S. aureus* to prevent clinical mastitis.

6 CHALLENGES AND LIMITATIONS OF ANTI-VIRULENCE THERAPY

Anti-virulence therapy represents an innovative alternative to control infectious diseases in humans and animals. However, as an emerging therapeutic strategy, its successful application in clinical and veterinary practice faces several challenges. A deep understanding of the target virulence factors in microbial pathogens and their role in pathogenicity is required including information about the redundancy of these elements. For example, the inhibition of a specific *S. aureus* adhesin, although important for bacterial colonization can be circumvented by alternative routes of infection (Mühlen and Dersch 2015). Another important factor to consider is the specificity of the target virulence factor. An anti-virulence therapy designed to target a specific pathogen (e.g., *S. aureus*) requires rapid and accurate diagnostic methods to be in place, which is still a distant reality in most livestock production systems. Moreover, although the virulence factors described here are primarily involved in *S. aureus* pathogenicity, the effect of anti-virulence therapies targeting more generic systems, such as virulence regulators, should be carefully investigated regarding their effect on the indigenous microbiota (Maura *et al.* 2016).

In addition, the clinical aspects and severity of the disease must be considered for the successful application of anti-virulence therapies. To treat acute infections, the simultaneous use of anti-virulence inhibitors and antibiotics is probably necessary. Anti-virulence compounds are especially attractive to treating chronic or non-life-threatening infections (Theuretzbacher and Piddock 2019). However, this concept may not apply to all etiologies as dysfunctional quorum sensing appears to be beneficial for *S. aureus* in chronic infections (Khan *et al.* 2015).

In the case of mastitis, as the anti-virulence therapies are not designed to eliminate the pathogen, an increased load of bacteria in the mammary gland can still promote inflammatory responses, which needs to be further evaluated *in vivo*. For example, when intramammary probiotics were used in cattle, induction of cellular inflammation was observed (Greene *et al.* 1991; Frola *et al.* 2013). Combinatorial therapy with the use of antimicrobials could be a more effective alternative in this case.

Anti-virulence compounds have been approved by the Food and Drug Administration (FDA) for protection against *Clostridium botulinum* (Arnon *et al.* 2006), *Bacillus anthracis* (Migone *et al.* 2009; Greig 2016), and *Clostridium difficile* (Lowy *et al.* 2010). However, the majority of the anti-virulence compounds discovered so far are still in the preclinical phase (Dickey *et al.* 2017) and other molecules have failed to give effective results when tested in the clinical phase (Armstrong *et al.* 1995; Baer *et al.* 2009; Johnson *et al.* 2014). The metrics for evaluating the success of anti-virulence drugs are complex and involve the analysis of pathophysiology parameters such as cell damage, inflammatory response, and disease severity (Maura *et al.* 2016). Due to the differences between murine and bovine immunity, good pre-clinical data may not result in a successful clinical trial. Furthermore, moving anti-virulence drugs into preclinical and clinical studies is expensive and the narrow activity of anti-virulence compounds against *S. aureus* may not be considered attractive enough for pharmaceutical investment.

CONCLUSION

Antimicrobial resistance (AMR) is considered by the scientific community as a likely contributor to the next pandemic, currently causing the death of approximately 1.2 million people annually worldwide (Murray *et al.* 2022). The anti-virulence approach stands out as a promising therapy to treat bacterial infections based on a mechanism

of action distinct from current therapies that depend on antimicrobial and immunomodulatory activity. Anti-virulence inhibitors are notable by virtue of stimulating a commensal state instead of a pathogenic one. In this review, we have highlighted approaches for the discovery and application of anti-virulence compounds against *S. aureus* with a focus on bovine mastitis, the costliest disease of dairy cattle.

In this context, several questions remain to be answered. Could an anti-virulence compound be effective as a prophylactic treatment *in vivo*? How effective can these molecules be with respect to the treatment of acute and chronic bovine mastitis? Could a combinatorial administration with antibiotics reduce the concentration of antimicrobials required to treat the disease? Would the presence of non-virulent *S. aureus* strains in the mammary gland still cause an inflammatory response? Could bacterial pathogens develop resistance to anti-virulence inhibitors? To address these questions, further *in vitro* and *in vivo* studies are required. While still in its infancy, the field focused on the study of anti-virulence compounds has expanded during the last number of years, and, in parallel, the potential of these compounds to become an important weapon to face bacterial infection has increased considerably.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data discussed in this review article was retrieved from publicly available databases and a full citation of the literature cited is provided.

AUTHOR CONTRIBUTION STATEMENT

YS and HM conceived the project. YS wrote the manuscript with inputs from PC and HM. YS, HM, and PC reviewed and edited the manuscript.

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CHAPTER 2

Iturins produced by *Bacillus* spp. inhibit the hemolytic activity of *S. aureus*

ABSTRACT

Antimicrobial resistance is considered a silent pandemic, causing millions of deaths annually worldwide. *Staphylococcus aureus* has become resistant to many available antibiotics, causing long-lasting infections in humans and animals. In livestock production, bovine mastitis caused by *S. aureus* has a major economic impact on the dairy sector. With the crucial need for new therapies, anti-virulence strategies have gained attention as alternatives to antibiotics. Here we aimed to identify novel compounds that inhibit hemolysin production/activity by *S. aureus*, a virulence factor associated with mastitis severity. For this, we screened strains of *Bacillus*, a bacterial genus that is known to produce several biologically active compounds. Our results demonstrate that lipopeptides produced by *Bacillus* spp. have anti-hemolytic activity against *S. aureus*. The compounds were capable of inhibiting the hemolytic activity of *S. aureus* by up to 100% depending on the concentration tested. After purification of the lipopeptides, iturins showed the highest anti-hemolytic activity. Analyzes to evaluate the mechanism of action indicated that lipopeptides did not decrease the expression of hemolysin-encoding genes and showed effects at the post-translational level. Iturins were not able to prevent hemolysin oligomerization *in vitro*, but molecular docking studies confirmed the ability of these compounds to bind to hemolysin, both inside the pore structure or at external residues involved in the target cells' binding and lysis. Therefore, our study revealed and characterized for the first time an anti-hemolytic activity of lipopeptides, compounds previously known for their cytotoxic effect against erythrocytes, highlighting the potential application of lipopeptides as an anti-virulence therapy to control bovine mastitis caused by *S. aureus*.

1 INTRODUCTION

In 2019, a total of 4.95 million deaths were associated with antimicrobial resistance (AMR) worldwide (Murray *et al.* 2022). *S. aureus* is listed as the second leading pathogen responsible for deaths associated with therapy resistance, only behind *Escherichia coli* (Murray *et al.* 2022). Indeed, *S. aureus* resistant to methicillin was responsible for over 100,000 deaths attributed to AMR in 2019 (Murray *et al.* 2022). This bacterium is also a major cause of infections in several animal hosts and negatively impacts both animal and public health since animals can act as a staphylococci reservoir (Haag *et al.* 2019).

In agriculture, *S. aureus* is amongst the main pathogens responsible for bovine mastitis, one of the most frequent and costly diseases of the dairy sector (Halasa *et al.* 2007; Zaatout *et al.* 2020b). Antibiotic therapy is currently the main approach to treating bovine mastitis, but the acquisition of resistance by *S. aureus* reinforces the need for new approaches to prevent and control mastitis (Rainard *et al.* 2018). Furthermore, the ability of *S. aureus* to invade epithelial cells and form biofilms is correlated with its persistence and difficult eradication from the mammary gland (Bardiau *et al.* 2014). Therefore, treatments with improved efficacy in eliminating/preventing *S. aureus* colonization are pivotal. In addition, methicillin-resistant *S. aureus* has been listed by the World Health Organization since 2017 in the high priority group for which new therapies should be developed (Tacconelli *et al.* 2017).

Anti-virulence therapy has emerged as a way to decrease bacterial pathogenicity thus preventing/treating infectious diseases while imposing a lower selective pressure on the pathogen toward resistance development (Sully *et al.* 2014; Quave *et al.* 2015; Vale *et al.* 2016a). The main anti-virulence targets in *S. aureus* are the bacterial membrane, the quorum sensing (QS) system, biofilm formation, and toxin production (Fleitas Martinez *et al.* 2019). In bovine mastitis, toxin production plays an important role in *S. aureus* pathogenicity (Cote-Gravel and Malouin 2019). A recent study demonstrated that a host-adapted *S. aureus* strain causing chronic mastitis showed increased alpha-hemolysin secretion, which was correlated with an improved ability of *S. aureus* to penetrate and disseminate into udder tissue (Mayer *et al.* 2021).

Although efforts are being made in an attempt to block/prevent alpha-hemolysin activity, the potential of bacterial metabolites as anti-virulence compounds is still underexplored. These metabolites can usually be produced with high yields and have

several competitive advantages, including lower cost, and low environmental impact, and the biosynthetic genes are often amenable to genetic manipulation allowing improvements in the production process. In this context, *Bacillus* is a bacterial genus easily cultivated *in vitro* that produces several secondary metabolites, mainly lipopeptides, with a wide range of industrial applications (Ongena and Jacques 2008; Zhao *et al.* 2017).

Lipopeptides produced by *Bacillus* spp. are often classified as surfactins, iturins, and fengycins according to their specific peptide chain and fatty acid structures (Ongena and Jacques 2008). In general, these compounds act on microbial surfaces decreasing interfacial tension and disrupting membrane structures, which explains their antimicrobial activity (Zhao *et al.* 2017). In addition, some studies have investigated the potential of lipopeptides in impairing bacterial virulence and an earlier study showed that a lipopeptide produced by *Bacillus subtilis* decreased the formation of *S. aureus* biofilms by 90% (Rivardo *et al.* 2009). More recently it was demonstrated that *Bacillus* spp. could abolish *S. aureus* colonization in humans. The effect was associated with the production of fengycins by a probiotic *Bacillus* that could eliminate *S. aureus* by inhibiting its quorum sensing system (Piewngam *et al.* 2018). Quorum sensing also positively upregulates hemolysin production (Le and Otto 2015), but studies evaluating the potential of lipopeptides as anti-hemolytic agents are lacking. Here we hypothesize that lipopeptides produced by *Bacillus* spp. interfere with the production of hemolysins and reduce the hemolytic activity of *S. aureus* strains associated with bovine mastitis. Therefore, this study aimed to identify *Bacillus* spp. with anti-hemolytic activity and characterize the effect of their lipopeptides on the hemolytic activity of *S. aureus*.

2 MATERIALS AND METHODS

2.1 Microorganisms and growth conditions

Ninety strains of *S. aureus* were obtained from the Mastitis Pathogens Culture Collection at Embrapa Gado de Leite (Juiz de Fora, Minas Gerais State, Brazil) and used in this study. The bacterial strains were previously isolated from cows with mastitis and identified by using standard biochemical procedures (Brito *et al.* 1999; Brito and Brito 1999). Moreover, *S. aureus* O11, a highly hemolytic strain isolated from

a ewe with severe mastitis, was also used in this study (Le Marechal *et al.* 2011). *S. aureus* strains were cultivated under aerobic conditions in Brain Heart Infusion broth (BHI) or Tryptic Soy Broth (TSB) for 18 h at 37 ºC.

Thirty-three cultures of *Bacillus* spp. isolated from soil, plants, water, and mastitic milk in different regions of Brazil (Supplementary Table 1) were used in this study for the screening of compounds that attenuate the hemolytic activity of *S. aureus*. The *Bacillus* spp. strains were cultivated in Tryptic Soy Broth (TSB) for 24 h at 30 ºC under agitation (200 rpm).

2.2 Hemolysin production

To assess the hemolytic activity of *S. aureus*, the strains were cultivated overnight at 37 ºC in a 96-well plate containing BHI broth. The cultures were then transferred to a sheep blood agar base Mueller-Hinton (NewProv, Pinhais, Brazil) using a colony replicator. The inoculated media was incubated at 37 ºC for 24 h and then kept overnight at 4 ºC. The presence of a complete hemolytic lytic zone after 24 h incubation at 37 ºC indicated the production of alpha-hemolysin while an incomplete lytic zone that evolved to complete lysis of the red blood cells after overnight incubation at 4 ºC was interpreted as the production of beta-hemolysins (Da Silva *et al.* 2005). Experiments were performed with two technical and three biological replicates.

2.3 Interference in hemolysin production

Three *S. aureus* strains characterized as alpha-hemolysin producers were selected for this assay. The alpha-hemolytic staphylococci and all the bacilli strains were grown in TSB medium. *S. aureus* strains were incubated at 37 ºC for 24 h and *Bacillus* spp. was grown at 30 ºC for 24 h under agitation. After growth, *Bacillus* supernatants were filtered through a 0.22 µm membrane, and 50 µL of the sterile supernatant was added to 150 µL of TSB broth inoculated with *S. aureus* at an initial OD of 0.05. The cell suspension was incubated at 37 ºC for 24 h. The OD_{600} of the cultures was measured to verify if *Bacillus* supernatants interfered with *S. aureus* growth. The samples were then centrifuged (10,000 g, 10 min, 4 ºC) and 100 µL of the supernatants were added to a suspension containing 25 µL of washed sheep erythrocytes diluted into 875 µL of Phosphate Buffer Saline (PBS). The mixture was

incubated at 37 ºC for 30 min. Following a step of centrifugation (5500 g, 1 min, 4 ºC), the OD_{543} of the supernatants was measured to quantify the release of hemoglobin (Tang *et al.* 2019). The hemolytic activity of *S. aureus* assessed in the absence of the supernatants was used as the positive control. We also verified if the compounds produced by *Bacillus* spp. were cytotoxic by evaluating the hemolytic activity of *Bacillus* spp. supernatants alone. Phosphate-buffered saline (PBS) was used as the negative control. Experiments were performed using two technical and two biological replicates. The Shapiro-Wilk test was used to assess data normality and the Mann-Whitney test at 95% confidence was applied to make pairwise inferences. Statistical analyzes were performed using GraphPad Prism software version 8.4.3.

2.4 Production of lipopeptides by *Bacillus* spp.

For the *Bacillus* spp. strains that demonstrated anti-hemolytic effects, the production of lipopeptides was evaluated over time. The strains were grown in TSB media with an initial OD of 0.1 for 120 h at 30 ºC with agitation (200 rpm). Samples were collected at 0, 3, 6, 12, 24, 48, 72, 96, and 120 hours of incubation. The production of lipopeptides was analyzed using the oil displacement assay with modifications (Morikawa *et al.* 2000). For this, 20 µL of petroleum was added to 70 mL of distilled water and 10 µL of the culture supernatants were added to the water/oil interface. The production of lipopeptides was quantified by measuring the oil displacement zone. The cultivation time that resulted in higher production of lipopeptides was selected for the extraction of these compounds.

2.5 Extraction of lipopeptides and evaluation of anti-hemolytic activity

To evaluate if lipopeptides produced by *Bacillus* spp. were causing the anti-hemolytic activity against *S. aureus*, the lipopeptides were extracted from *Bacillus* spp. supernatants using the acid precipitation methodology (Sharma *et al.* 2015). Briefly, the cultures with an initial OD_{600} at 0.1 were grown in TSB media at 30 ºC under 200 rpm for 48 h. Following a centrifugation step (10,000 g, 15 min, 4 ºC), the lipopeptides were precipitated by adjusting the pH of the supernatants to 2.0 using 6 M HCl. The samples were incubated overnight at 4 ºC. The acid precipitate was recovered by centrifugation (10,000 g, 15 min, 4 ºC) and resuspended in distilled water by

neutralizing the pH to 7.0 using 6 M NaOH. The crude extract containing lipopeptides was then lyophilized and a stock solution of 5 mg/mL was prepared and heat sterilized (121 ºC/20 min).

The activity of the crude extracts containing lipopeptides against the hemolytic activity of *S. aureus* was evaluated in a range of concentrations made by two-fold serial dilutions (1,000 µg/mL to 7.31 µg/mL). The three *S. aureus* strains previously selected as well as *S. aureus* O11, a highly hemolytic strain isolated from a severe mastitis case in ewes (Le Marechal *et al.* 2011), were used in this assay. *S. aureus* with an initial OD of 0.05 was grown in 96-well plates (200 µL per well) in the presence of 50 µL of the crude extracts. The samples were incubated at 37 ºC for 24 h. After incubation, the plates were centrifuged at 4,000 g for 10 min and 50 µL of the supernatants were added to 200 µL of diluted erythrocytes from sheep blood. Washed erythrocytes from sheep blood were diluted 36x as described previously. The plates were incubated at 37 ºC for 1 h to allow the activity of hemolysins and then centrifuged (4,000 g /1 min). The hemoglobin released in the supernatant was measured spectrophotometrically at OD_{543}. The hemolytic activity of *S. aureus* in the absence of the supernatants was assessed as the positive control and PBS was used as the negative control. The toxic effect of the lipopeptide extracts was also evaluated in different concentrations. The experiment was performed using two technical and two biological replicates. To compare the effect of concentration and treatment, two-way ANOVA and Tukey's multiple comparisons test were performed using GraphPad Prism 8.4.3.

2.6 Effect of lipopeptide extracts in the expression of genes involved in hemolysin production

Due to the availability of the genome sequence and the high hemolytic activity of *S. aureus* O11, this strain was selected for this and the following experiments. *S. aureus* O11 was cultured in TSB broth containing the crude extract of lipopeptides from one of each *Bacillus* species, *B. velezensis* 87 and *B. subtilis* TR47II, at 31.25 µg/mL and 250 µg/mL, respectively. These concentrations were the minimum to abolish the hemolytic phenotype (100% inhibition of hemolysis). The bacterial cultures were grown for 24 h at 37 ºC.

The RNA of *S. aureus* cultures treated or not (control) with the crude extract of lipopeptides was extracted using Trizol Reagent® (Sigma-Aldrich, San Luis, EUA)

according to the manufacturer's instructions. The cell lysis step was optimized by adding zirconia beads of 0.1 mm to the tubes followed by shaking for 1 min using a mini bead beater (Biospec Products, Bartlesville, USA). The extracted RNA was treated with DNase (Promega, Madison, USA) to remove any contaminant DNA. The treated RNA was then converted to its complementary DNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, USA). All reactions were performed following the manufacturer's instructions.

To quantify the relative expression of genes involved in hemolysin production, primer pairs were designed for each of the target genes using the Primer3Plus tool (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser *et al.* 2007). The quality of the sequences was verified using OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer?returnurl=%2Fcalc%2Fanalyzer). The target genes and primer sequences used in this study are listed in Supplementary Table 2.

The RT-qPCR reactions were performed using the SYBR Green qPCR Master Mix (2×) (BioRad, California, EUA), as described by the manufacturer, using 25 ng/µL of cDNA. The amplification reaction was set to an initial denaturation at 95 ºC for 10 min followed by 40 cycles of denaturation at 95 ºC for 30 s and annealing and extension at 60 ºC for 1 min. The melting curve was performed with a denaturation step at 95 ºC for 30 s, initial hold at 60 ºC/1 min, followed by an increment in the temperature to 95 ºC at a ramp rate of 1 ºC/min. All reactions were performed in duplicate with three biological replications.

StepOne Real-Time PCR System (Applied Biosystems, Massachusetts, EUA) was used to perform the RT-qPCR analysis. For each primer, the efficiency curve was plotted using the average Ct and $\log_{10}$ of control cDNA concentration. The relative gene expression was calculated based on the equation of the regression line obtained. The 16S rRNA was used as the reference gene.

To analyze if the expression of the genes in the control differed from the expression of the same genes in the treatments, the Mann-Whitney test at 95% confidence level was applied. Statistical analyzes were performed using GraphPad Prism software version 8.4.3.

2.7 Effect of lipopeptides on hemolysin activity

To account for potential post-translational interactions of lipopeptides with *S. aureus* hemolysin, anti-hemolytic activity assays were carried out using cell-free supernatants from *S. aureus*. *S. aureus* O11 was grown in TSB medium for 24 h at an initial OD of 0.05. The culture was centrifuged (10,000 g, 5 min) and the supernatant was treated with lipopeptides extracts from *B. velezensis* 87 and *B. subtilis* TR47II using concentrations varying from 1,000 µg/mL to 125 µg/mL. The samples were incubated at 37 ºC for 1 h. Fifty µL of the treated supernatants were then incubated with 200 µL of washed sheep erythrocytes diluted 36X at 37 ºC for 1 h. The microplate containing the samples was centrifuged at 4,000 g per 1 min. The supernatant was transferred to a new microplate and the hemoglobin released was measured spectrophotometrically at OD_{543}.

Controls were performed using non-treated *S. aureus* supernatants. Statistical analysis was done in GraphPad Prism software version 8.4.3. A non-parametric t-test was applied and a significant difference between treatments and control was considered at a 95% confidence level.

2.8 Lipopeptides purification

One hundred milligrams of the lipopeptide crude extracts were resuspended in 50 mL of Milli Q water to give a 2 mg/mL solution. This solution was applied to a 5 g, 20 mL C18 SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with 20 mL methanol and 30 mL of Milli Q water. The column was washed with 40 mL of 25% ethanol and peptides were eluted in 20 mL of 70% 2-propanol plus 0.1% trifluoroacetic acid (TFA), referred here as isopropanol (IPA) and 85% of IPA for extracts from *B. velezensis* 87 and *B. subtilis* TR47II, respectively. The IPA was removed from the C18 SPE IPA eluent by lyophilization (Genevac HT-4X, Genevac Ltd, Ipswich, UK). The samples were resuspended in 40% acetonitrile and applied to a semi-prep Proteo Jupiter C12 RP-HPLC column (250 x 10 mm, 4µ, 90Å) running a 40-100% acetonitrile 0.1% TFA gradient over 65 minutes where buffer B was 90% acetonitrile 0.1% TFA. The eluent was monitored at 214 nm and fractions were collected at 1 minute intervals. Fractions of interest were pooled and checked for masses of interest using a Bruker Ultraflex MALDI TOF Mass Spectrometry in positive ion reflectron mode.

2.9 Anti-hemolytic activity of the purified lipopeptides

Fractions identified as iturins, fengycin and surfactins were dried and resuspended in methanol to obtain a 5 mg/mL solution. To test the activity of the fractions, *S. aureus* was grown in TSB medium at 37 ºC for 24 h. The culture was centrifuged and the supernatant was incubated with the iturins, fengycins and surfactins at a final concentration ranging from 1,000 µg/mL to 7.81 µg/mL (two-fold serial dilutions) for 1 h at 37 ºC. Fifty µL of the samples were then incubated with 450 µL of sheep blood diluted 36X in PBS and incubated for 1 h at 37 ºC to allow the hemolysins to act. The samples were centrifuged at 4,000 rpm for 1 min, the supernatant was transferred to a 96-well microplate and the OD_{543} was measured using a Synergy HT spectrophotometer (Biotek, Vermont, USA). To compare the concentration effect of the purified lipopeptides in the hemolytic activity of *S. aureus*, the results were subjected to two-way ANOVA analysis, performed using GraphPad Prism 8.4.3.

2.10 Oligomerizarion assay

To evaluate if the purified lipopeptides prevent hemolysis by impairing α-hemolysin oligomerization, an SDS-PAGE was performed as described by Dong et al. (2013) with some modifications. The active lipopeptide was mixed with 2.5 µg of alpha hemolysin (Sigma-Aldrich, St. Louis, USA) at 0.5, 0.25, and 0.125 mg/mL. To promote oligomerization, 5 mM of deoxycholate was added to the samples and the mixtures were incubated at 22 ºC for 20 min (Dong *et al.* 2013b). Then, TruPAGE LDS sample buffer 4x (Merck, Darmstadt, Germany) was added to the samples at 1x final concentration and the mixtures were incubated at 50 ºC for 10 min (Dong *et al.* 2013b). Twenty-five µL of each reaction mixture was loaded onto NuPAGE 12% Bis-Tris Gel (Invitrogen, Massachusetts, EUA). Ten microliters of the SeeBlue Plus2 Pre-stained (Invitrogen, Massachusetts, EUA) were applied as the protein standard. The gel was run at 100V for 50 minutes using MES buffer (Invitrogen, Massachusetts, EUA). Gels were stained using the EZblue Gel Staining Reagent (Sigma, Missouri, EUA) overnight.

2.11 Molecular docking

Molecular docking experiments were performed to evaluate if iturins could be preventing hemolysis by interacting with the heptameric structure of hemolysin or with its receptor on erythrocytes, known as protein ADAM-10. For ADAM-10 receptor, a recent study showed that the E665 residue is essential for the binding and consequent activity of hemolysin (von Hoven *et al.* 2022). Therefore, the grid box was designed to focus on this residue. For hemolysin, three grid boxes were designed to cover the amino acid residues described in the literature to interact with other anti-hemolytic compounds (Melo *et al.* 2016; Liu *et al.* 2020; Ghoneim *et al.* 2022; Wan *et al.* 2022). The grid boxes were built using the following amino acid residues: Grid 01 - Tyr102, Ar104, Asn105, Ile107, Asp108, Thr109, Glu111, Tyr112, Met113, Ser114, Leu 116, Tyr 118, Ile 142, Gly 143 and His 144; Grid 02 - Gly126, Asp127, Asp128, and Ile132; Grid 03 - Asn176, Gln177, Asn 178, Trp179, Gly 180, Pro 181, Tyr182, Gln194, Leu195, Met197, Lys198, Thr199, and Agr200. AutoDockTools v1.5.7 (Morris *et al.* 2009) was used to construct the grid boxes, prepare the target protein and convert the file to .pdbqt format for further analysis in Autodock Vina.

Five different structures of iturin A (ligands) with masses corresponding to those found in the MALDI-TOF analyzes, were obtained from PubChem in 2D .sdf format. The variants obtained with their characteristics are listed in Table 1. The 3D structures were generated using MarvinSketch (Csizmadia 1999) and converted to .pdbqt extension using PyRx (https://pyrx.sourceforge.io/). The docking analysis was then performed by Autodock Vina v.1.1.2 (Trott and Olson 2010). Finally, Chimera software version 1.16 was used to visualize the results and generate the final image (Pettersen *et al.* 2004).

Table 1. Iturin variants obtained from PubChem

Variant	**Formula**	**Molecular weight**	**PubChem CID**
Iturin A	$C_{48}H_{74}N_{12}O_{14}$	1,043.2 g/mol	102287549
Iturin A1	$C_{47}H_{72}N_{12}O_{14}$	1,029.099 g/mol	101589794
Iturin A2	$C_{48}H_{74}N_{12}O_{14}$	1,043.2 g/mol	9988651
Iturin A4	$C_{49}H_{76}N_{12}O_{14}$	1,057.2 g/mol	11062109
Iturin A C-15	$C_{49}H_{76}N_{12}O_{14}$	1,057.2 g/mol	101589795

3 RESULTS

3.1 Hemolysin production by *S. aureus* isolates from bovine mastitis

The hemolytic activity of *S. aureus* on sheep blood agar plates revealed that most of the *S. aureus* cultures (n = 62, 69%) produced beta-hemolysins, indicated by the presence of incomplete hemolysis after overnight incubation at 37 ºC (Figure 1A, Supplementary Figure 1). In turn, nineteen isolates (~21%) produced alpha-hemolysins and were able to completely lyse sheep erythrocytes (Figure 1A, Supplementary Figure 1). In the current study, only nine *S. aureus* isolates did not present hemolytic activity on sheep blood agar plates (Figure 1A, Supplementary Figure 1). Most of the beta-hemolytic *S. aureus* strains produced a hemolysis zone smaller than 10 mm and were categorized as having low hemolytic potential. *S. aureus* 4051 was the only strain that produced a hemolysis zone with a diameter greater than 13 mm and was therefore classified as having high hemolytic potential (Figure 1B, Supplementary Figure 1).

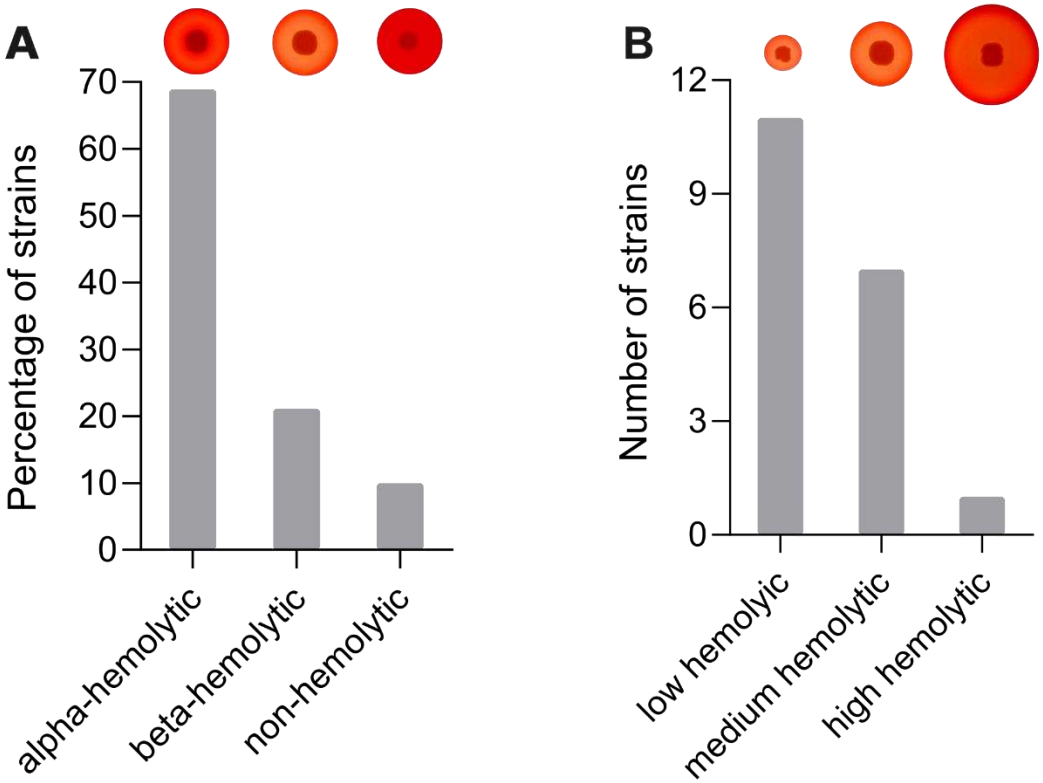

Figure 1. Hemolytic activity of *S. aureus* strains isolated from cows with mastitis on sheep blood agar plates. A) Percentage of strains showing alpha, beta, and non-hemolytic properties. B) Number of beta-hemolytic strains categorized as showing low (<10 mm), medium (between 10 and 13 mm), and high hemolytic (>13 mm) activity based on the diameter of their hemolysis zone.

3.2 Effect of *Bacillus* spp. supernatants in the hemolytic activity of *S. aureus*

Three beta-hemolytic *S. aureus* strains (4051, 4347, and 4628) were selected for this experiment. Preliminary analyzes revealed that the supernatants of 14 *Bacillus* spp. strains analyzed in this study (strains 12, 13, 14, 210, 221, 93, 204, 201, 21, 27, 32, 55, 86, 90, and 94) are cytotoxic for sheep erythrocytes. Therefore, these isolates were excluded from the following analysis. The other *Bacillus* spp. reduced the hemolytic activity of *S. aureus* by up to 93% compared to the controls. In contrast, some *Bacillus* strains increased the hemolytic activity of *S. aureus* by up to 28% depending on the strain tested. However, most *Bacillus* cell-free supernatants also affected the growth of *S. aureus,* and only *Bacillus velezensis* 18, *Bacillus subtilis* 140, and *Bacillus velezensis* 87 reduced the hemolytic activity without causing inhibition in *S. aureus* growth (Figure 2). On average, strains 18, 140, and 87 decreased the hemolytic activity of *S. aureus* by 52%, 43%, and 65%, respectively (Figure 2). Moreover, *B. velezensis* 18 and *B. velezensis* 87 decreased the hemolytic activity of two out of the three *S. aureus* strains analyzed (Supplementary Figure 2). *B. velezensis* 18 reduced the hemolytic activity of *S. aureus* 4051 and *S. aureus* 4347 by 67% and 52%, respectively while *B. velezensis* 87 decreased the hemolytic activity of these strains by 92% and 86%, respectively (Supplementary Figure 2). It is also important to note that in addition to the antimicrobial activity of some *Bacillus* strains against *S. aureus*, the hemolytic activity of *S. aureus* was also strongly inhibited, indicating that these strains are potentially useful candidates for further studies focused on the development of alternative antimicrobial therapies against *S. aureus*.

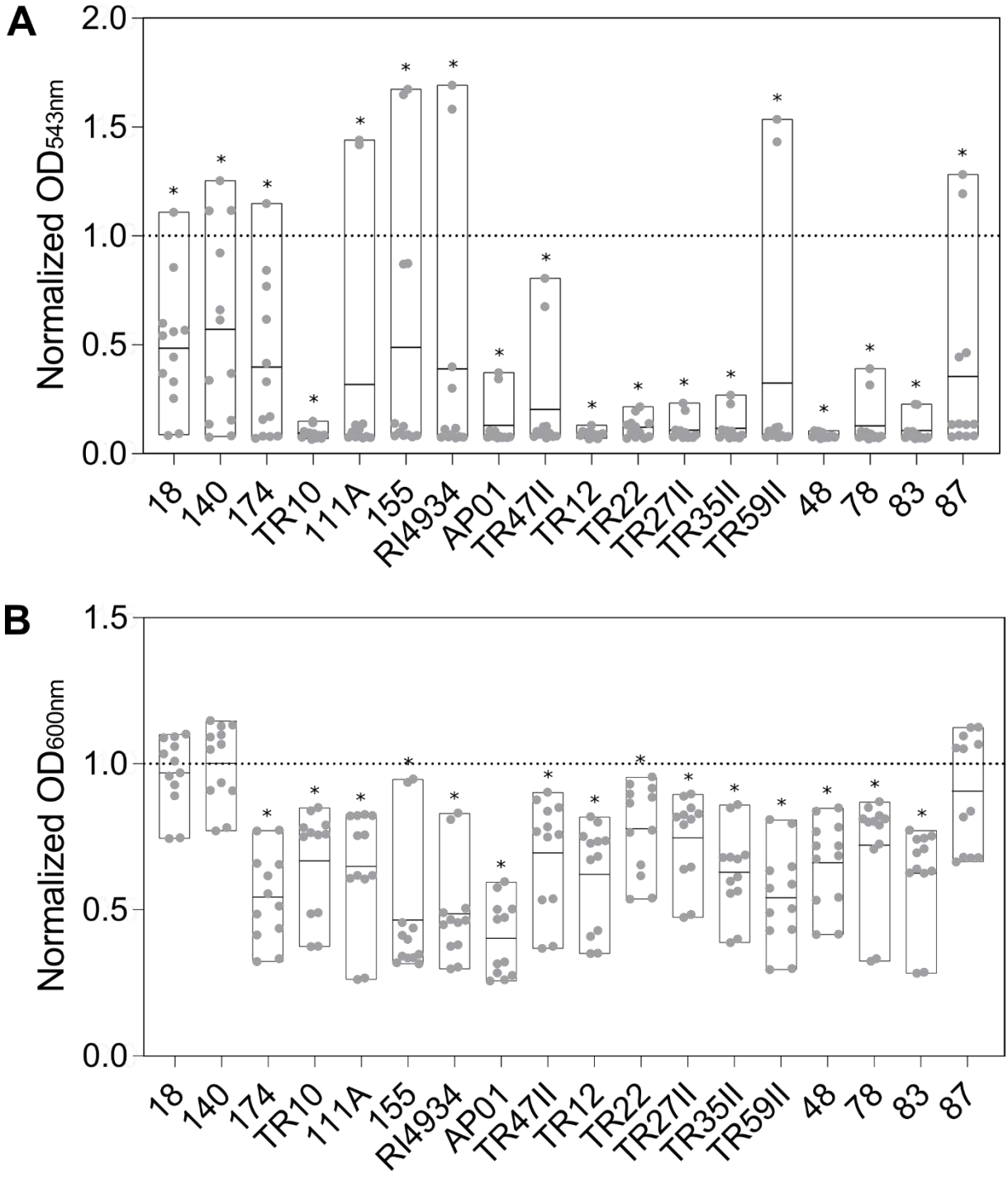

Figure 2. Effect of *Bacillus* spp. supernatants on *S. aureus* hemolysis (A) and growth (B). The optical density (OD) was normalized in comparison with the controls (set to 1 – dotted line). The floating bars show the minimum, maximum, and mean OD considering all the replicates of the three *S. aureus* strains analyzed (*S. aureus* 4051, *S. aureus* 4347, and *S. aureus* 4628) represented in gray dots. The line inside the bars represents the mean normalized OD and the asterisks indicate a significant difference in the treatments compared to the control at a 95% confidence level.

3.3 Lipopeptide production and anti-hemolytic activity

To investigate if the lipopeptides produced by *Bacillus* spp. were responsible for the anti-hemolytic activity detected in the supernatants, we selected the two *Bacillus* strains showing the best anti-hemolytic activity without affecting *S. aureus* growth (*Bacillus velezensis* 18, *Bacillus velezensis* 87). To evaluate if the effect on growth could be due to other compounds in the supernatants or a matter of concentration of bioactive molecules, we selected a *Bacillus* strain (*Bacillus subtilis* TR47II) that showed anti-hemolytic activity and also affected bacterial growth. The concentration of lipopeptides in the supernatants of *Bacillus* spp. was higher in the stationary phase and peaked after 48 h of incubation (Supplementary Figure 3). It should be noted that *Bacillus subtilis* TR47II produces a higher concentration of metabolites with biosurfactant activity, as evidenced by the difference in the diameter of the oil displacement halo compared to the other strains (Supplementary Figure 3C).

The supernatants of *Bacillus* spp. (48 h, 30 ºC, 200 rpm) were subjected to acid precipitation and the anti-hemolytic activity of the crude extract was tested at different concentrations. The concentration of crude extracts containing lipopeptides that reduced the hemolytic activity of *S. aureus* varied according to the species of *Bacillus* and *B. subtilis* TR47II was the most effective in inhibiting the hemolytic activity of *S. aureus*. The lowest concentration of lipopeptides (7.81 µg/mL) reduced the hemolytic activity of *S. aureus* by more than 40% depending on the test strain and the hemolytic activity of all tested *S. aureus* strains was completely inhibited at 31.25 µg/mL (Figure 3). *B. velezensis* 18 and *B. velezensis* 87 also showed strong anti-hemolytic activity but at higher concentrations (≥ 250 µg/mL) with up to 100% of inhibition of *S. aureus* hemolysis (Figure 3A-D). *B. velezensis* 87 reduced by more than 80% the hemolytic activity of *S. aureus* 4051 and *S. aureus* O11 at 62.5 µg/mL and 31.25 µg/mL, respectively. It was also observed that at high concentrations (≥ 125 µg/mL) the lipopeptides produced by *B. subtilis* TR47II showed cytotoxic effects against sheep erythrocytes.

The concentration of lipopeptides significantly ($p < 0.001$) affected their anti-hemolytic effect against all tested *S. aureus* strains. Lipopeptides produced by different *Bacillus* strains varied significantly in their activity against the tested *S. aureus* strains ($p < 0.001$ for *S. aureus* strains 4051 and O11, and $p = 0.013$ for *S. aureus* strains 4347 and 4628). However, multiple comparison analysis showed that depending on

the concentration and the *Bacillus* and *S. aureus* strains analyzed, the anti-hemolytic effect of the lipopeptides did not differ statistically ($p > 0.05$), especially for lipopeptides isolated from *B. velezensis* 18 and *B. velezensis* 87 (Figure 3).

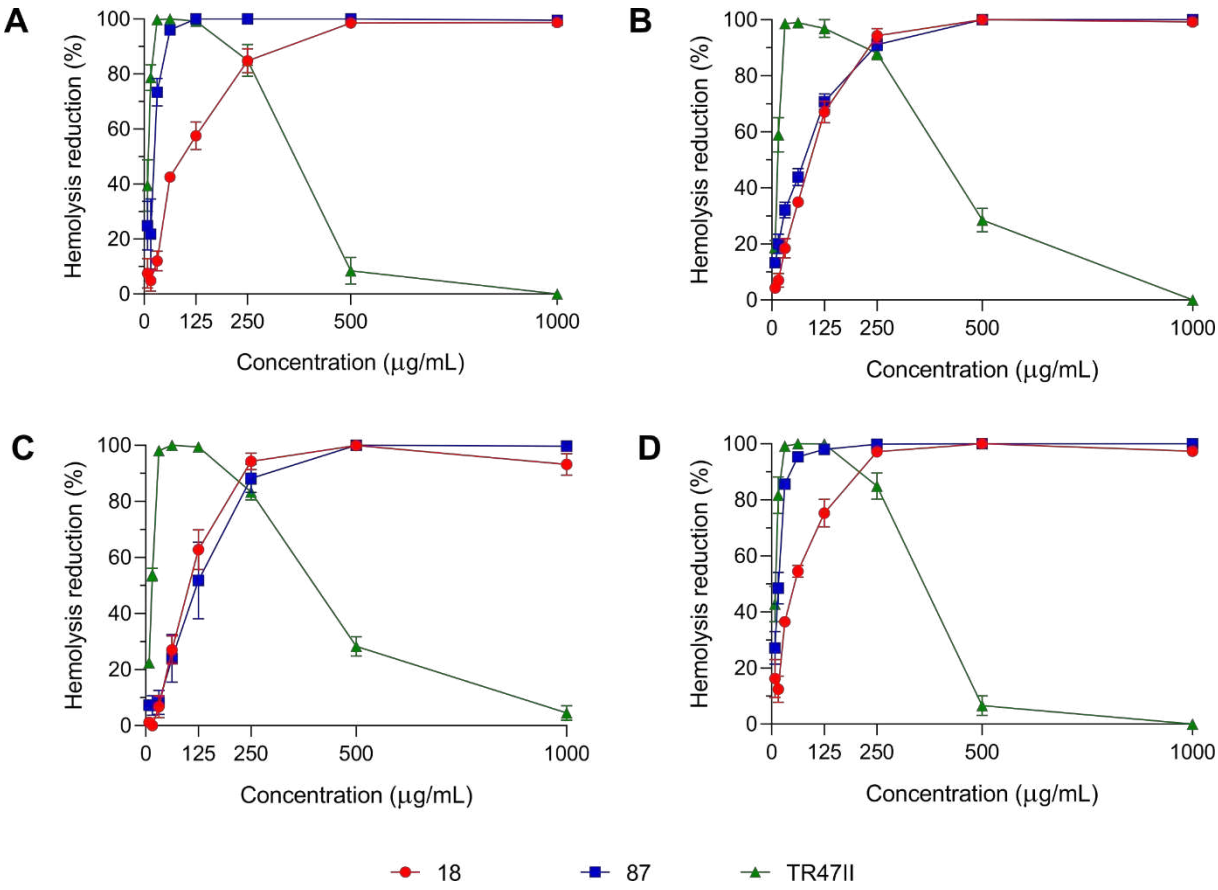

Figure 3. Effect of crude extracts containing lipopeptides from *Bacillus* spp. on the hemolytic activity of *S. aureus* 4051 (A), *S. aureus* 4347 (B), *S. aureus* 4628 (C), and *S. aureus* O11 (D). Each strain of *Bacillus* is color-coded as indicated in the figure. Bars show the standard error of the mean.

3.4 Effect of lipopeptide crude extracts from *Bacillus* spp. on the expression of *S. aureus* genes associated with hemolysin production

Real-time PCR experiments were performed to evaluate if the anti-hemolytic activity of lipopeptides was due to a reduced expression of the genes involved in hemolysin production. The expression of all hemolysin genes evaluated in this study increased after the treatments with lipopeptide extracts from *B. velezensis* 87 and *B. subtilis* TR47II except for the gene encoding delta hemolysin (*hld*), which showed no

difference to the control when cells were treated with extracts from *B. subtilis* TR47II (Figure 4). The quorum sensing genes (*agrA* and *agrC*) also showed increased expression across treatments. In general, the level of expression was higher for treatments with *B. velezensis* 87. Some genes, such as *hla*, *hlg* and *agrC* had increased expression with both lipopeptide extracts, while other genes only increased expression when *S. aureus* was treated with extracts from *B. velezensis* 87 (Figure 4).

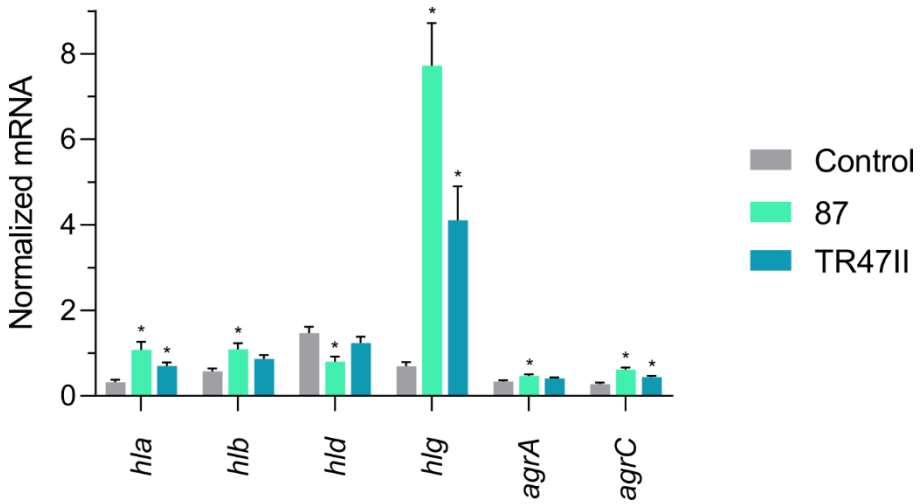

Figure 4. Effect of lipopeptide extracts on the expression of genes involved in the synthesis of hemolysins by *S. aureus* O11. *B. velezensis* 87 and *B. subtilis* TR47II are represented by green and blue bars, respectively, and the control is shown in dark gray. Error bars indicate the standard error of the mean. Asterisks represent a significant difference between treatments and the control by the Mann-Whitney test at a 95% confidence level.

3.5 Post-translational anti-hemolytic effect of lipopeptides

Most genes involved in hemolysin production evaluated in the current study showed an increase in expression when *S. aureus* cells were treated with lipopeptide extracts. We, therefore, hypothesized that the anti-hemolytic activity of the lipopeptides produced by *Bacillus* strains was due to direct inhibition of hemolysin activity, not synthesis. When *S. aureus* O11 supernatants were treated with lipopeptides produced by *B. velezensis* 87 at 500 µg/mL and *B. subtilis* TR47II at 125 µg/mL the hemolytic activity of *S. aureus* O11 decreased approximately 90% and 83%, respectively (Figure 5).

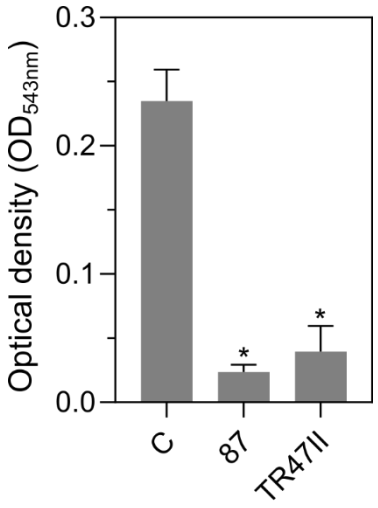

Figure 5. Effect of lipopeptides on *S. aureus* hemolysin activity. The release of hemoglobin by *S. aureus* O11 was measured after supernatants were treated with lipopeptides from *B. velezensis* 87 and *B. subtilis* TR47II at 500 µg/mL and 125 µg/mL, respectively. C represents the hemolytic activity of *S. aureus* O11 supernatant in the absence of treatments (Control). Asterisk means a significant difference between the treatments and the control evaluated using Mann Whitney t-test.

3.6 Lipopeptides purification

The purification of lipopeptides produced by *B. velezensis* 87 and *B. subtilis* TR47II using reserve-phase chromatography showed that compounds with different degrees of hydrophobicity were eluted along the acetonitrile gradient (Figure 6). Initially, all of the peaks were pooled for mass spectra analysis, and compounds eluted at the beginning of the chromatogram (fractions 23-26) were identified as iturins, while compounds eluted around 38-41 minutes were fengycins and very hydrophobic compounds eluted with 100% acetonitrile were identified as surfactins. The identification of the compounds was confirmed using MALDI-TOF mass spectrometry. *B. velezensis* 87 produced more fengycins while extracts obtained from *B. subtilis* TR47II showed a higher concentration of iturins (Figure 6). The two major HPLC peaks corresponding to the different classes of lipopeptides were collected and concentrated individually for the *in vitro* assays. The MALDI-TOF results showed that *B. velezensis* 87 and *B. subtilis* TR47II produce different variants of iturins, with only the iturin with 1043.7 Da in common. The fengycins and surfactins produced by both strains were virtually identical,

but more surfactins variants were detected in the extracts obtained from *B. velezensis* 87 (Table 2).

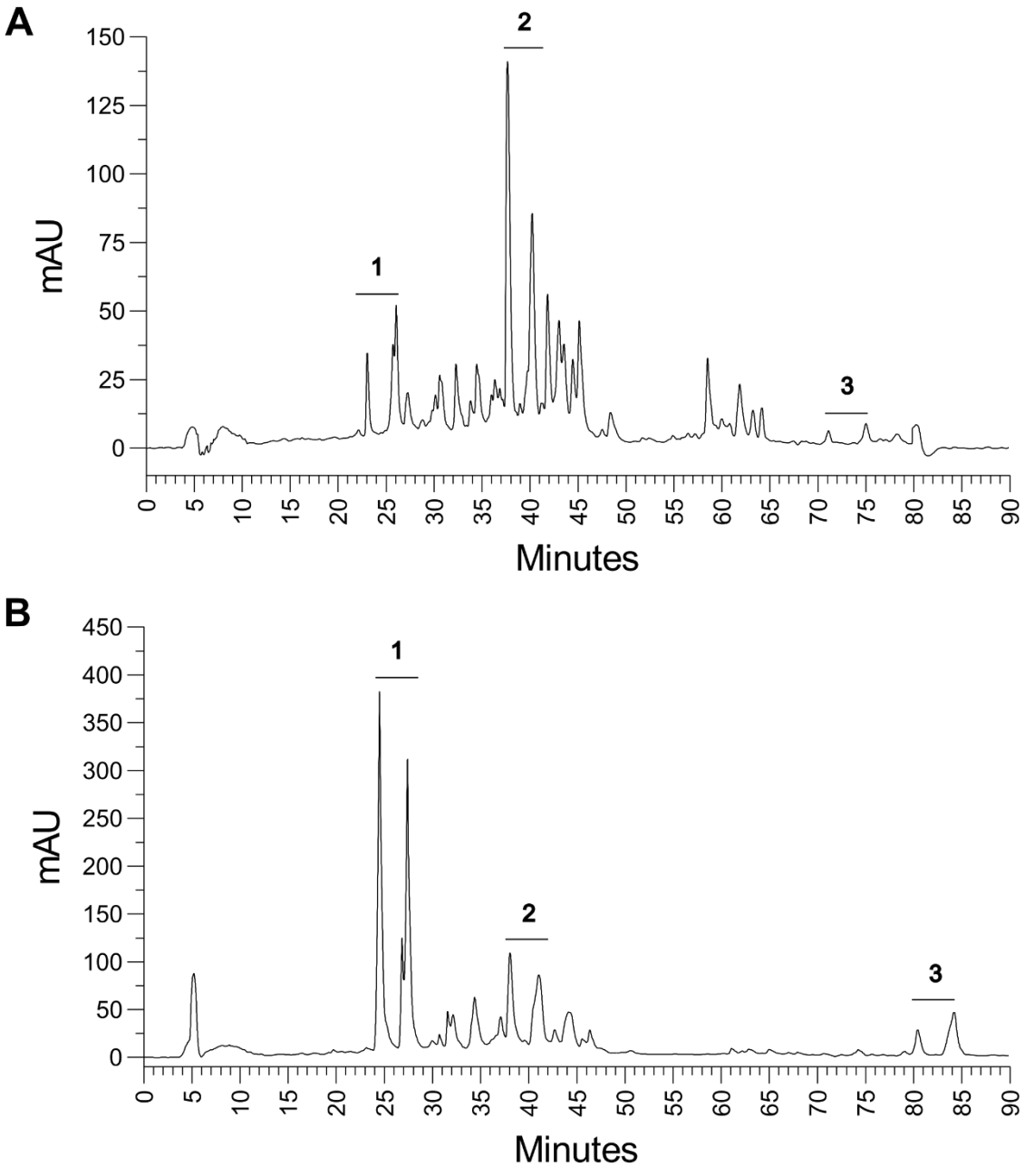

Figure 6. Chromatogram of lipopeptides produced by the species of *Bacillus* analyzed in this study. A) Chromatogram of lipopeptides produced by *B. velezensis* 87 and B) Chromatogram of lipopeptides produced by *B. subtilis* TR47II. Numbers 1, 2, and 3 show the two major peaks of iturins, fengycins, and surfactins, respectively. The peptides were

analyzed in a C12 chromatography column and eluted using a gradient of acetonitrile + TFA 0.1%.

Table 2. Mass spectra of lipopeptides purified using reverse phase HPLC

Bacillus velezensis 87		
Fraction	**Main peaks**	**Putative Class**
23	1043.7; 1029.0; 1081.7	Iturins
26	1079.7; 1095.7; 1043.0	
38	1463.72	Fengycins
41	1477.69; 1491.72	
71	1044.8; 1060.8; 1022.8	Surfactins
75	1058.8; 1074.8	
Bacillus subtilis TR47II		
Fraction	**Main peaks**	**Putative Class**
23	1021.7; 1043.7; 1059.7	Iturins
26	1035.8; 1057.7; 1073.7	
38	1463.8	Fengycins
41	1477.7; 1491.8	
81	1044.8; 1060.7	Surfactins
85	1058.8	

3.7 Activity of fengycins, iturins and surfactins

Among the lipopeptides produced by the *Bacillus* spp. strains analyzed in this study, iturins showed, in general, the highest anti-hemolytic activity. Iturins produced by *Bacillus velezensis* 87 decreased the hemolytic activity of *S. aureus* from 12%-61% depending on the concentration of lipopeptides (Figure 7A). The iturins produced by *Bacillus subtilis* TR47II were more effective in lower concentrations, reducing the hemolytic activity of *S. aureus* at 31.25 µg/mL by up to 76%. However, cytotoxic activity was observed above 125 µg/mL for the variant with 1057.74 Da and ≥ 250 µg/mL for the iturin with 1043.67 Da (Figure 7B). Fengycins produced by *B. velezensis* 87 and *B. subtilis* TR47II only presented anti-hemolytic activity at 1000 µg/mL and above 62.5 µg/mL, reaching a maximum of 43% and 41% reduction in hemolysis, respectively (Figure 7C and 7D). Fengycins produced by *B. velezensis* 87 showed cytotoxic effects in a range of concentrations (Figure 7D). Surfactins produced by *B. velezensis* 87 were not effective in preventing hemolysis, with only surfactin 1044.82 Da showing an 11%

reduction at 500 µg/mL. Indeed, surfactins from *B. velezensis* 87 were highly cytotoxic for erythrocytes in some concentrations (Figure 7E). In contrast, surfactins purified from *B. subtilis* TR47II were less toxic and had better anti-hemolytic activity at several concentrations, with the 1058.78 Da surfactin causing a 38% reduction in *S. aureus* hemolysis at 250 µg/mL (Figure 7F).

The hemolytic activity of *S. aureus* O11 was significantly affected ($p < 0.0001$) by different concentrations of iturins, fengycins, and surfactins produced by *B. velezensis* 87 and *B. subtilis* TR47II. Considering all concentrations tested, the two variants of lipopeptides within each lipopeptide class also significantly differ in activity, except for fengycins produced by *B. subtilis* TR47II ($p = 0.188$).

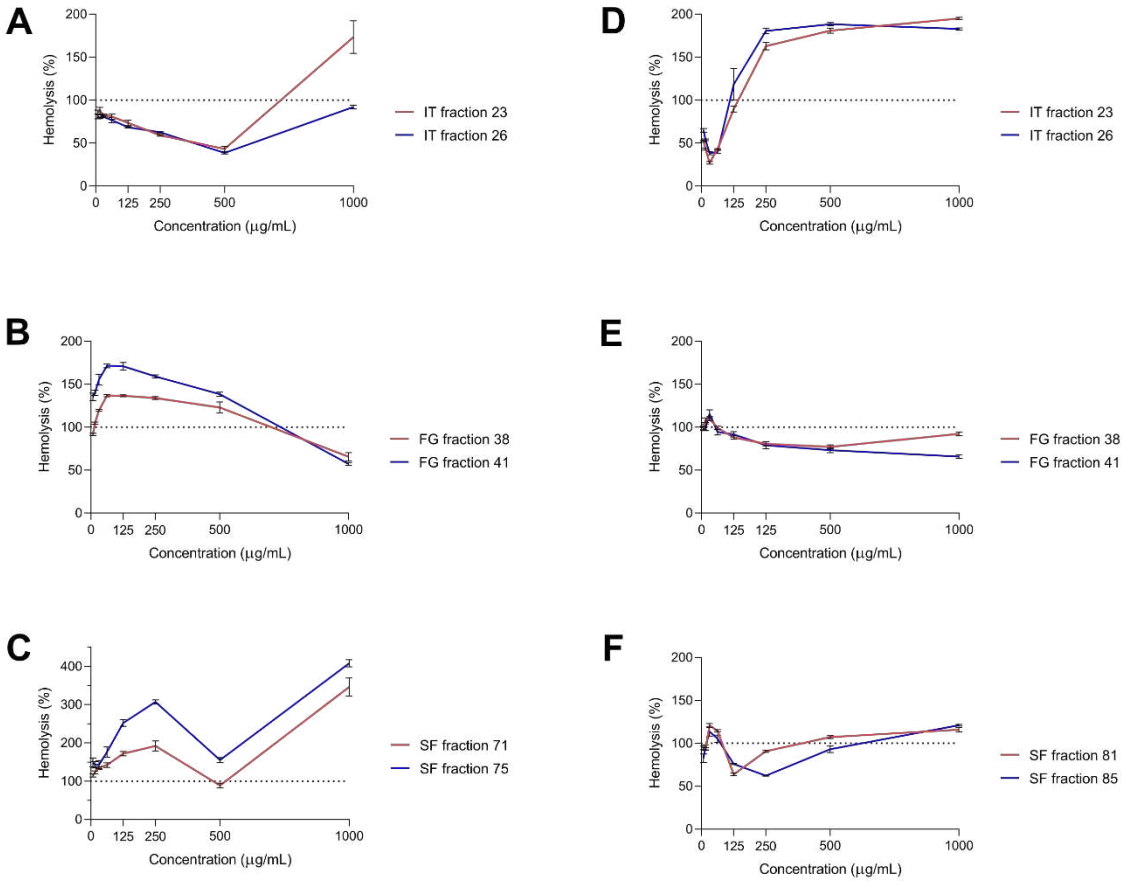

Figure 7. Hemolytic activity of *S. aureus* O11 supernatants in the presence of purified lipopeptides from *Bacillus* spp. The panel shows the activity of iturins (IT - A and B), fengycins (FG - C and D), and surfactins (SF - E and F), produced by *Bacillus velezensis* 87 (A, B, and C) and *Bacillus subtilis* TR47II (D, E, and F). The hemolytic activity of the samples was normalized by the hemolytic activity of non-treated *S. aureus* O11

supernatant (set to 100% - dotted lines). The lipopeptides were purified using RP-HPLC and their masses were identified using MALDI-TOF MS.

3.8 Oligomerization assay

The SDS-PAGE analysis showed that iturins produced by both *B. velezensis* 87 and *B. subtilis* TR47II did not affect α-hemolysin oligomerization in any of the concentrations tested, as confirmed by the presence of hemolysin heptamers (higher molecular weight band) in all the treatments. This was also demonstrated by the same pattern of bands obtained in the positive control (hemolysin alone) and the treatments and the absence of bands in the negative controls (treatment alone) (Figure 8).

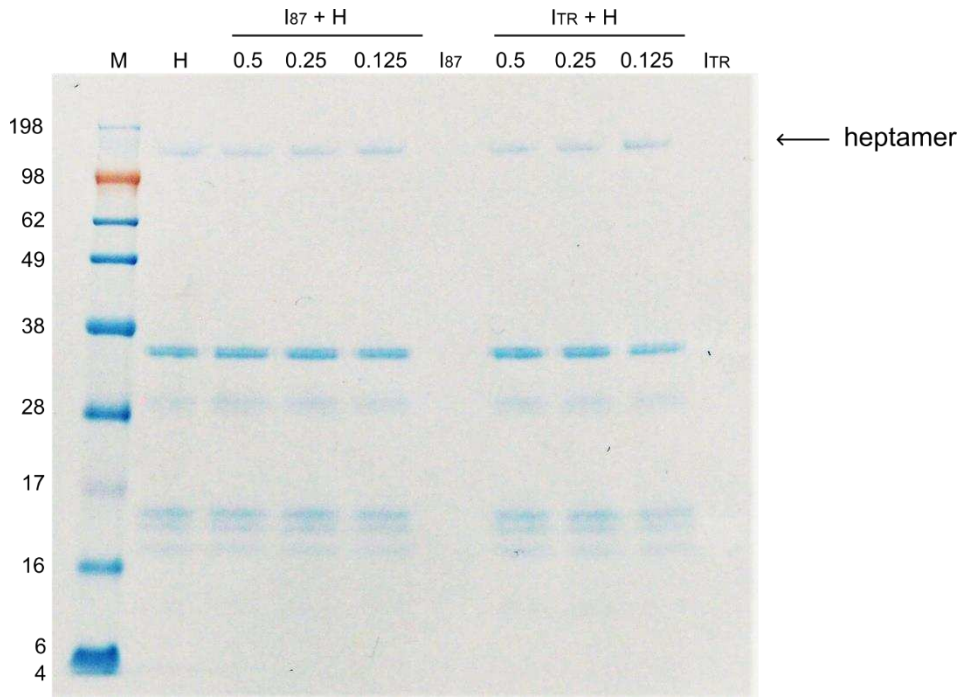

Figure 8. Oligomerization assay of α-hemolysin in the presence of iturins. Iturins from *B. velezensis* 87 and *B. subtilis* TR47II were tested in different concentrations. Hemolysin (H) alone was used as a positive control and iturins from both *Bacillus* species were used as a negative control (I_{87} and I_{TR}). SeeBlue Plus2 Pre-stained was used as a protein standard (M). The experiment was done using a Nu-Page gel stained with EZBlue.

3.9 Molecular docking

The molecular docking of the ADAM-10 receptor, present on the erythrocytes membrane, with the iturin variants, revealed that all iturin structures are capable of forming hydrogen bonds with Glu 665. Nonetheless, the binding affinity was low, ranging from -2.7 to -2.9 kcal/mol for the best models obtained from Autodock Vina (Supplementary Table 3). The variants were capable of forming 3 to 4 hydrogen bonds with ADAM-10, 2 of them with the important residue Glu 665 for Iturin A, Iturin A1, and Iturin A2. Iturin A C-15, in turn, showed the best binding affinity (-2.9 kcal/mol) with 4 hydrogen bonds with ADAM-10, being one with Glu 665.

The iturins showed the capacity to bind to hemolysin, by forming hydrogen bonds with residues from the designed grid box 01 (Supplementary Table 4). The binding affinity of iturins with hemolysin was higher than with ADAM-10, ranging from -6.6 kcal/mol to -8.3 kcal/mol according to the best models for each variant. Three to seven hydrogen bonds were established between iturins and hemolysin, most of them with external residues of the heptameric structure (Figure 9). Iturin A4 showed the best binding affinity and had the highest number of hydrogen bonds with hemolysin, showing its potential as a ligand *in vivo*. It is also important to highlight the second best model categorized by Autodock Vina for Iturin A1 where the ligand bonds inside the pore structure of hemolysin, which could partially explain the blocking of its activity (Figure 10).

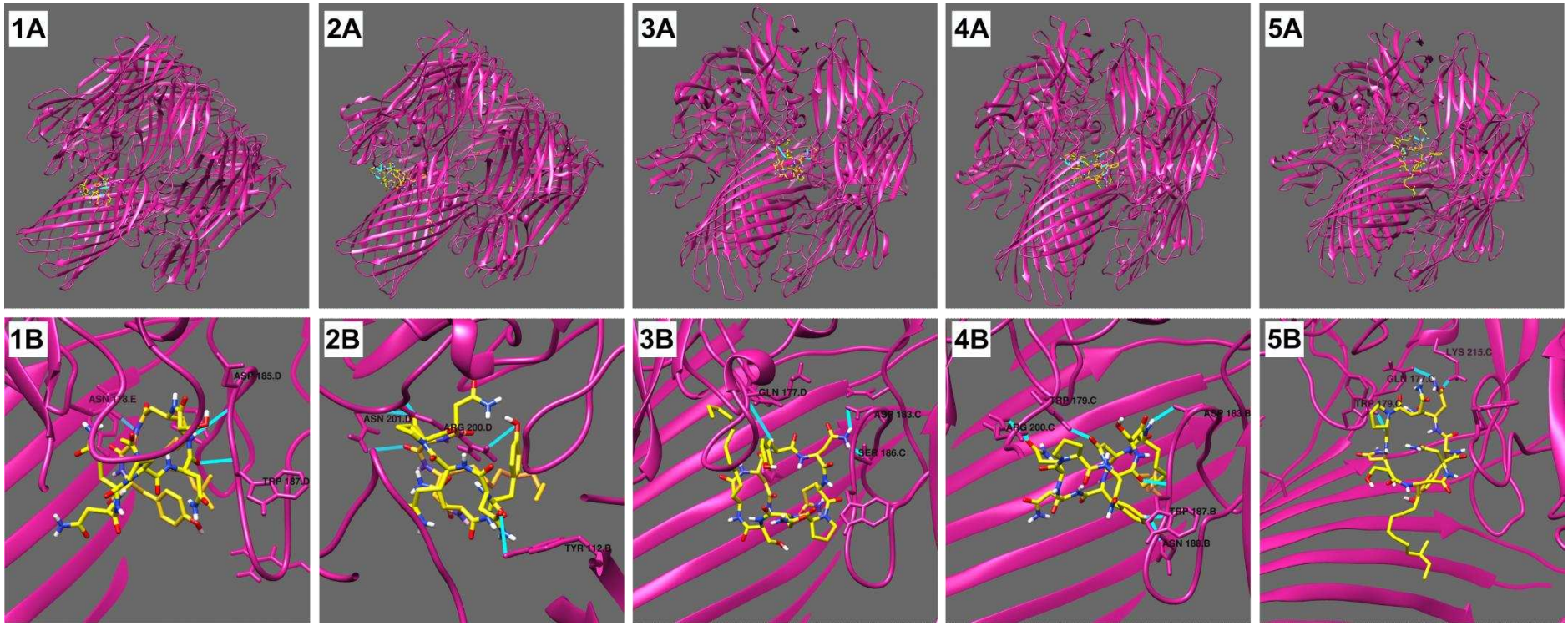

Figure 9. Best molecular models between hemolysin and iturins categorized by AutoDock Vina. Top panels (1A-5A) show the complete heptameric structure of hemolysin interacting with iturins; Bottom panels (1B-5B) show the interactions between the compounds highlighting the hydrogen bonds in light blue. The iturin variants represented in panels 1, 2, 3, 4, and 5 are the following: Iturin A, Iturin A1, Iturin A2, Iturin A4, Iturin A C-15. The docking analyzes were performed by AutoDock Vina v.1.1.2 and images were generated using UCSF Chimera.

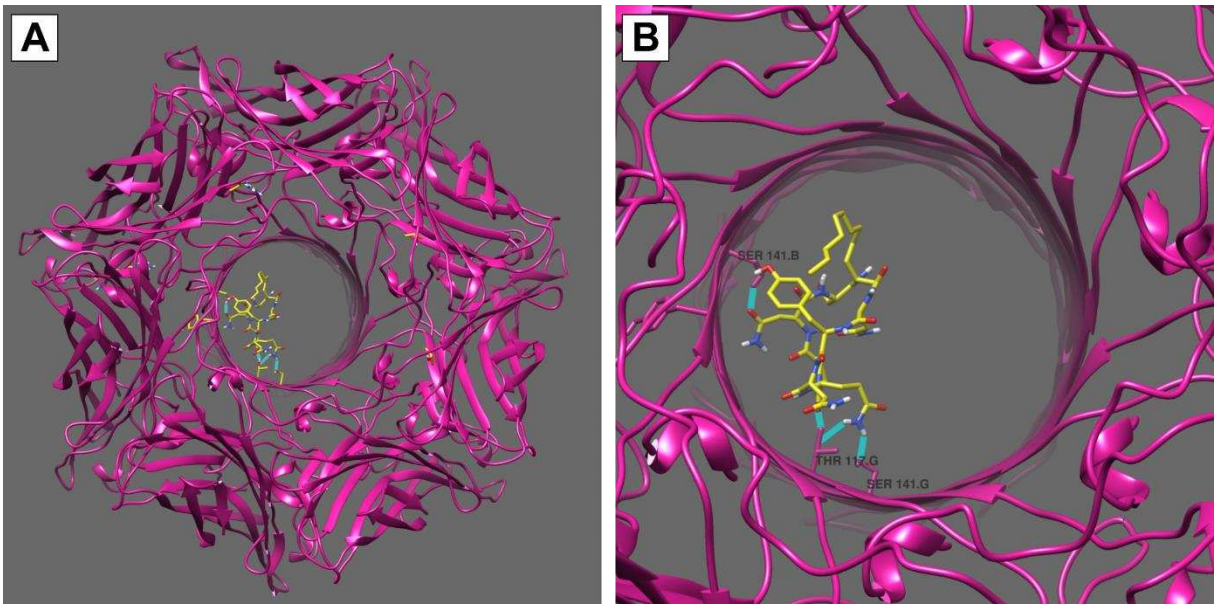

Figure 10. Molecular docking model proposed for hemolysin and Iturin A1. In A the entire structure is shown and in B the interaction between hemolysin and Iturin A1 through hydrogen bonds is highlighted in light blue. The docking analyzes were performed by AutoDock Vina v.1.1.2 and images were generated using UCSF Chimera.

4 DISCUSSION

S. aureus is an important pathogen for both humans and animals and its resistance to multiple antibiotics has increased the need for therapeutic alternatives to treat infectious diseases caused by this bacterium. As bactericidal therapies have been associated with the development of resistance, therapeutic strategies that affect the ability of bacteria to cause disease without killing the microorganism may be advantageous by delaying the adaptative process (Sully *et al.* 2014; Quave *et al.* 2015; Vale *et al.* 2016a). In this way, the anti-virulence strategy has emerged as an alternative therapy to treat *S. aureus* infections, in an attempt to control *S. aureus* pathogenicity, while offering a possible long-term effective medicine.

In bovine mastitis, toxin secretion plays a role in the invasion, penetration, and destruction of the mammary gland tissue by *S. aureus* (Cote-Gravel and Malouin 2019). A study has shown that cows infected with a double *hla* and *hlb* mutant strain were able to eliminate the pathogen and had only mild symptoms compared to severe mastitis caused by the parental strain (Kenny *et al.* 1992). Alpha hemolysin is the best-studied hemolysin produced by *S. aureus*, consisting of a small pore-forming cytotoxin secreted

as a 33 kDa water-soluble monomer that assembles into a heptamer to form a pore in a wide variety of host cells (Berube and Wardenburg 2013; Divyakolu *et al.* 2019b). Although several anti-hemolytic compounds have been investigated, including antibodies (Foletti *et al.* 2013; Francois *et al.* 2018), nanoparticles (Henry *et al.* 2015; Chen *et al.* 2018; Keller *et al.* 2020) and natural compounds (Karginov *et al.* 2007; Dong *et al.* 2013a; Wang *et al.* 2015b), most studies are directed towards human pathogens and none of these treatments evolved to clinical trials.

In this study, we found that lipopeptides, specially iturins, produced by *Bacillus velezensis* and *Bacillus subtilis* have anti-hemolytic effects against *S. aureus* isolated from bovine mastitis in a dose-dependent manner. Our findings indicate that lipopeptides decrease hemolysin activity rather than its production and that iturins do not interfere with hemolysin oligomerization. However, molecular docking studies showed that iturins are capable of binding to the hemolysin structure *in silico*, which could potentially explain their effect on hemolysin activity.

Several lipopeptides have demonstrated antimicrobial activity against *S. aureus*, as is the case of the antibiotic daptomycin used to treat severe Gram-positive infections, the first molecule of this class to reach commercial application (Morikawa *et al.* 2000). Lipopeptides have also demonstrated anti-biofilm activity (Quinn *et al.* 2012; De Zoysa *et al.* 2020) and anti-quorum sensing effect (Piewngam *et al.* 2018) against *S. aureus*. Fengycin produced by a probiotic *Bacillus* strain inhibited the quorum sensing system of *S. aureus* and prevented its colonization in the human intestine (Piewngam *et al.* 2018). Despite these advances, the current study is the first to demonstrate a direct anti-hemolytic effect of lipopeptides in *S. aureus* without affecting bacterial growth.

Our findings indicate that the expression of genes involved in hemolysin production, including *hla*, *hlb* and *hlg* increase when *S. aureus* was treated with lipopeptides extracted from *B. velezensis* 87 and *B. subtilis* TR47II. Furthermore, the expression of the genes *agrA* and *agrC*, which positively regulates the expression of toxins (Le and Otto 2015), were upregulated in our treatments. However, although these genes significantly increased in our treatments, it should be noted that only *hlg* had an expressive increase compared to the control. The increase in hemolysin gene expression may be due to a stress response mediated by sigma B, which regulates gene transcription in *S. aureus* during stress potentially increasing *hla* expression (Chen *et al.* 2011). In *B. subtilis*, for example, high amounts of surfactin increased the production of

stress proteins under the control of alternative sigma factors, such as sigma B and sigma W (Lilge *et al.* 2022). Hemolysin-encoded genes (*hlb* and *hlg*) were also upregulated during alcohol-induced stress (Korem *et al.* 2010). The increase in expression of these proteins also does not confirm an increased production or activity of the respective toxins. The *hld* gene, which encodes a delta-toxin, was the only gene downregulated under the conditions evaluated in the current study. Delta toxin is transcribed together with RNAIII, the effector of the agr system (Le and Otto 2015). Our results also indicated that the quorum sensing genes of *S. aureus* were upregulated. Therefore, the reduction in the expression of *hld* might be overcome by an increased expression of the quorum sensing genes. In general, these results do not support the observed anti-hemolytic phenotypes, suggesting that lipopeptides could be affecting hemolysin activity rather than its production.

We verified that the hemolytic activity of *S. aureus* decreased significantly in relation to the control when *S. aureus* cell-free supernatants were treated with lipopeptides from *Bacillus*, indicating that these molecules could inhibit enzymatic activity. To better understand the mechanism of action, we purified the lipopeptides using reverse-phase chromatography under an eluting gradient of acetonitrile. Previous studies indicated that due to their different degrees of hydrophobicity, iturins are eluted first in reverse-phase chromatography, followed by fengycins and surfactins (Kim *et al.* 2010; Yang *et al.* 2015). Our results demonstrated a higher concentration of fengycin and iturin in *B. velezensis* 87 and *B. subtilis* TR47II extracts, respectively, which explains the higher toxicity of the *B. subtilis* TR47II crude extracts (Vanittanakom *et al.* 1986). Surfactins were produced in smaller quantities by both bacteria under the tested conditions. The identification of the fractioned lipopeptides was confirmed by mass spectrometry analysis.

Iturin, fengycin, and surfactin are families of lipopeptides with a remarkable structural heterogeneity given the diverse peptide sequence, the nature of the peptide cyclization, and the variable length and branching of the fatty acid chain (Ongena and Jacques 2008). Iturins are composed of a heptapeptide linked to a beta-amino fatty acid chain of 14-17 carbons in length (Raaijmakers *et al.* 2010; Zhao *et al.* 2017). Iturin variants include iturin A and C, bacillomycin D, F and L, mycosubtilin, mixirin, subtulene A, and mojavensin A (Cochrane and Vederas 2016). Fengycins are decapeptides linked to a beta-hydroxyl fatty acid chain of 14 to 21 carbon units. The fengycins are

categorized into fengycin A, B, C and Z and plispastatins A and B based on the difference in their sequence and structure (Cochrane and Vederas 2016). Lastly, surfactins have their heptapeptide linked to a betahydroxy fatty acid chain composed of 12 to 17 carbons (Theatre *et al.* 2021). The variants found for the surfactin group are esperin, lichenysin, pumilacidin and surfactin (Ongena and Jacques 2008).

Our findings indicate several masses corresponding to lipopeptide variants among the fractions analyzed. Most of the predominant masses were produced by both *Bacillus* strains, including 1043 Da iturin, 1463 and 1477 Da fengycin and 1044 and 1058 Da surfactins. Only the second fraction of iturins varied among the analyzed species of *Bacillus*, with 1079 Da being more prevalent in *B. velezensis* while 1057 Da was more prevalent in *B. subtilis* (Supplementary Figure 4 and Supplementary Figure 5). It has been reported that the 1043 Da iturin can represent a C14 iturin A2 $(M+H)^+$ while both 1079 and 1057 Da may represent a C15 A3/A4/A5 chain with Na^+ and H^+, respectively (Pathak and Keharia 2014). For fengycin, 1463 and 1477 Da were the most prevalent masses in the first and second fractions, respectively, and may represent C16 and C17 fengycins in their protonated form (Yang *et al.* 2015). For surfactin, the 1044 and 1058 Da stood out, possibly corresponding to a C14 and C15 surfactin with a Na^+ addition (Pathak and Keharia 2014).

Lipopeptides from the same class had a similar impact on *S. aureus* hemolysis regardless of the *Bacillus* strain. However, the activity varied between producers depending on the concentration. This difference in activity may be due to the presence of different variants in the lipopeptide fractions from *B. velezensis* 87 and *B. subtilis* TR47II. The higher cytotoxicity observed for the surfactins produced by *B. velezensis* 87 could be due to the presence of a more hemolytic variant. Surfactins are known for their cytotoxicity against erythrocytes, but the degree of cytotoxicity varies according to variants (Jiang *et al.* 2016; Fei *et al.* 2020). Iturins are also generally known to cause hemolysis in a dose-dependent manner that differs between variants (Quentin *et al.* 1982; Aranda *et al.* 2005). However, previous observations indicated that some iturins, such as iturin C, do not show hemolytic potential (Quentin *et al.* 1982).

In contrast to the hemolytic potential of lipopeptides described in the literature, to the best of our knowledge, this is the first report describing an anti-hemolytic activity of lipopeptides, especially iturins, which prevented hemolysis by *S. aureus* in a wide range of concentrations. We evaluated the mechanism of action of these compounds and

found that lipopeptides interfere with the activity of hemolysins. Although the compounds did not act by preventing the oligomerization of hemolysin, molecular docking studies revealed a good binding affinity of iturins with the heptameric structure of hemolysin.

The iturins were able to bind to the heptameric structure of hemolysin through interactions with external residues, including Trp 179, Asp 183, Asp 185, and Arg 200. Arg 200 is implicated in membrane binding and cell lysis, while Asp 183 and Asp 185 are involved in cell lysis (Walker and Bayley 1995; Gouaux 1998). Indeed, several studies have demonstrated the ability of anti-virulence compounds to bind to Trp 179 and Arg 200, showing the importance of these residues for hemolytic activity (Liu *et al.* 2020; Ghoneim *et al.* 2022; Wan *et al.* 2022). Another proposed binding model for Iturin A1 and hemolysin shows the interaction of the ligand with the protein within the pore structure, which could be a possible mechanism by which iturin blocks the hemolytic activity. Likewise, an anti-hemolytic effect of isatin-Schiff copper(II) complexes was attributed to the blockage of the channel formed by alpha-hemolysin caused by interactions with the complexes (Melo *et al.* 2016). In general, this analysis indicates that all iturin variants are capable of binding to hemolysin, some by more than one mechanism, corroborating with the greater activity of the crude extract containing the mixture of lipopeptides in relation to the purified compounds.

The molecular docking studies also revealed a binding affinity of iturins with the hemolysin receptor ADAM-10. The docking was focused on Glu 665, a residue known to be important for hemolysin binding in ADAM-10 (von Hoven *et al.* 2022). Therefore, although the binding score was low, the interaction of iturins with Glu 665 is plausible and with biological implications on hemolysin activity. In addition, the ability of iturin to bind to other residues of the receptor that have not yet been studied regarding their importance for the hemolytic activity should be further investigated.

Therefore, in addition to the biological functions already described for iturins such as anti-fungal (Cho *et al.* 2009; Han *et al.* 2015), anti-bacterial (Asaka and Shoda 1996; Leclère *et al.* 2005) and anti-cancer (Dey *et al.* 2015; Dey *et al.* 2017), this study reveals, for the first time, that these molecules also have anti-hemolytic potential. This study provides novel insights into the use of iturins/lipopeptides as anti-virulence agents against *S. aureus*. Further studies aiming to evaluate the effectiveness of these compounds in the prevention of *S. aureus* colonization in bovine mammary epithelial

cells are warranted to demonstrate their potential application as an alternative approach to treat bovine mastitis.

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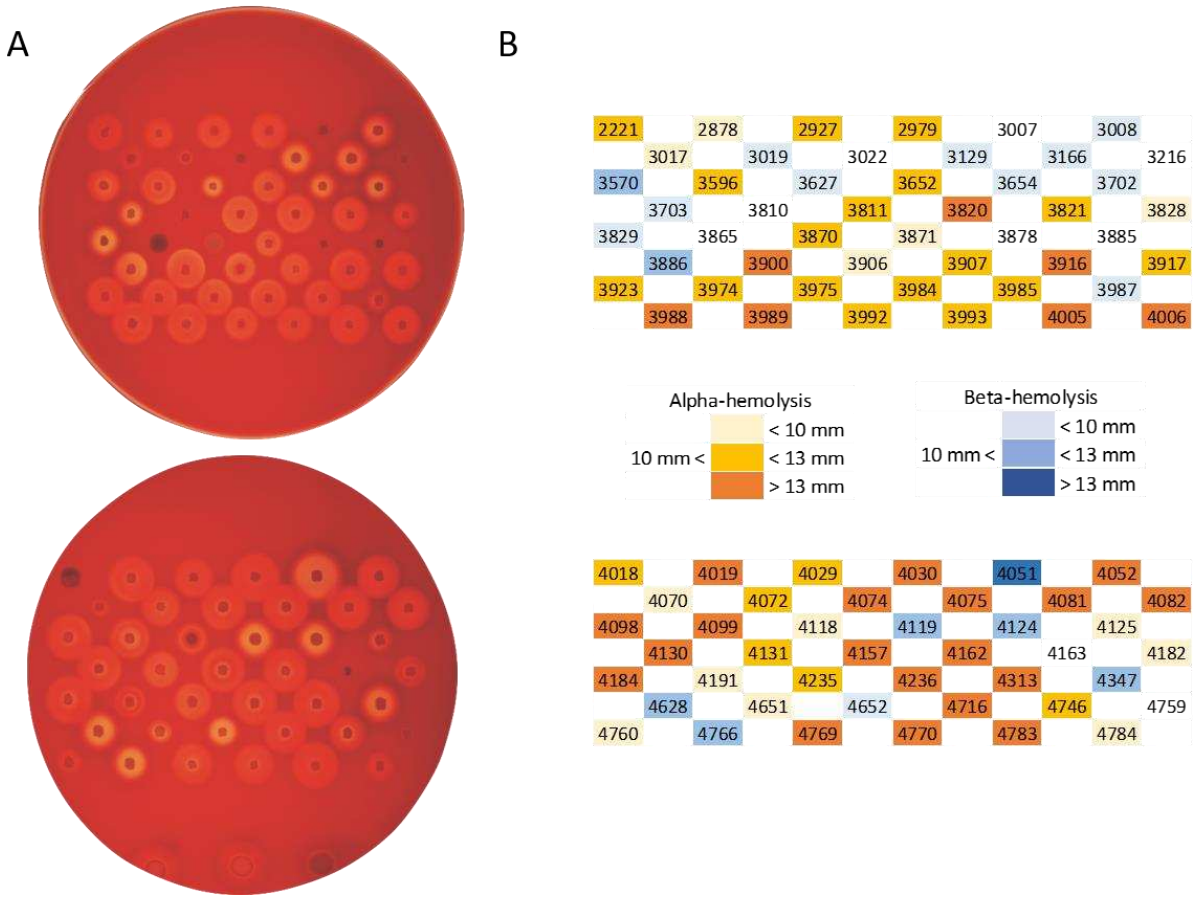
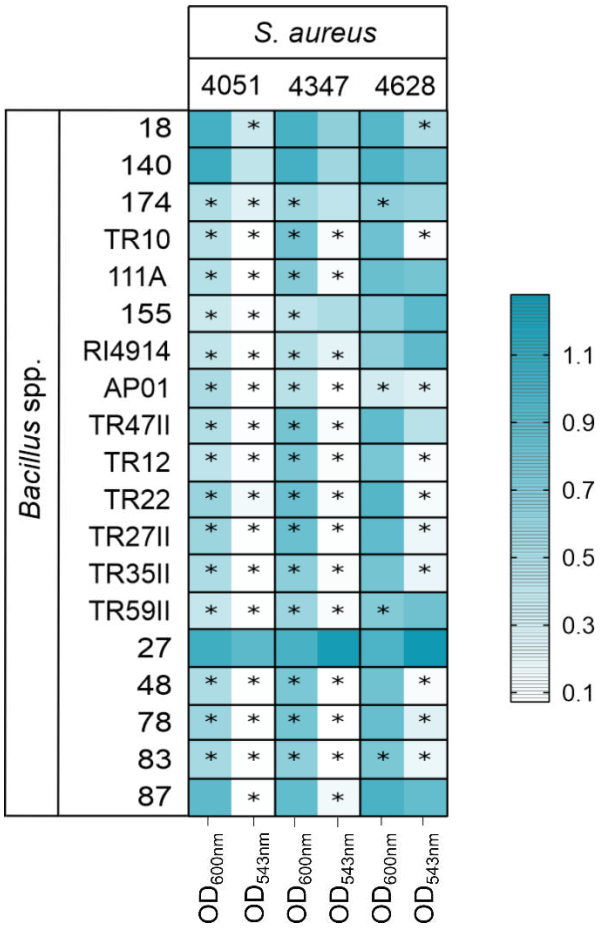
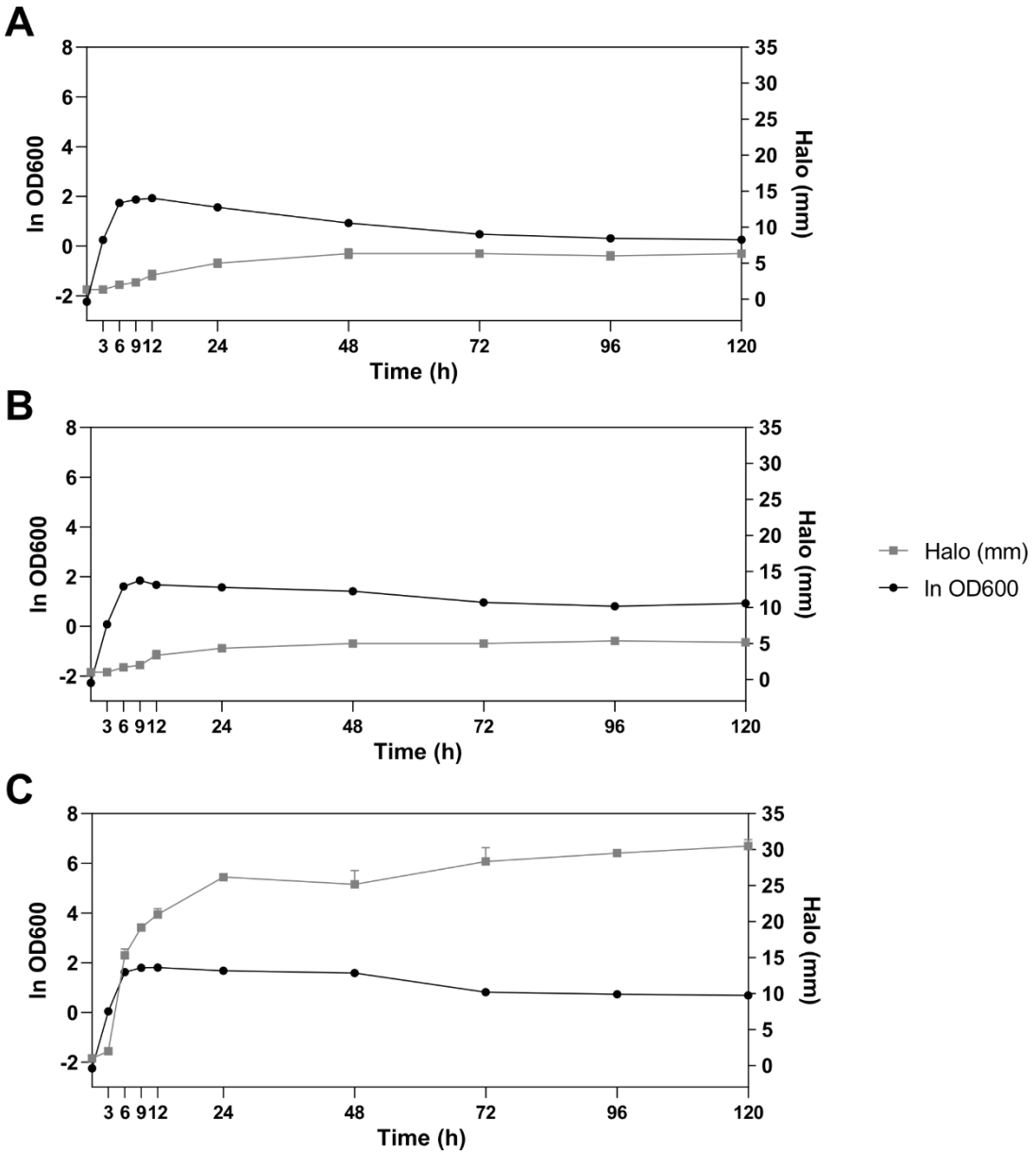
SUPPLEMENTARY FIGURES



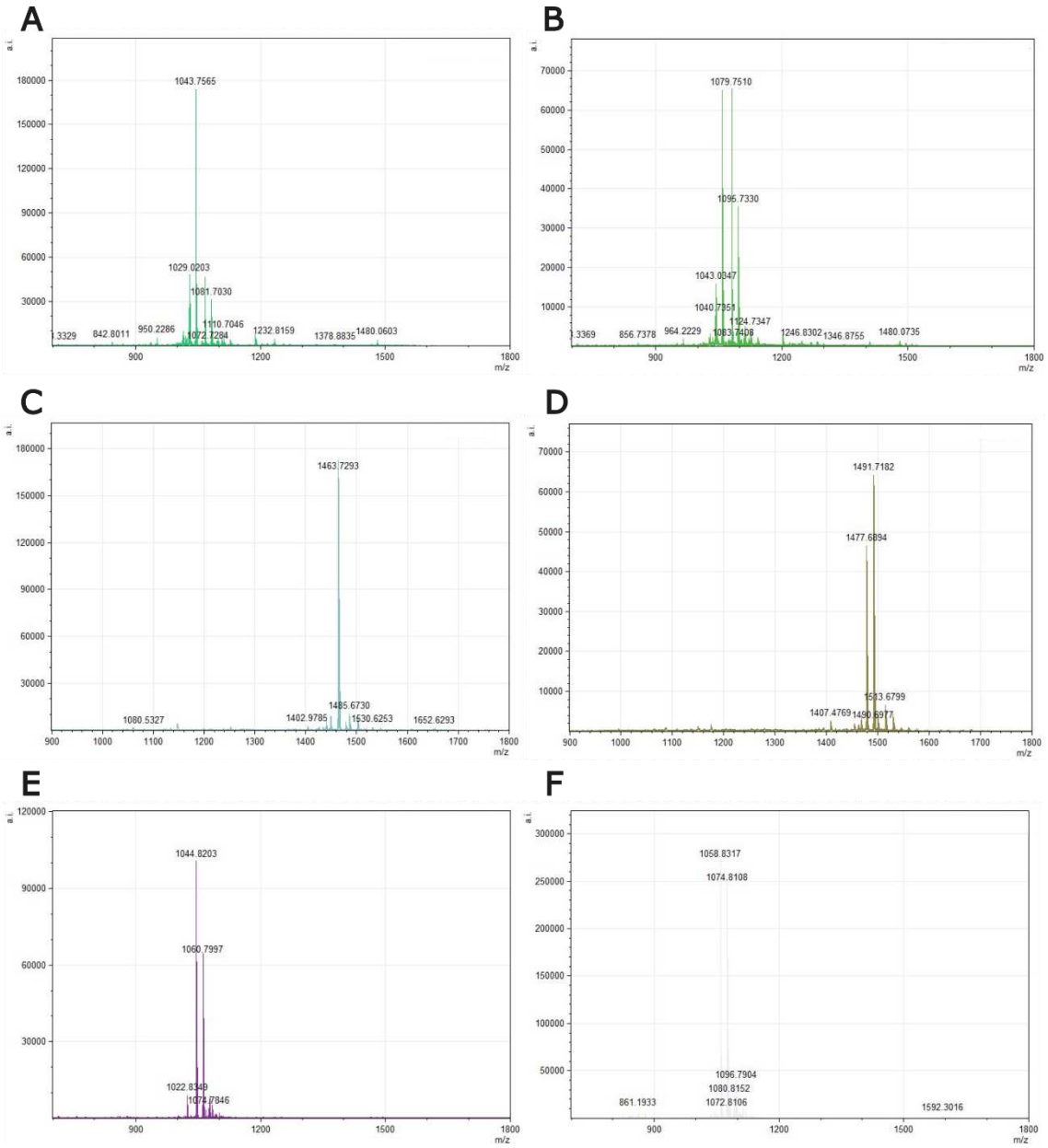
Supplementary Figure 1. Hemolytic activity of *S. aureus* isolated from cows with mastitis in sheep blood agar. A) hemolytic activity of the isolates after incubation at 37 °C for 24 h and then at 4 °C overnight. B) identification of the cultures distributed in the sheep blood agar categorized by the type of hemolysis (alpha or beta) and the size of the hemolytic zone.



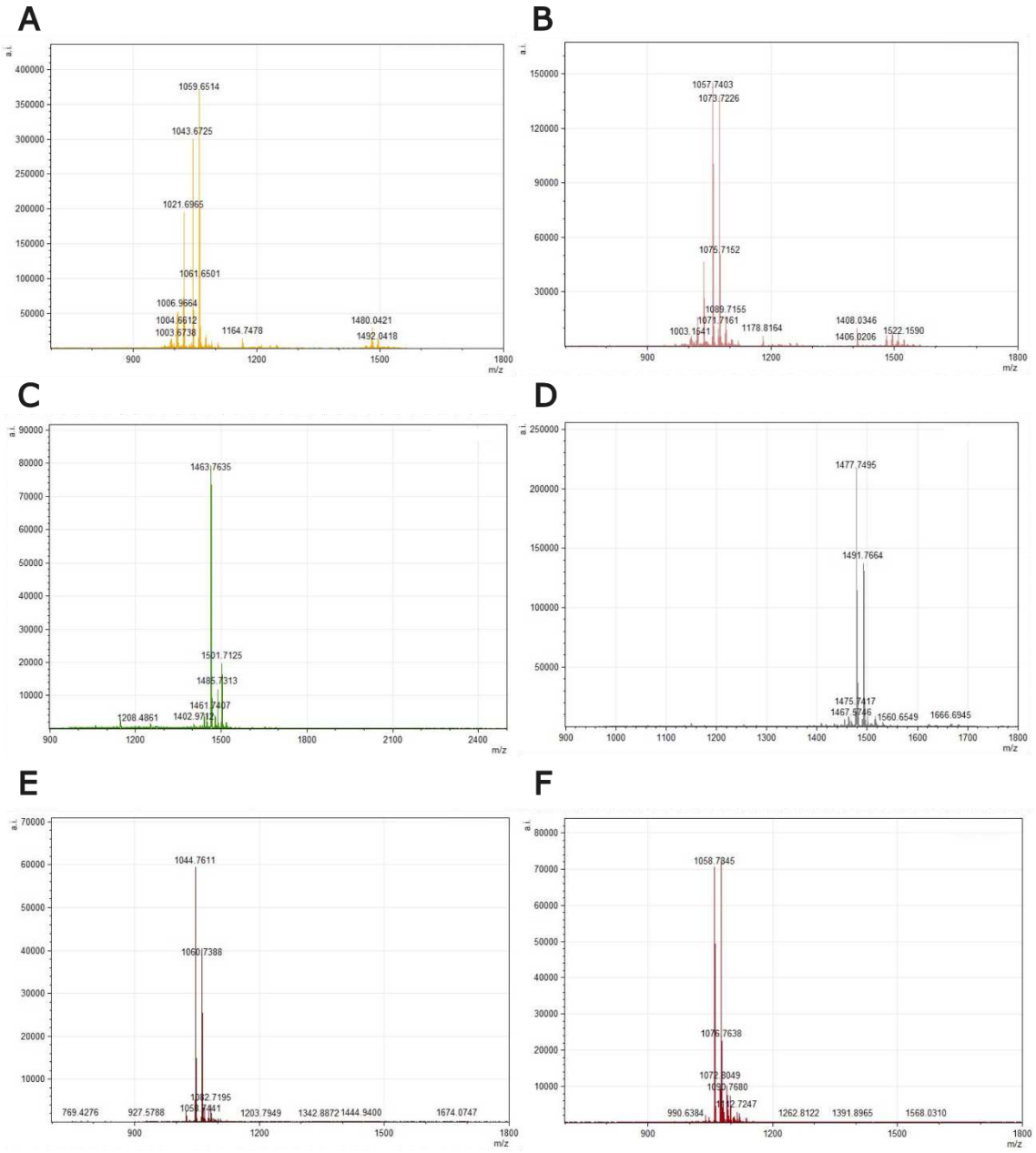
Supplementary Figure 2. Heatmap showing the effects of *Bacillus* supernatants on the growth (OD_{600nm}) and hemolytic activity of *S. aureus* (OD_{543nm}). *Bacillus* strains are shown on the left side of the figure and *S. aureus* strains are on the top. Only *Bacillus* supernatants that did not show hemolytic activity (toxic effect) were included in this analysis. The OD of the controls (without treatment) was normalized to 1.0. Asterisks show a significant difference ($p < 0.05$) in hemolytic activity between the treated cultures and the control assessed by the Mann-Whitney test.



Supplementary Figure 3. Growth curve and production of lipopeptides by *Bacillus velezensis* 18 (A), *Bacillus velezensis* 87 (B), and *Bacillus subtilis* TR47II (C). Lipopeptide production was estimated using the crude oil displacement assay and expressed by halo diameter (square symbols). Bacterial growth (OD_{600nm}) is represented by a line connecting filled circles. The experiment was performed using three biological replicates. Bars indicate the standard error of the mean.



Supplementary Figure 4. MALDI-TOF mass spectrometry of lipopeptides from *Bacillus velezensis* 87 purified by RP-HPLC. A and B, represent, respectively, fractions 23 and 26; C and D represent fractions 38 and 40, and E and F represent fractions 71 and 75.



Supplementary Figure 5. MALDI-TOF mass spectrometry of lipopeptides from *Bacillus subtilis* TR47II purified by RP-HPLC. A and B, represent, respectively, fractions 24 and 27; C and D represent fractions 38 and 41, and E and F represent fractions 81 and 84.

SUPPLEMENTARY TABLES

Supplementary Table 1. *Bacillus* strains used in this study

Bacteria	Method of identification	Source	State/Country	Reference (DOI)
B. velezensis 48	rRNA 16S and rpoB sequencing	Potato field	MG/Brazil	-
B. velezensis 78	rRNA 16S and rpoB sequencing	Mango roots	MG/Brazil	-
B. velezensis 83	rRNA 16S and rpoB sequencing	Mango roots	MG/Brazil	-
B. velezensis 87	rRNA 16S and rpoB sequencing	Washing of coffee planting soil	MG/Brazil	-
B. cereus/thuringiensis 32	rRNA 16S and rpoB sequencing	Forest of the coffee nursery	MG/Brazil	-
B. cereus/thuringiensis 55	rRNA 16S and rpoB sequencing	Potato field	MG/Brazil	-
B. cereus/thuringiensis 90	rRNA 16S and rpoB sequencing	Washing of coffee planting soil	MG/Brazil	-
B. cereus/thuringiensis 94	rRNA 16S and rpoB sequencing	Leaf litter of the coffee nursery	MG/Brazil	-
B. toyonensis 21	rRNA 16S and rpoB sequencing	Forest of the coffee nursery	MG/Brazil	-
B. toyonensis 86	rRNA 16S and rpoB sequencing	Washing of coffee planting soil	MG/Brazil	-
B. altitudinis 27	rRNA 16S and rpoB sequencing	Forest of the coffee nursery	MG/Brazil	-
Bacillus cereus 12	Fatty acid methyl esters (FAME) analysis	Bovine mastitis	MT/Brazil	-

Bacillus cereus 13	Fatty acid methyl esters (FAME) analysis	Bovine mastitis	MT/Brazil	-
Bacillus cereus/thuringiensis 14	Fatty acid methyl esters (FAME) analysis	Bovine mastitis	MT/Brazil	-
B. subtilis LBBMA RI4914	Fatty acid methyl esters (FAME) analysis	Production water of oil exploration field	ES/Brazil	10.1016/j.fuel.2016.04.080
B. subtilis LBBMA 111A	Fatty acid methyl esters (FAME) analysis	Mangrove region contaminated with oil	RJ/Brazil	-
B. subtilis LBBMA AP01	Fatty acid methyl esters (FAME) analysis	Contamination of an agar plate used for isolation of phytopathogenic fungi	MG/Brazil	-
B. subtilis TR47II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis LBBMA 155	Fatty acid methyl esters (FAME) analysis	Mangrove region contaminated with oil	RJ/Brazil	-
B. subtilis TR10	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR12	rRNA 16S sequencing and fatty acid methyl	Soil	ES/Brazil	10.1007/s00792-015-0740-7

	esters (FAME) analysis			
B. subtilis TR22	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR27II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR35II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR59II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. velezensis 18	rRNA 16S sequencing	*Hevea brasiliensis* stalk	AM/Brazil	-
B. wiedmannii 93	rRNA 16S sequencing	*Hevea brasiliensis* leaf	AM/Brazil	-
B. subtilis 140	rRNA 16S sequencing	*Hevea brasiliensis* leaf	AM/Brazil	-
B. tequilensis 174	rRNA 16S sequencing	*Hevea brasiliensis* root	AM/Brazil	-
B. cereus 201	rRNA 16S sequencing	*Hevea brasiliensis* root	AC/Brazil	-

Bacillus sp. 204	rRNA 16S sequencing	*Hevea brasiliensis* root	AC/Brazil	-
Bacillus sp. 210	rRNA 16S sequencing	*Hevea brasiliensis* root	AC/Brazil	-
B. thuringiensis 221	rRNA 16S sequencing	*Hevea brasiliensis* leaf	AM/Brazil	-

Supplementary Table 2. Target genes and corresponding primer sequences used in the RT-PCR assay

Gene	Direction	Sequence	Product length (bp)
agrA	F	5'- CGT GGC AGT AAT TCA GTG TAT G -3'	83
	R	5'- TGG GCA ATG AGT CTG TGA GA -3'	
agrC	F	5'- GAA ATA CCA GAT GAA GTA ACT CGC A -3'	127
	R	5'- ATG CAA CTC GAA TGA TAG GAT C -3'	
hla	F	5'- AGAGATTCTTGGAACCCGGTATATG -3'	146
	R	5'- ATAACTGTAGCGAAGTCTGGTGAA -3'	
hlb	F	5'- GGT TGT GGA TTC GAT AAT GAT AGC -3'	131
	R	5'- CGA TCA TGT CCA GCA CCA -3'	
hld	F	5'- GGA AGG AGT GAT TTC AAT GGC A -3'	80
	R	5'- TGT TCA CTG TGT CGA TAA TCC A -3'	
hlgC	F	5'- CCAATCAGCCCCATCACTCGGT -3'	130
	R	5'- CGCTTTGACGCCCCATAAAACACT -3'	

Supplementary Table 3. Best model result of the molecular docking between ADAM-10 and iturin variants performed by AutoDock Vina

PubChem CID	Binding Affinity (kcal/mol)	Num. of Hbonds	Residues
CID_9988651	-2,7	3	2x Glu665 Asn669
CID_11062109	-2,7	3	Ser663 Asn669 Glu 665
CID_101589794	-2,7	3	2x Glu665 Glu668
CID_101589795	-2,9	4	Ser663 Glu665 Glu668 Asn669
CID_102287549	-2,8	4	Ser663 2x Glu665 Asn669

Supplementary Table 4. Results of the molecular docking between hemolysin and iturins variants performed by AutoDock Vina

	Model 01			Model 02			Model 03			Model 04			Model 05		
PubChem CID	**Binding Affinity (kcal/mol)**	**Num. of Hbonds**	**Residues**	**Binding Affinity (kcal/mol)**	**Num. of Hbonds**	**Residues**	**Binding Affinity (kcal/mol)**	**Num. of Hbonds**	**Residues**	**Binding Affinity (kcal/mol)**	**Num. of Hbonds**	**Residues**	**Binding Affinity (kcal/mol)**	**Num. of Hbonds**	**Residues**
CID_9988651	-6,6	3	Gln177 Asp183 Ser 186	-6,6	3	Gln177 Asp183 Trp187	-6,4	4	Gln177 Asp183 Asn173 Trp187	-6,4	3	Gln177 His144 Lys198	-6,3	4	Thr155 Asp227 Ser225 Arg104
CID_11062109	-8,3	7	2x Arg200 Asp183 Trp 179 2x Trp187 Asn188	-7,4	2	Thr155 Asp227	-7,3	2	Asn188 Asp185	-7,3	2	Gln177 Asn173	-7,3	2	Trp187 Tyr118
CID_101589794	-7,1	5	Tyr 112 2x Arg200 2x Asn201	-6,9	4	2x Thr117 2x Ser141	-6,8	1	Thr155	-6,6	1	Thr155	-6,5	2	2x Arg104
CID_101589795	-7,7	3	Lys 215 Gln 177 Trp 179	-7,6	3	Asn 178 Arg 200 Tyr 182	-7,5	4	Trp 187 Ser 186 Gln 177 Trp 179	-7,5	2	Asp227 Thr155	-7,4	4	Trp 187 His 144 Gln 177 Asn 201
CID_102287549	-7,1	3	Asn 178 Trp 187 Asp 185	-6,6	3	Trp 187 Asn 178 Tyr 112	-6,6	3	Lys 198 Arg 200 His 144	-6,5	2	Trp 187 Pro 181	-6,4	5	Lys 215 Pro 181 Gly 180 Tyr 112 Hys 144

CHAPTER 3

Exopolysaccharides produced by *Bacillus* spp. inhibit biofilm formation of *S. aureus* from bovine mastitis

ABSTRACT

Staphylococcus aureus is one of the most common etiological agents associated with contagious mastitis in dairy cattle, a disease with strong economic impacts on the dairy sector worldwide. *S. aureus* isolated from bovine mastitis not only can harbor multiple genes that confer resistance to antibiotics but also can form biofilms, which makes the treatment of bovine mastitis even more challenging. Microbial biofilms can increase bacterial resistance to antimicrobials, leading to the persistence of bacteria in both biotic and abiotic environments. Natural compounds produced by *Bacillus* spp. have shown a wide range of biological effects, including anti-biofilm activity. Here we investigate anti-biofilm compounds produced by *Bacillus* spp. against *S. aureus* isolated from bovine mastitis focusing on compounds that do not affect bacterial growth in an attempt to prevent the development of resistance. The results showed that supernatants from three *Bacillus* strains were able to reduce *S. aureus* biofilm without affecting its growth. The anti-biofilm activity was associated with exopolysaccharides (EPS) secreted by *Bacillus* spp. The EPS decreased *S. aureus* biofilm formation in a dose-dependent manner, reaching the highest inhibition (83%) at 1 mg/mL. The EPS also had some biofilm disruption activity, probably explained by the increased expression of the genes *nuc* and *aur* in the cells treated with EPS. The characterization of the active compounds produced by *B. velezensis* 87 and *B. subtilis* TR47II showed the production of an EPS of 31.2 and 33.7 kDa, respectively, composed mainly of glucose and mannose and with similar functional groups as revealed by FT-IR. In conclusion, we prospected and characterized new EPS produced by *Bacillus* spp. and showed, for the first time, the potential applications of EPS as an anti-biofilm compound to treat infections of the bovine intramammary gland caused by *S. aureus*.

1 INTRODUCTION

Bovine mastitis is an inflammatory disease of the mammary gland caused by physical trauma or microorganisms (Cheng and Han 2020). The disease affects milk production and leads to premature culling or prolonged treatments, strongly affecting the economy of the dairy sector (Halasa *et al.* 2007; Aghamohammadi *et al.* 2018). Among several microorganisms that cause bovine mastitis, *S. aureus* is considered one of the most common etiological agents, usually associated with subclinical and chronic mastitis (Pereira *et al.* 2011). Its persistence in the bovine udder is attributed to the ability of *S. aureus* to survive within epithelial cells and to form a biofilm (Bardiau *et al.* 2014).

Biofilms are well-organized structures composed of cells embedded in an extracellular matrix adherent to a surface (Gomes *et al.* 2016b). In *S. aureus*, the extracellular matrix is composed mainly of the polysaccharide intercellular adhesin (PIA) and microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which are required in the adhesion step of biofilm formation (Campos *et al.* 2022). Studies have described the important role of biofilm in the persistence and resistance of *S. aureus* in the bovine mammary gland (Veh *et al.* 2015; Grunert *et al.* 2018). Veh et al. (2015) demonstrated that strains with phenotypes of strong biofilm formation are likely to persist during the dry period. Moreover, a 3-year longitudinal study from a single dairy herd revealed that strains producing more biofilm had higher prevalence and persistence within the host (Grunert *et al.* 2018).

Although antibiotics remain the main therapeutic option for treating bovine mastitis (Cheng and Han 2020), bacteria inside biofilms can be 10 to 1000 times more resistant to antibiotics than the same strain grown in a planktonic state (Melchior *et al.* 2006). Therefore, new therapeutic options are required to tackle *S. aureus* biofilms in the context of bovine mastitis. Anti-biofilm treatments have emerged as a possible adjuvant to antibiotic therapy with a focus on the inhibition of bacterial attachment or the stimulation of biofilm dispersal using, for example, surface-coating agents, matrix-degrading enzymes or compounds that interfere with networks of regulatory genes in bacteria (Bhattacharya *et al.* 2015). In this context, several compounds such as phytochemicals, antimicrobial peptides, nanoparticles, enzymes, bacteriophages,

antibodies, and a range of bacterial metabolites have shown anti-biofilm activity against *S. aureus* (Suresh *et al.* 2019; Mishra *et al.* 2020).

The use of bacterial metabolites as therapeutic agents has some industrial benefits such as simplicity of production, low costs, and potentially low environmental impact. In this context, strains of *Bacillus* are known for their industrial applications (Lyngwi *et al.* 2014) and their capacity to sporulate (heat stability). Additionally, some strains have probiotic features (safety) (Elshaghabee *et al.* 2017; Mingmongkolchai and Panbangred 2018), and secondary metabolites produced by *Bacillus* spp. have shown anti-biofilm activity against pathogens (Nithya *et al.* 2011; Kalpana *et al.* 2012; Spano *et al.* 2013; Ceresa *et al.* 2020).

Studies have revealed an anti-biofilm effect of EPS produced by *Bacillus* spp. against pathogens, including *S. aureus* (Sayem *et al.* 2011; Spano *et al.* 2016). Most EPS act by modifying the physical characteristics of abiotic surfaces or bacterial cells (Rendueles *et al.* 2013), modulating gene expression (Kim *et al.* 2009) or interfering with multivalent carbohydrate-protein interactions (Wittschier *et al.* 2007). Moreover, biosurfactants produced by *Bacillus* species have anti-biofilm activity (Giri *et al.* 2019; Englerova *et al.* 2021), and can reduce the expression of biofilm-related genes in *S. aureus* (Englerova *et al.* 2021). In addition, alpha-amylase produced by *Bacillus subtilis* (Kalpana *et al.* 2012) and 4-phenylbutanoic acid produced by *Bacillus pumilus* (Nithya *et al.* 2011) also showed anti-biofilm activity against *S. aureus*. However, studies are lacking regarding the potential of *Bacillus* metabolites to inhibit the biofilm formation of *S. aureus* in the context of bovine mastitis.

We hypothesized that compounds secreted by *Bacillus* spp. can prevent biofilm formation by *S. aureus* strains isolated from cows with mastitis. Thus, the objective of this work was to investigate the anti-biofilm potential of compounds secreted by *Bacillus* spp. against *S. aureus* isolated from mastitic dairy cows. We aimed to screen anti-biofilm compounds that do not affect bacterial growth since these compounds might impose a lower selective pressure towards resistance development in comparison to antibiotics (Sully *et al.* 2014; Quave *et al.* 2015; Vale *et al.* 2016b).

2 MATERIALS AND METHODS

2.1 Microorganisms and growth conditions

In this study, ninety *S. aureus* strains isolated from cows with mastitis were evaluated for their ability to form biofilm and subsequently used to screen for anti-biofilm compounds. The strains belong to the Mastitis Pathogens Culture Collection of Embrapa Dairy Cattle (Juiz de Fora, Minas Gerais State, Brazil). In addition, *S. aureus* O46, a strong biofilm producer isolated from a ewe with mild mastitis, was used in this study (Le Marechal *et al.* 2011). *S. aureus* was grown in Tryptic Soy Broth (TSB) medium and incubated for 18 h at 37 ºC under aerobic conditions.

Thirty-three isolates of *Bacillus* spp. obtained from soil, plants, water, and mastitic milk in different regions of Brazil (Supplementary Table 1) were screened for the production of anti-biofilm compounds against *S. aureus*. *Bacillus* spp. strains were routinely grown in Tryptic Soy Broth (TSB) for 24 h at 30 ºC under agitation (200 rpm).

2.2 Biofilm formation

The biofilm formation of *S. aureus* strains was analyzed using the crystal violet assay (Stepanovic *et al.* 2000). In general, bacteria were grown in 96-well plates containing 200 µL of BHI broth at 37 ºC for 24 h. Thereafter, the medium was removed and the wells were washed three times with sterile physiological saline solution (0.85% NaCl, w/v). In the last wash, the plates were shaken to remove non-adherent bacteria. The remaining bacteria were fixed in the plate using 200 µL of 99% methanol for 15 min. Next, the plates were emptied and left to dry in a safety cabinet. The adherent cells were stained using 200 µL of 2% crystal violet for 5 min. The reagent was discarded and the excess dye was washed under tap water. After the plate dried, the cells were resuspended with 200 µL of 33% glacial acetic acid and the OD_{570nm} was measured in a Multiskan GO microplate spectrophotometer (Thermo Scientific, Vantaa, Finland). *S. aureus* strains were classified as non-adherent, weakly adherent, moderately adherent, or strongly adherent according to the criteria proposed by Stepanovic et al. (2000). The experiment was performed with two biological and three technical replicates.

2.3. Anti-biofilm effect of *Bacillus* spp. supernatants against *S. aureus*

S. aureus strains classified as strong biofilm producers were selected to evaluate the capacity of *Bacillus* spp. in inhibiting this phenotype. For this assay, *S. aureus* was grown in TSB medium at 37 ºC for 24 h while *Bacillus* spp. strains were grown in TSB medium at 30 ºC for 24 h under 200 rpm of agitation. A volume of 150 µL of *S. aureus* inoculum adjusted to have approximately 10^6 CFU/mL was transferred to a 96-well plate and mixed with 50 µL of filtered supernatants of *Bacillus* spp. The supernatants were filtered using a polyvinylidene difluoride (PVDF) membrane of 0.22 µm. After 24 h incubation, the optical density was measured spectrophotometrically at 600 nm to determine if the supernatants inhibited bacterial growth. The crystal violet assay was then performed as described above to quantify biofilm formation. *S. aureus* cultures not treated with *Bacillus* supernatants were used as a positive control whereas TSB broth was used as the negative control (Isaac *et al.* 2017). The experiment was performed with three biological and two technical replicates. Shapiro-Wilk test was used to assess data normality and the Mann-Whitney test at 95% confidence was applied to make pairwise inferences. Statistical analyzes were performed using GraphPad Prism software version 8.4.3.

2.4 Effect of crude extracts containing lipopeptides and exopolysaccharides from *Bacillus* spp. in the biofilm formation of *S. aureus*

To analyze if lipopeptides or exopolysaccharides present in *Bacillus* supernatants were responsible for the anti-biofilm activity against *S. aureus*, both extracts were obtained and their activities were compared with the activities of the supernatants. The isolates *Bacillus velezensis* 18*, Bacillus altitudinis* 27 and *Bacillus velezensis* 87 were selected for this assay based on their capacities to inhibit the *S. aureus* biofilm without inhibiting bacterial growth. *B. subtilis* TR47II, a strain that significantly inhibited the growth of *S. aureus*, was also selected to evaluate if the inhibition was due to a concentration effect or to compounds distinct from those showing anti-biofilm properties. The *Bacillus* strains were grown with an initial OD_{600nm} of 0.1 in TSB medium at 30 ºC under 200 rpm for 24 h. After growth, the cultures were centrifuged (9,800 g, 20 min) and the supernatants were filtered and separated into three aliquots of identical volume. One aliquot was kept as the cell-free supernatant

and the other two were subjected to acid and ethanol precipitation as described below to obtain lipopeptides and exopolysaccharides, respectively.

For extraction of lipopeptides, the compounds were precipitated by adjusting the pH of the supernatants to 2.0 using 6 M HCl. The samples were incubated overnight at 4 ºC. The acid precipitate was recovered by centrifugation (5,000 g, 20 min, 4 ºC) and resuspended to the same initial volume with distilled water at pH 7.0 (Sharma *et al.* 2015). Exopolysaccharides (EPS) were obtained as described by Spano et al. (2013), with modifications. Two volumes of cold absolute ethanol were added to the filtered supernatants and the samples were incubated overnight at 4 ºC. Subsequently, the aliquots were centrifuged at 5,000 g for 30 min, and the precipitate was washed two times with cold absolute ethanol and resuspended in the same initial volume using hot water (80-90 ºC) (Spano *et al.* 2013). Fractions from all treatments were separated into two aliquots and half of them were autoclaved (121 ºC/15 min) to evaluate if this method of sterilization would affect activity as filtration of the fractions using a polyvinylidene difluoride (PVDF) membrane of 0.22 µm was difficult to achieve.

S. aureus 3865, *S. aureus* 3906, and *S. aureus* 3917 were selected for this assay due to their decreased formation of biofilm when treated with the *Bacillus* supernatants. *S. aureus* O46, a strain isolated from sheep with mastitis and considered a strong biofilm producer, was also added to this analysis. The experiment was performed in 96-well plates using 150 µL of *S. aureus* with OD_{600nm} adjusted to 0.05 in TSB supplemented with 2% of glucose and 50 µL of the following treatments: 1) *Bacillus* supernatants, 2) *Bacillus* extracts containing lipopeptides, and 3) *Bacillus* extracts containing exopolysaccharides, filter-sterilized or autoclaved.

2.5 Concentration effect of crude extracts containing exopolysaccharides in the biofilm formation of *S. aureus*

To evaluate the production of exopolysaccharides (EPS) over time, *Bacillus* strains were grown in TSB media with an initial OD_{600nm} of 0.1 for up to 120 h at 30 ºC with agitation (200 rpm). Samples were collected at 0, 3, 6, 12, 24, 48, 72, 96, and 120 hours of incubation to measure the OD_{600nm} and the production of EPS. EPS yield was evaluated by using the phenol-sulfuric method to measure the concentration of carbohydrates, the major component of EPS. The EPS was precipitated using ethanol

as described above and the carbohydrate quantification was performed using the phenol-sulfuric assay (Masuko *et al.* 2005). Glucose was used as the standard. The incubation time that resulted in higher production of EPS was used in the subsequent analyzes.

The concentration effect of EPS extracts on *S. aureus* biofilm formation was evaluated by growing *B. velezensis* 18, *B. altitudinis* 27, *B. velezensis* 87, and *B. subtilis* TR47II in TSB media with an initial OD_{600} at 0.1 for 24 h at 30 ºC under agitation (200 rpm). The EPS was precipitated from the supernatants as previously described. The precipitates were lyophilized and a stock solution of 5 mg/mL was prepared and heat-sterilized (121 ºC/15 min). *S. aureus* strains 3865, 3906, 3917, and O46 were inoculated with an initial OD_{600nm} of 0.5 in TSB medium supplemented with glucose 2% and containing increasing concentrations of the EPS extracts (1.96 µg/mL to 1000 µg/mL). The cultures were incubated for 24 h at 37 ºC and the biofilm formation was evaluated using the crystal violet assay. The experiment was performed using two technical and two biological replicates.

2.6 Effect of *Bacillus* spp. EPS in the expression of genes involved in biofilm production/dispersion in *S. aureus*

S. aureus strain O46 was used in this analysis due to its strong biofilm formation phenotype and availability of its genome sequence as a reference to evaluate the effect of EPS from *Bacillus* spp. in the expression of biofilm-related genes (Supplementary Table 2). *S. aureus* O46 was grown in TSB broth containing the crude extract of exopolysaccharides from *B. velezensis* 87 and *B. subtilis* TR47II at 1000 µg/mL, the concentration that caused the greatest decrease of biofilm production by *S. aureus*. The culture was grown for 24 h at 37 ºC and the whole culture (mixture of planktonic and sessile cells) was used for RNA extraction.

Trizol Reagent® (Sigma-Aldrich, San Luis, EUA) was used to extract the RNA of *S. aureus* treated and non-treated with EPS. The extraction was performed according to the manufacturer's instructions, except that the cell lysis step was optimized by adding 0.5 g of 0.1 mm zirconia beads into the tube, which was subjected to mechanical disruption for 1 min using a bead beater (Biospec Products, Bartlesville, USA). The extracted RNA was treated with DNase (Promega, Madison, USA) and then converted to its complementary DNA using the High-Capacity cDNA Reverse

Transcription Kit (Thermo Fisher Scientific, Waltham, USA). All reactions were performed as described by the manufacturers.

For each one of the genes selected for this analysis, primer pairs were designed using the Primer3Plus tool (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser *et al.* 2007). The quality of the sequences obtained was analyzed using OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer?returnurl=%2Fcalc%2Fanalyzer). Primer sequences as well as the expected product length are listed in Supplementary Table 2.

The RT-qPCR reactions were performed using 25 ng/µL of the cDNA and the SYBR Green qPCR Master Mix (2×) (BioRad, California, EUA), according to the manufacturer's instructions. The amplification reaction was the following: initial denaturation at 95 ºC for 10 min followed by 40 cycles of denaturation at 95 ºC for 30 s and annealing and extension at 60 ºC for 1 min. The melting curve was obtained using the following reaction: denaturation step at 95 ºC for 30 s, initial hold at 60 ºC/1 min, followed by an increase in the temperature to 95 ºC at a ramp rate of 1 ºC/min. All reactions were performed with three biological and two technical replicates. The RT-qPCR analysis was carried out using the StepOne Real-Time PCR System (Applied Biosystems, Massachusetts, EUA).

For each primer, the average Ct and $\log_{10}$ of cDNA concentration (control) were used to plot the efficiency curve. The relative gene expression was then calculated using the equation of the line obtained and the 16S rRNA as a reference gene. Gene expression comparisons were done using the Mann-Whitney test at a 95% of level confidence. All statistical analyzes were done in GraphPad Prism software version 8.4.3.

2.7 Effect of exopolysaccharides on biofilm disruption

To verify if the crude extracts containing exopolysaccharides could disrupt biofilms, *S. aureus* O46 at an initial OD_{600nm} of 0.05 was grown in a 96-well plate containing TSB + glucose 2%. The plate was incubated for 24 h at 37 ºC. After biofilm formation, the planktonic cells were removed, the biofilm was washed with sterile saline solution, and the exopolysaccharide extracts from *B. velezensis* 87 and *B. subtilis*

TR47II were added to the wells at a final concentration of 1000 µg/mL (prepared in TSB medium). The plate was incubated for another 24 h and the biofilm was subsequently revealed using the crystal violet method. Non-treated *S. aureus* cultures were used as the positive control. Statistical analysis was done in GraphPad Prism software version 8.4.3. A non-parametric t-test was applied and differences between treatments and control were considered significant at $\alpha = 0.05$.

2.8 Scanning electron microscopy (SEM) analysis

Stainless-steel coupons were added to a 12-well plate where *S. aureus* O46 was grown in TSB medium + 2% glucose at an initial OD_{600} of 0.05 in the presence of crude EPS extracts from *Bacillus velezensis* 87 and *Bacillus subtilis* TR47II at 1000 µg/mL (final concentration). *S. aureus* O46 grown in the absence of the crude EPS extracts was used as the positive control. The coupons were removed from the culture after 3, 6, 12, and 24 h of incubation at 37 ºC and washed with sterile PBS to remove non-adherent cells. The biofilm was fixed in the coupons using glutaraldehyde 2.5% for 1 h and dehydrated using an increasing concentration of ethanol (50, 70, 80, 90 and 100%) per 15 min (Song *et al.* 2019). The stainless-steel coupons were then attached to SEM stubs using carbon tape and sputter-coated with gold at 80 mA for 1 min using an Emitech K575X sputter coater (Quorum Technologies, Lewes, UK). Gold-coated samples were analyzed using a Gemini field emission scanning electron microscope (ZEISS, Jena, Germany) at an accelerating voltage of 2 kV and a working distance of 5.2–7.1 mm. The images were acquired using the secondary electron detector of the instrument. To ensure that the images were representative at least three different areas of each sample were analyzed.

2.9 Characterization of the exopolysaccharides

To avoid the interference of proteins in the exopolysaccharides analyzes, *Bacillus* spp. supernatants were subjected to acid precipitation prior to the ethanolic precipitation. The culture was grown for 24 h at 30 ºC and then centrifuged at 10,000 g for 15 min. Thereafter, 5% trichloroacetic acid (TCA) was added to *Bacillus* spp. supernatants and the mixtures were kept at 4 ºC for 2 h (Cao *et al.* 2020b). The

precipitate was removed by centrifugation (15,000 g/40 min) and two volumes of cold absolute ethanol were added to the protein-free supernatants to precipitate the EPS. After overnight incubation at 4 ºC the samples were centrifuged at 15,000 g/40 min to recover the EPS and the pellet was resuspended in hot water and freeze-dried. The carbohydrate, protein, and nucleic acid contents of exopolysaccharides extracts from *Bacillus* spp. were evaluated using the phenol-sulfuric assay (Masuko *et al.* 2005), the Bradford assay (Bradford 1976) and the measurement of OD at 260 nm (Sayem *et al.* 2011), respectively.

2.9.1 Monosaccharide composition of the exopolysaccharides

The monosaccharide composition of the exopolysaccharides from *Bacillus* spp. was evaluated after acid hydrolysis. Briefly, 1 mL of 2 M trifluoroacetic acid (TFA) was added to 1–2 mg of the samples and kept at 120 ºC for 3 h. Samples were dried with a stream of nitrogen at 40 ºC for complete evaporation of the TFA and re-suspended in deionized water. The monosaccharides were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an ICS-6000 (Dionex, Califórnia, EUA) equipped with a CarboPac™ PA1 column (Dionex, Califórnia, EUA). Standards containing mixtures of fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid, and glucuronic acid at known concentrations were used for calibration. All experiments were carried out in triplicate.

2.9.2 Estimation of exopolysaccharides molecular weight

The molecular weight of the exopolysaccharides in the extracts obtained from *Bacillus* spp. was estimated by size-exclusion chromatography using high-performance liquid chromatography (HPLC-SEC) following a method adapted from Gómez-Mascaraque *et al.* (2019). The samples and standards were dissolved at 1 mg/mL in the mobile phase, which was composed of a 10 mM Na_2HPO_4-NaH_2PO_4 solution containing 150 mM NaCl (pH 7.4). Samples and standards were filtered through 0.45 µm pore syringe filters (Sartorius, Gottingen, Germany) and 20 µL of each sample/standard solution was separated using an HPLC system and a flow rate of 0.5

mL/min (L *et al.* 2019). The HPLC system consisted of a Waters 2695 separations module and a Waters 2414 refractive index detector (Waters, Massachusetts, USA). An OHpak SB-806 HQ column (Shodex, Tóquio, Japan) equilibrated at 40 ºC was used for separation. Calibration was performed using pullulan standards (P-82 kit, Shodex, Tóquio, Japan).

2.9.3 Analysis of exopolysaccharides functional groups

The functional groups of EPS from *Bacillus velezensis* 87 and *Bacillus subtilis* TR47II were analyzed by Fourier-transform infrared spectroscopy (FTIR) using a Brucker invenio S instrument with a Pike Miracle ATR cell MD440 (6 mm aperture). Freeze-dried EPS samples were analyzed from 4000 cm^{-1} to 400 cm^{-1}. The analysis was carried out in triplicate and the results were averaged to obtain the final spectra. Water and atmosphere compensation followed by normalization from 1200 - 800 cm^{-1} was performed on the raw spectra.

3 RESULTS

3.1 Biofilm formation by *S. aureus* strains isolated from bovine mastitis

Among the 90 *S. aureus* isolates evaluated, seven (isolates 3865, 3870, 3988, 4070, 3906, 3992, and 3917) were classified as strong biofilm producers (Figure 1). The average OD_{570nm} of these cultures varied from 0.53 to 0.87 (negative control = 0.07). Moreover, 12 isolates were classified as moderate biofilm producers, with OD_{570nm} varying between 0.21 and 0.34. The majority of the isolates (n = 58) were classified as weak biofilm producers ($0.11 < OD_{570nm} < 0.20$) and 13 isolates were classified as non-adherent (Figure 1).

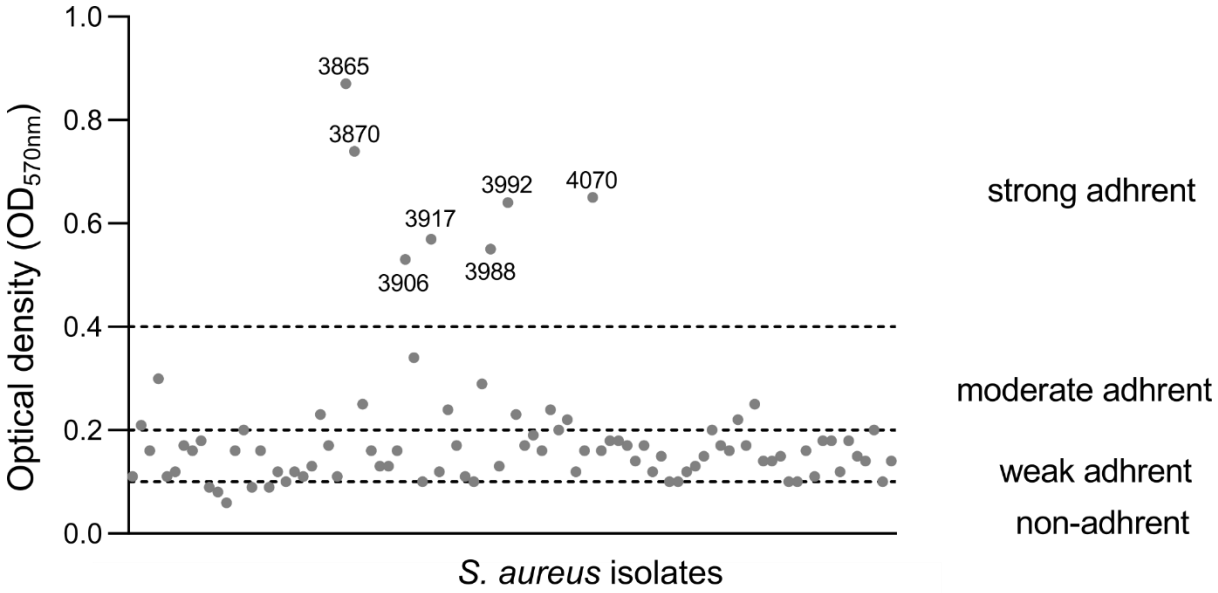


Figure 1. Biofilm production by a panel of *S. aureus* strains isolated from bovine mastitis. The optical density (OD_{570nm}) of each culture (filled dots) measured after the crystal violet assay is represented. The bacteria were classified as non-adherent, weak, moderate, and strong adherent according to Stepanovic et al. (2000).

3.2 Attenuation of biofilm formation by *S. aureus* using *Bacillus* spp. cell-free supernatants

Five strains of *S. aureus* (3865, 3870, 3988, 3906, and 3917) that were classified as strong biofilm producers were selected for this experiment. The cell-free supernatants obtained from the 33 strains of *Bacillus* spp. evaluated in this study showed distinct effects on biofilm production by *S. aureus*. Some cell-free supernatants inhibited *S. aureus* growth, making it difficult to assess whether these cultures can directly inhibit the formation of biofilm. Moreover, the activity of *Bacillus* supernatants also differed among the *S. aureus* strains evaluated (Figure 2). For example, all supernatants significantly inhibited the growth of *S. aureus* 3870. Although some *Bacillus* supernatants stimulated the biofilm formation of *S. aureus* strains, this was the case for only 3 *Bacillus* strains that significantly increased the biofilm formation of *S. aureus* 3988 and *S. aureus* 3906. Importantly, the cell-free supernatants of *Bacillus tequilensis* 174 and *Bacillus subtilis* 111A strongly stimulated (up to 519 %) the biofilm formation of *S. aureus* 3988 and *S. aureus* 3906, and were excluded from the study. Nonetheless, several *Bacillus* spp. supernatants decreased biofilm formation of *S.*

aureus without affecting bacterial growth. This was especially evident for *S. aureus* 3865 and *S. aureus* 3917, where fourteen and ten supernatants significantly decreased the biofilm formation of these strains, respectively. Indeed, compounds secreted by *Bacillus velezensis* 18, *Bacillus altitudinis* 27, and *Bacillus velezensis* 87 significantly decreased the biofilm formation of the majority of the *S. aureus* strains by approximately 40% without affecting bacterial growth (Figure 2).

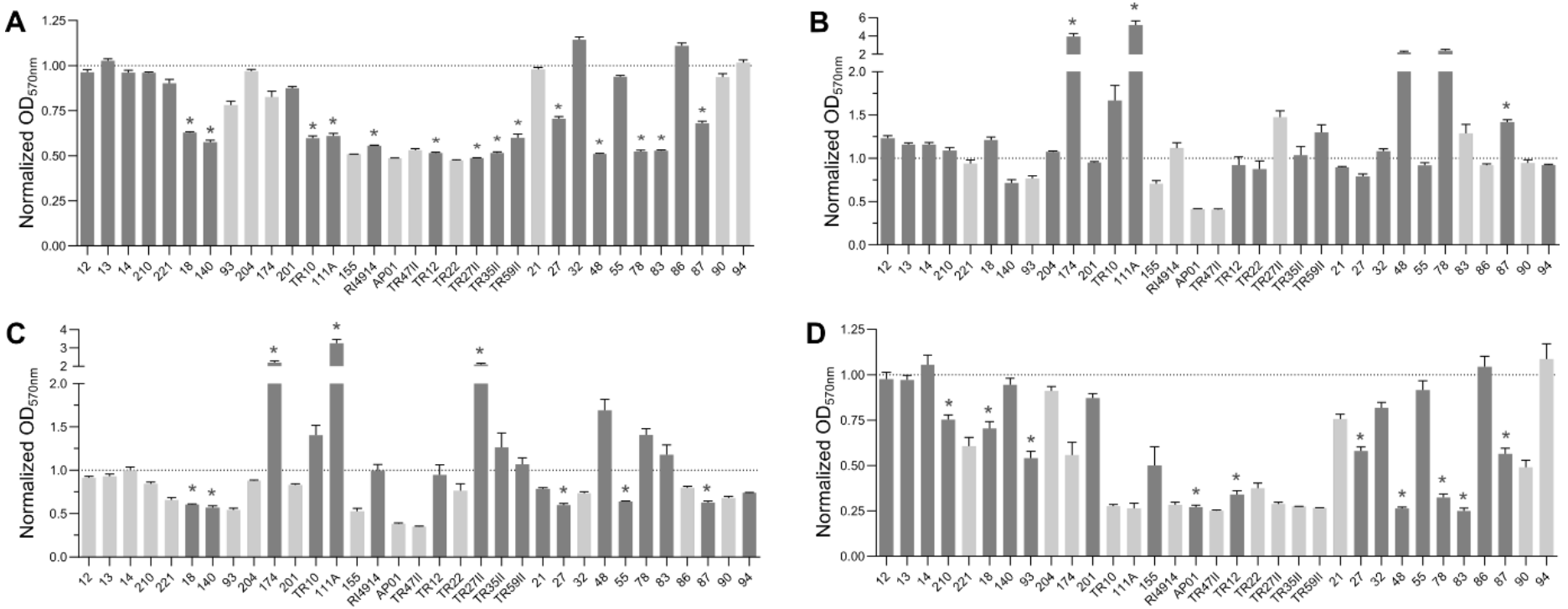


Figure 2. Effect of *Bacillus* cell-free supernatants on *S. aureus* biofilm formation. Five strains of *S. aureus* were analyzed: 3865 (A), 3988 (B), 3906 (C), 3917 (D). The strain 3870 is not shown because all *Bacillus* supernatants significantly reduced its growth. Light gray bars represent the formation of *S. aureus* biofilms by strains in which *Bacillus* spp. supernatants also showed a significant effect on bacterial growth. Dark grey bars represent *S. aureus* biofilm formation of strains in which growth was not affected by *Bacillus* supernatants. Asterisks show a significant difference ($p < 0.05$) in biofilm formation between treatment and control (set to 1) for dark gray bars, assessed by the Mann-Whitney test.

3.3 Effect of lipopeptides and exopolysaccharides from *Bacillus* spp. in biofilm formation by *S. aureus*

We compared the effect of *Bacillus* cell-free supernatants and the lipopeptides and exopolysaccharides obtained from these supernatants to obtain insights about the compounds responsible for the anti-biofilm activity. We also evaluated if the sterilization method (filtration and autoclaving) affected the activity of the tested compounds and supernatants. The effect of the different treatments varied according to the *Bacillus* and the *S. aureus* strains analyzed (Supplementary Figure 1). Overall, exopolysaccharides from all *Bacillus* spp., either filtered or autoclaved, were effective in preventing biofilm formation by *S. aureus* (Figure 3).

Filtered lipopeptides from *B. velezensis* 18, *B. altitudinis* 27, and *B. subtilis* TR47II also showed anti-biofilm activity against *S. aureus* strains. However, after autoclaving, lipopeptides from *B. subtilis* TR47II lost their anti-biofilm activity. Supernatants from all *Bacillus* spp. analyzed also lost their ability to prevent biofilm formation by *S. aureus* after autoclaving (Figure 3). Nonetheless, it is important to highlight that filter-sterilized cell-free supernatants of some *Bacillus* strains showed the highest biofilm inhibition observed in this study, and for the majority of the *S. aureus* strains analyzed. For example, supernatants from *Bacillus subtilis* TR47II reduced biofilm formation of *S. aureus* 3865 and *S. aureus* 3917 by 99.99% and 99.98%, respectively (Supplementary Figure 1).

Filtered exopolysaccharides from *Bacillus subtilis* TR47II showed the highest anti-biofilm activity among the extracts, reducing biofilm formation of *S. aureus* by approximately 65%. Moreover, the autoclaved exopolysaccharide extract from *Bacillus subtilis* TR47II maintained up to 90% of its activity when compared to the filtered extracts. Exopolysaccharide extracts from other *Bacillus* strains inhibited the formation of biofilm by *S. aureus* by approximately 40%, but this activity decreased to approximately 22% when the extracts were autoclaved. The activity of filtered EPS did not differ significantly from the activity of filtered supernatants, according to Tukey multiple comparisons test.

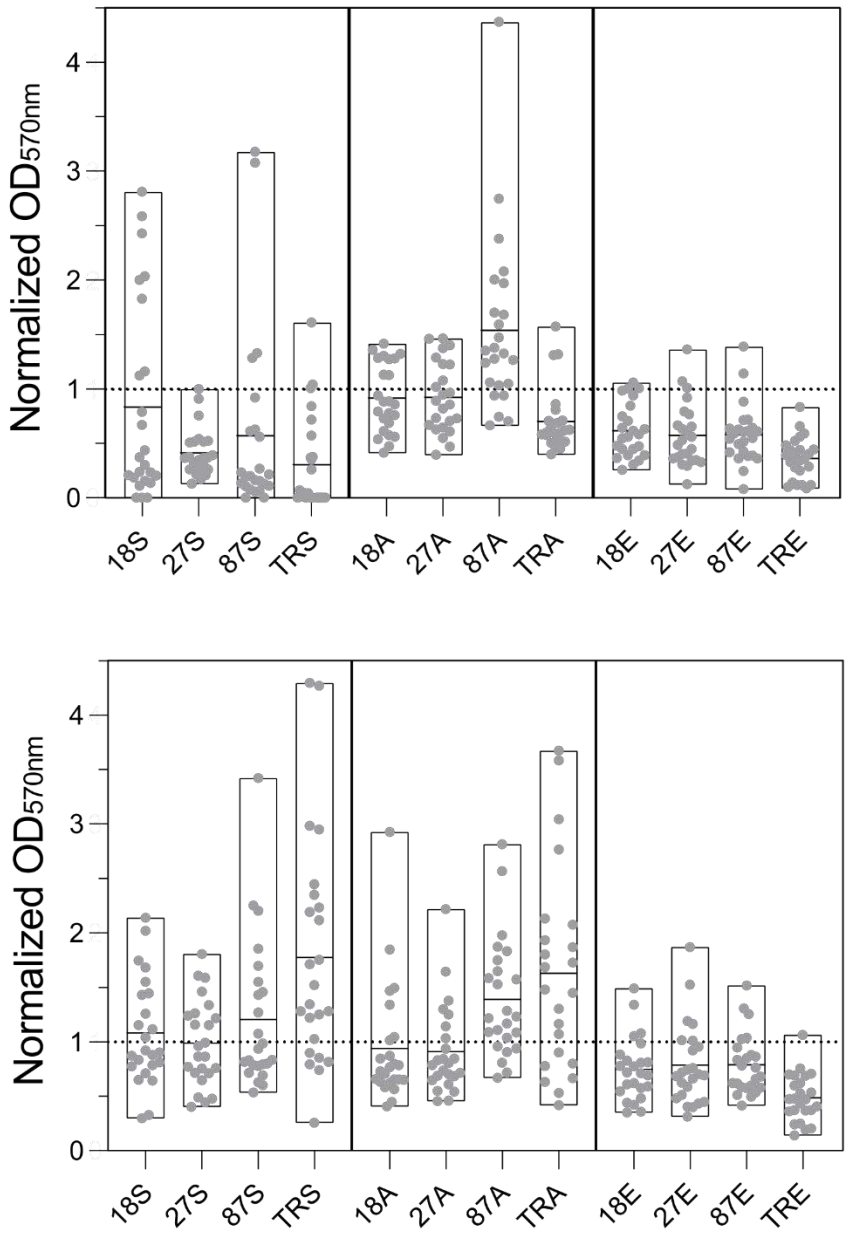


Figure 3. Effect of *Bacillus* cell-free supernatants (S), lipopeptides (L), and exopolysaccharides (E) from *Bacillus* spp. on biofilm formation by *S. aureus*. Panel A illustrates the activity of filtered extracts while panel B shows the activity of autoclaved extracts. The floating bars show the minimum, maximum, and mean OD_{570nm} considering all the replicates of the four *S. aureus* strains analyzed (*S. aureus* 3865, *S. aureus* 3906, *S. aureus* 3017, and *S. aureus* O46) represented in gray dots. The optical density of the control (non-treated) was set to 1 as indicated by the dotted line and all the data were normalized by the control for comparison purposes.

3.4 Concentration effect of exopolysaccharides (EPS) produced by *Bacillus* spp. on *S. aureus* biofilm

Cultivation of *Bacillus* yielded a higher production of EPS during the stationary phase. In general, all *Bacillus* strains evaluated in the current study showed higher EPS production after 24 h or 48 h of incubation (Supplementary Figure 2). In turn, after 72 h the amounts of EPS produced by *Bacillus* spp. tended to decrease for all strains tested.

Thereafter, the activity of exopolysaccharides in the biofilm formation of *S. aureus* was evaluated in different concentrations, using autoclaved extracts since obtaining EPS by filtration was difficult to achieve. In general, the anti-biofilm effect was more pronounced at higher concentrations of EPS (Figure 4). At lower concentrations, most EPS extracts stimulated the formation of bacterial biofilms. The EPS extracted from *B. subtillis* TR47II at 1,000 µg/mL reduced the formation of biofilm by 56% in *S. aureus* 3865 (Figure 4A). *S. aureus* 3906 also had its capacity to form biofilm reduced by 56% when treated with the highest concentration of the EPS produced by *B. velezensis* 87 (Figure 4B). *S. aureus* 3917 was less susceptible to the EPS treatment with the culture being able to maintain 72% of its original biofilm even at the highest concentration of EPS produced by several *Bacillus* strains (Figure 4C). *S. aureus* O46, the strongest biofilm producer among the tested strain, was the most susceptible to the anti-biofilm activity of EPS extracts from *Bacillus* spp. EPS produced by *Bacillus subtilis* TR47II reduced the biofilm formation of *S. aureus* O46 by 18% when the EPS concentration was 125 µg/mL and the inhibition of biofilm production increased to 83% at an EPS concentration of 1,000 µg/mL (Figure 4D). In conclusion, EPS obtained from *B. subtilis* TR47II demonstrates the strongest anti-biofilm activity against *S. aureus* strains, especially within the concentration range of 500 and 1,000 µg/mL.

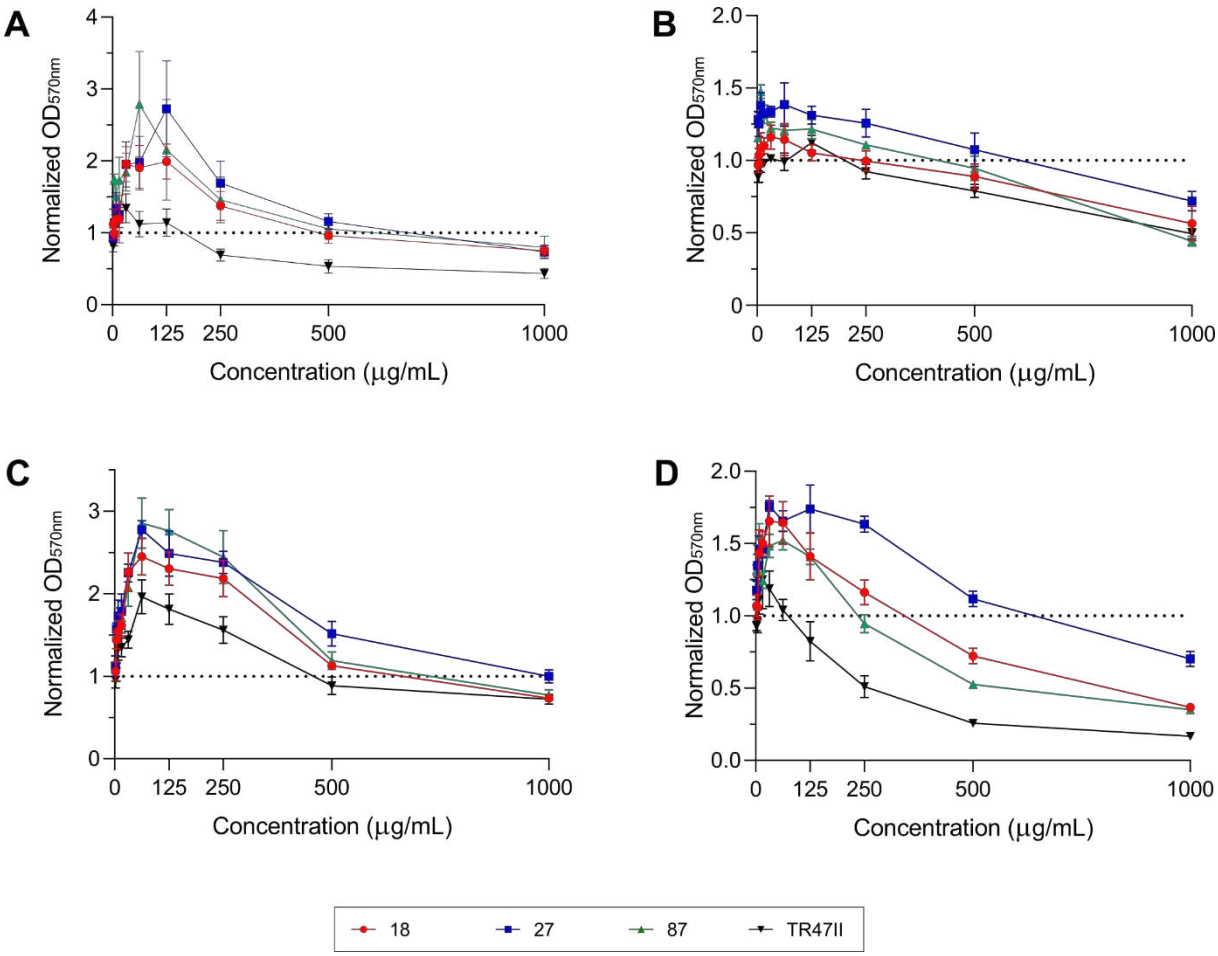


Figure 4. Concentration effect of crude extract containing EPS from *Bacillus* in the biofilm formation of *S. aureus* 3865 (A), *S. aureus* 3906 (B), *S. aureus* 3917 (C) and *S. aureus* O46 (D). The biofilm formation of the controls was normalized to 1.0. Each color represents one strain of *Bacillus* as shown in the figure legend. Bars indicate the SEM (standard error of the mean) of two technical and two biological replicates.

3.5 Effect of exopolysaccharides from *Bacillus* on *S. aureus* biofilm analyzed by Scanning Electron Microscopy

To phenotypically characterize the effect of EPS on *S. aureus* biofilm, we performed an SEM analysis. The results revealed, in accordance with the previous analyzes, a reduction in biofilm formation when *S. aureus* was grown in the presence of EPS from *Bacillus* spp. (Figure 5). The effect was consistent across time points and was detected as early as 3 h of bacterial growth, indicating that the initial attachment of bacteria to the solid surface might be affected. On stainless steel coupons, the EPS from *Bacillus velezensis* 87 was more efficient than EPS from *Bacillus subtilis* TR47II

in preventing the biofilm formation of *S. aureus*. The results also revealed that the structure of *S. aureus* cells is preserved in the treatments, suggesting absence of antimicrobial activity.

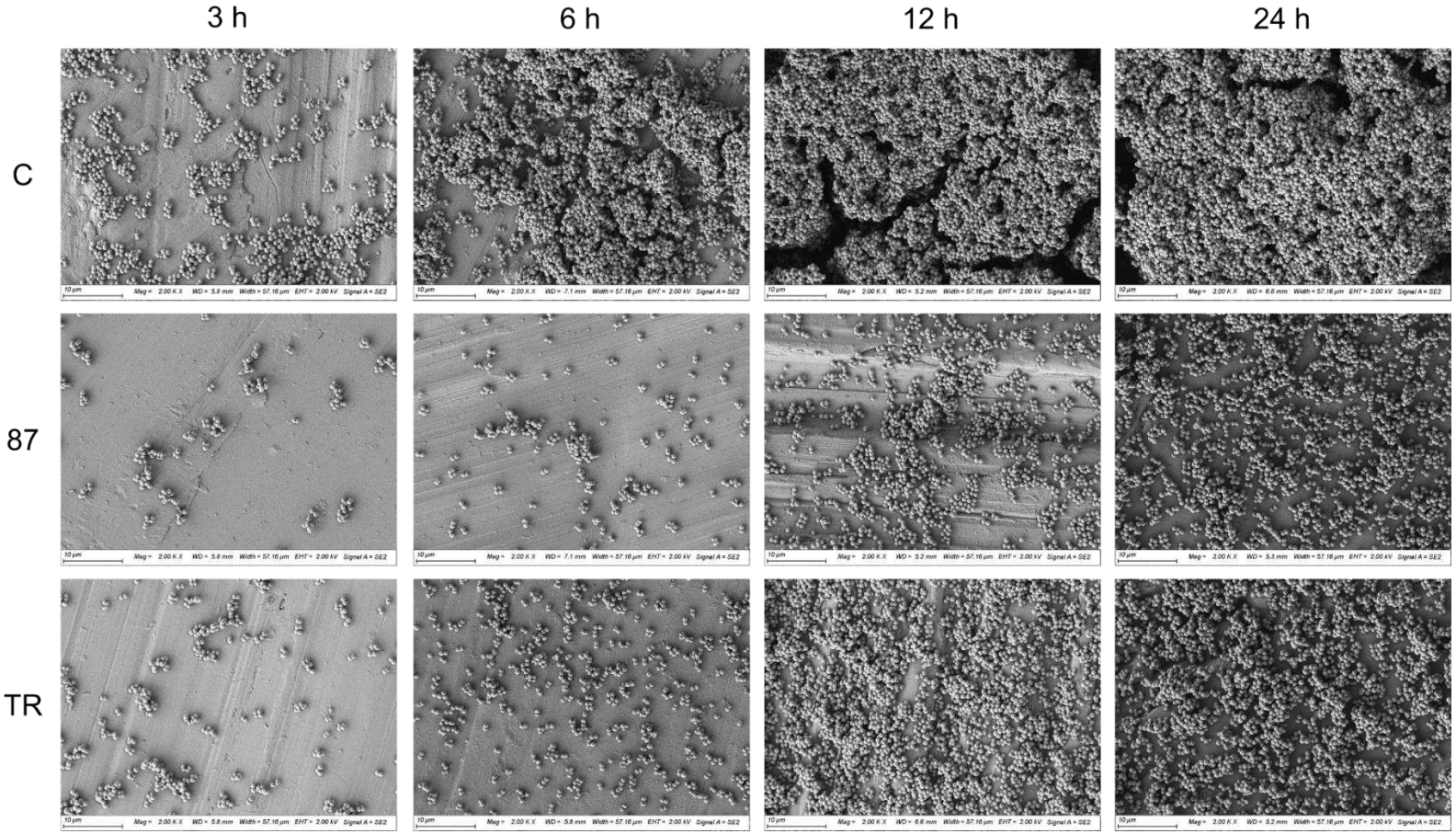


Figure 5. Biofilm formation of *S. aureus* on stainless steel coupons analyzed by Scanning Electron Microscopy. The effect of exopolysaccharides from *Bacillus velezensis* 87 and *Bacillus subtilis* TR47II on *S. aureus* biofilm was evaluated in four-time points (3 h, 6 h, 12 h, and 24 h). The control (C) represents the biofilm formation of *S. aureus* over time in the absence of treatments.

3.6 Effect of exopolysaccharides from *Bacillus* spp. in the expression of genes involved in biofilm formation/disruption in *S. aureus*

To investigate if the anti-biofilm effect of exopolysaccharides was due to the interference in the transcription of biofilm-related genes, an RT-qPCR analysis was performed. In general, genes that promote biofilm formation were up-regulated in the treatment, however, a similar tendency was observed in genes involved in biofilm degradation. The expression of *icaA* and *icaC* genes, for example, which encode intercellular adhesion proteins, increased at a significant level following treatment with exopolysaccharide extracts from *B. velezensis* 87 and *B. subtilis* TR47II. Other genes encoding proteins important to cell adhesion such as elastin binding protein (*ebps*) and fibronectin binding protein A (*fnbA*) did not present statistical differences between the control and the treatments. The quorum sensing gene *agrC* as well as the gene encoding laminin binding protein (*eno*) increased expression at a 95% confidence level after the treatment with EPS from *B. subtilis* TR47II. Genes encoding proteins important for biofilm degradation (*nuc* and *aur*) were more expressed in the treatments, except for the *nuc* gene in the treatment with EPS from *B. velezensis* 87. The expression of the aureolysin gene (*aur*) was significantly higher in the treatment with exopolysaccharides extract from *B. subtilis* TR47II. In turn, the *psm* gene responsible for the synthesis of phenol-soluble modulins, which has a role in biofilm dispersion, did not differ in expression ($p < 0.05$) between the control and the treatments (Figure 6).

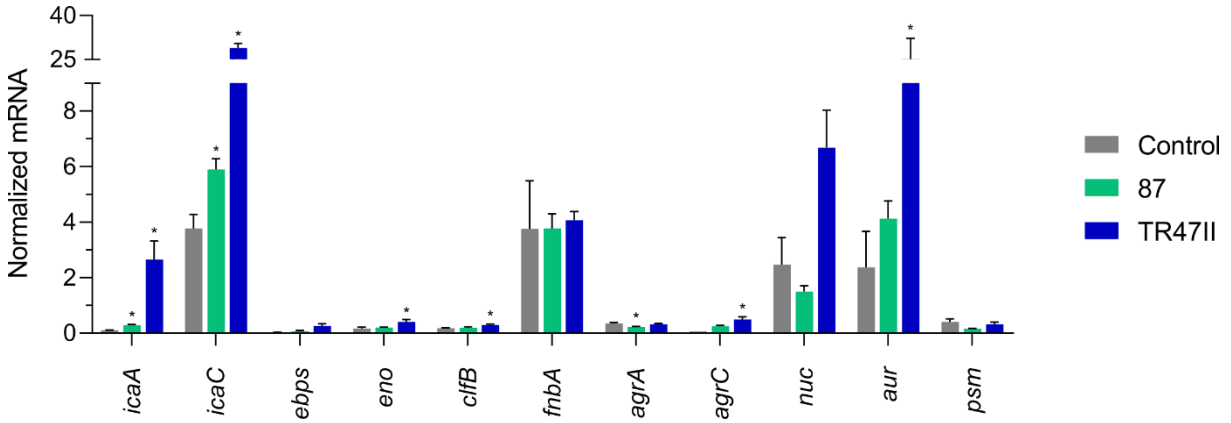


Figure 6. Effect of exopolysaccharides extracts from *Bacillus* spp. in the expression of genes involved in the biofilm phenotype of *S. aureus*. *B. velezensis* and *B. subtilis* strains are represented in green and blue, respectively, and the control is shown in gray. Error bars indicate the standard error of the mean between the biological replicates. Asterisks

represent a significant difference assessed by the Mann-Whitney test between the treatments and the control at a 95% confidence level.

Since RT-qPCR analysis showed increased expression of genes involved in biofilm disruption when *S. aureus* was treated with EPS, we phenotypically assessed the effect of EPS in biofilm removal using the crystal violet assay. The experiment revealed that after biofilm formation, exopolysaccharides from *B. velezensis* 87 and *B. subtilis* TR47II at 1000 µg/mL were capable of reducing the biofilm biomass of *S. aureus* O46 (Figure 7). The treatment with EPS from *B. subtilis* TR47II decreased the biofilm of *S. aureus* O46 by 36.4% resulting in a significant difference at a 95% confidence level in relation to the control (Figure 7).

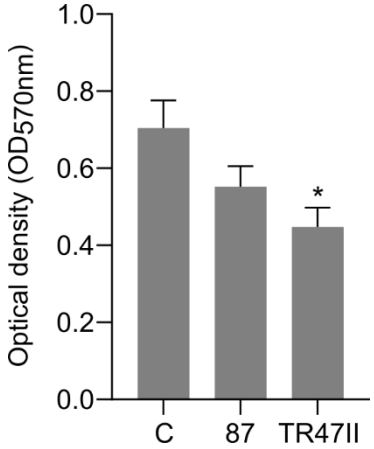


Figure 7. Measurement of biofilm disruption after the treatment of *S. aureus* biofilm with exopolysaccharides extracts from *Bacillus* spp. The biofilm was pre-formed for 24 h before the assay. C = control; 87 = *B. velezensis* 87; TR47II = *B. subtilis* TR47II. Error bars indicate the standard error of the mean between the replicates. Asterisks denote a significant difference between the treatments and the control evaluated using the Mann-Whitney test at a 95% confidence level.

3.7 Characterization of the active exopolysaccharides

To characterize the EPS with anti-biofilm activity against *S. aureus*, we performed analyzes to determine the composition of the compounds, their molecular weight, and functional groups. The carbohydrate, protein, and nucleotide contents were measured in the EPS samples, revealing approximately 48 to 63% of carbohydrates,

4% of proteins, and 0.1% of nucleotides (Table 1). The composition of both exopolysaccharides was similar, although a smaller amount of carbohydrate was detected in the EPS from *B. subtilis* TR47II, according to Dubois's method.

Table 1. Composition of exopolysaccharides extracts from *Bacillus* spp.

Samples	**Carbohydrate (%)**	**Protein (%)**	**Nucleotide (%)**
B. velezensis 87	63 ± 3.8	3.92 ± 0.18	0.17 ± 0.15
B. subtilis TR47II	48 ± 8.2	3.59 ± 0.12	0.13 ± 0.09

The sugar composition of the exopolysaccharides revealed a glucose-mannose based structure for both *Bacillus* species. Glucosamine, galactose, arabinose, fucose, and xylose were also detected in smaller amounts (Table 2). Rhamnose, arabinose, fructose, galactosamine, galacturonic acid, and glucuronic acid were not detected in the samples or were only present in trace amounts. The sum of all sugar units detected indicates a concentration of approximately 50% of carbohydrates in the EPS samples.

Table 2. Carbohydrate composition of EPS produced by *B. velezensis* 87 and *B. subtilis* TR47II

Sugar units	**87** (ug/mg)	**TR** (ug/mg)
Fucose	<2	<2
Arabinose	6 ± 0	6 ± 0
Glucosamine	15 ± 1	18 ± 1
Galactose	12 ± 1	11 ± 2
Glucose	202 ± 3	235 ± 2
Mannose	228 ± 2	255 ± 7
Xylose	<2	<2
Total	465 ± 7	529 ± 12

The values report means (n = 3) ± standard deviation.

The molecular weight of the exopolysaccharides, estimated by HPLC-SEC, revealed structures of 31.2 ± 0.1 kDa and 33.7 ± 0.02 kDa for *Bacillus velezensis* 87

and *Bacillus subtilis* TR47II, respectively, according to pullulan standards (Supplementary Figure 3). The FT-IR analyzes revealed that the two EPS samples also have similar spectra (Figure 8), with typical absorption bands of polysaccharides. The broad intense band at 3242 cm^{-1} corresponds to the stretching vibration characteristic of O-H bounds (Cao *et al.* 2020a). The weak absorption band around 2925 cm^{-1} is a characteristic peak of C-H deformation vibration (Rani *et al.* 2017). The peak at 1670 cm^{-1} can be associated with the elastic C=O vibration (Rani *et al.* 2017). The peaks at 1540-1536 cm^{-1} indicate N-H deformation and vibration of the NH_2 group (Cao *et al.* 2020a). The 1042 cm^{-1} peak corresponds to C-O-C and other C-O groups, indicating the presence of a pyranose ring (Vinothkanna *et al.* 2021). Moreover, absorption at 983 cm^{-1} indicates glycosyl residue, mainly pyranose form (Vinothkanna *et al.* 2021), 884 cm^{-1} indicates beta-type glycosidic linkage (Sun *et al.* 2014), 837 cm^{-1} corresponds to mannose units (Xu *et al.* 2019) and absorption at 618 cm^{-1} indicates that alkynes are present (Vinothkanna *et al.* 2021).

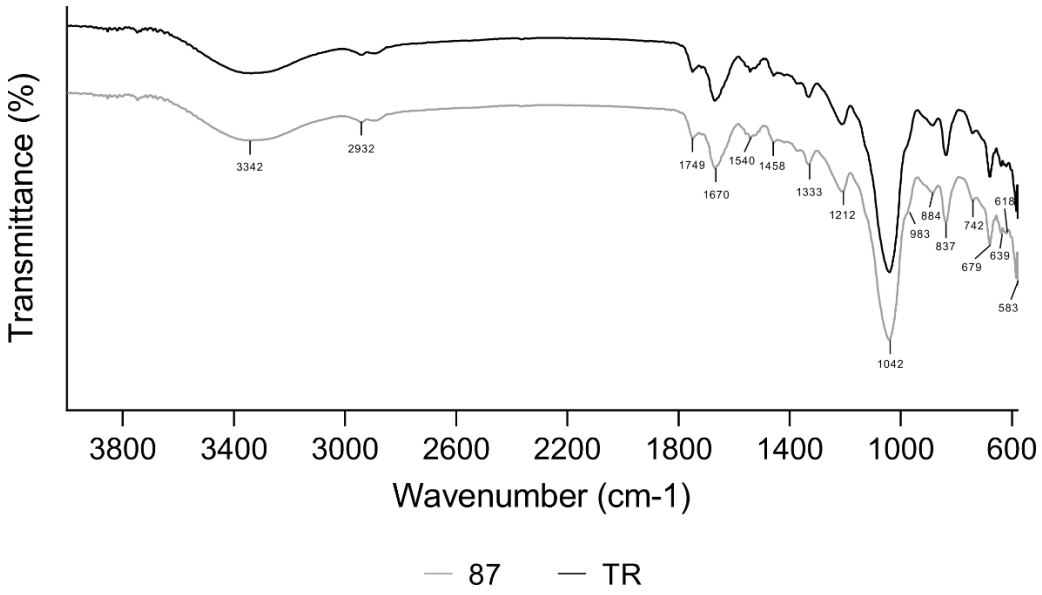


Figure 8. FT-IR spectra of exopolysaccharides from *Bacillus velezensis* 87 and *Bacillus subtilis* TR47II were obtained using Brucker invenio S instrument with a Pike Miracle ATR cell MD440.

4 DISCUSSION

S. aureus is one of the most prevalent and widespread bacterial pathogens, causing numerous skin and soft tissue infections as well as invasive diseases (Cheung

et al. 2021). Its pathogenicity in addition to the increasing resistance of this bacterium to currently available drugs led to the deaths of more than 100,000 people in 2019, attributed to infections caused by methicillin-resistant *S. aureus* (Antimicrobial Resistance 2022). The formation of biofilms by *S. aureus* also affects the effectiveness of antimicrobials (Otto 2018), leading to bacterial persistence and disease recurrence (Melchior *et al.* 2006).

The ability to form biofilms is a common feature among *S. aureus* isolated from bovine mastitis (Notcovich *et al.* 2018; Zaatout *et al.* 2020a). Most of the strains causing subclinical bovine mastitis in east coast Malaysia (60%) (Saeed *et al.* 2022) and northern Kazakhstan (48%) (Rychshanova *et al.* 2022) formed mostly moderate biofilms. On the other hand, the majority of *S. aureus* isolated from subclinical bovine mastitis in southern Xinjiang (61.5%) were classified as weak biofilm producers (Ren *et al.* 2020), in agreement with the current study. Although the amount of biofilm produced varies among *S. aureus* strains, the majority are capable of forming at least weak biofilms, suggesting the importance of this virulence factor for the pathogenicity of *S. aureus* in bovine mastitis.

In this study, we screened anti-biofilm compounds in *Bacillus* spp. supernatants and identified several isolates capable of decreasing the biofilm formation of *S. aureus*. However, several supernatants also showed antimicrobial activity. These *Bacillus* strains were excluded from the analysis for two reasons: 1) the effect on the biofilm could be a consequence of bacterial growth inhibition and, 2) we sought to find compounds with minimal or no antimicrobial activity to prevent selective pressure against *S. aureus*. The antimicrobial activity observed in *Bacillus* strains against *S. aureus* may be due to the production of bacteriocins or lipopeptides (Sharma *et al.* 2006; Barboza-Corona *et al.* 2009; Saggese *et al.* 2018; Rasiya and Sebastian 2021). We were able to identify three *Bacillus* strains that significantly decreased the biofilm formation of *S. aureus* without affecting its growth.

According to previous reports, *Bacillus* spp. are able to produce different bioactive compounds, mainly lipopeptides and exopolysaccharides, capable of inhibiting biofilm formation (Sayem *et al.* 2011; Spano *et al.* 2013; Giri *et al.* 2019; Englerova *et al.* 2021). Therefore, we focused on the extraction of these compounds from *Bacillus* supernatants and compared their anti-biofilm activity with the supernatants alone in an attempt to determine which compound was responsible for decreasing biofilm

production by *S. aureus*. Our results revealed that lipopeptides produced by some *Bacillus* strains were able to decrease the biofilm formation of certain *S. aureus* strains (Supplementary Figure 1). However, exopolysaccharides had a broader and more effective anti-biofilm activity and were selected for further studies. For some combinations of *S. aureus* - *Bacillus* strains, the anti-biofilm effect was greater when the supernatant was used instead of the crude extract containing purified lipopeptides or exopolysaccharides. This could be due to a possible combinatorial effect of these and other compounds.

In this study, the effect of EPS on biofilm formation of *S. aureus* was dose-dependent, with maximum activity at the highest concentration tested (1,000 µg/mL). The biofilm inhibition reached 83% at this concentration for EPS produced by *B. subtilis* TR47II against *S. aureus* O46, a strong biofilm producer. Exopolysaccharides produced by *Bacillus licheniformis* T14, a marine thermophilic bacterium, also showed the best anti-biofilm activity at the highest concentration tested (400 µg/mL), decreasing the biofilm formation of *S. aureus* 210 in 60% (Spano *et al.* 2016). Likewise, EPS produced by *Lactobacillus plantarum* YW32 isolated from Kefir grains in China, was most active at 5,000 µg/mL against *S. aureus* AC1, inhibiting 45.13% of the biofilm formation (Wang *et al.* 2015a). These observations indicate that a high concentration of EPS is needed to achieve a significant anti-biofilm effect and that EPS produced by the *Bacillus* strains investigated in the current study are highly effective in reducing the formation of biofilm by *S. aureus*.

To investigate the mechanism by which EPS decreases the formation of biofilm in *S. aureus*, the effect of the compounds on the expression of biofilm-related genes was evaluated. Although we found overexpression of genes encoding PIA and some adhesins in at least one of the treatments, the results also showed a trend towards an increased expression of genes encoding the degradative enzymes nuclease (*nuc*) and aureolysin (*aur*), for at least one of the treatments. Moreover, the EPS from *B. subtilis* showed a significant effect on *S. aureus* biofilm disruption. The enzymes encoded by the *nuc* and *aur* genes have been associated with biofilm dispersal and could partially explain the effects observed for the *Bacillus* supernatants against *S. aureus* (Kiedrowski *et al.* 2011; Lyngwi *et al.* 2014). The dispersal of biofilms by exopolysaccharides has been previously described. For example, a purified polysaccharide from *Vibrio* sp. dispersed *Pseudomonas aeruginosa* but not *S. aureus* biofilms (Jiang *et al.* 2011).

Additionally, an exopolysaccharide produced by *Kingella kingae* was able to disperse pre-formed *S. aureus* biofilms (Bendaoud *et al.* 2011).

However, these results alone do not explain the much greater anti-biofilm effect observed when *S. aureus* strains were grown in the presence of *Bacillus* EPS. As EPS was added to the 96 well-plates prior to the addition of *S. aureus* cultures in our experiments, the mechanism of action may involve chemical modifications of the abiotic surface. Previous experiments have directly demonstrated that bacterial polysaccharides with antibiofilm activity have biosurfactant activity (Kanmani *et al.* 2011; Rendueles *et al.* 2013) and can alter the properties of abiotic surfaces, including charge inversion when added to a cationic surface and increased hydrophobicity (Valle *et al.* 2006). Accordingly, when abiotic surfaces were pre-treated with EPS, the biofilm formation of several pathogens decreased. This was the case, for example, of microtiter plate wells pre-coated with culture supernatants containing EPS from *B. licheniformis* SP1 that inhibited biofilm formation of *E. coli* (Sayem *et al.* 2011). Similarly, glass surfaces pre-treated with EPS from *E. coli* inhibited the biofilm formation of *S. aureus* and other pathogens (Valle *et al.* 2006; Rendueles *et al.* 2011).

Lastly, EPS can also inhibit biofilm formation by altering the surfaces of bacterial cells. For example, cell-free supernatants of *B. licheniformis* SP1 decreased the cell surface hydrophobicity of *E. coli* and *P. fluorescens* (Sayem *et al.* 2011). Furthermore, cell aggregation of a range of Gram-positive and Gram-negative bacteria, a process that is mediated by cell-surface adhesins, was inhibited by a polysaccharide from *E. coli* K2 (Valle *et al.* 2006). Likewise, intercellular adhesion of *E. coli* cells was inhibited by EPS from *Lactobacillus acidophilus* (Kim *et al.* 2009), *Streptococcus phocae* PI80 (Kanmani *et al.* 2011), and *Vibrio* sp. A101 (Jiang *et al.* 2011). Therefore, multiple mechanisms may be involved in the anti-biofilm activity of the EPS characterized in this study, and further experiments are needed to obtain further insights into the mechanism of action of these molecules.

The characterization of the EPS structure showed that the anti-biofilm compounds produced by the *Bacillus* spp. used in this study have a molecular weight of approximately 30 kDa. This represents a small EPS compared with similar compounds with anti-biofilm activity (Valle *et al.* 2006; Jiang *et al.* 2011; Rendueles *et al.* 2011; Sayem *et al.* 2011; Spano *et al.* 2013). However, it should be noted that we used pullulan as the standard in the HPLC-SEC analysis whereas most previous studies used

dextrans as the standard for chromatographic analyzes. Moreover, the EPS analyzed in the current study were mainly composed of glucose and mannose, which is a unique feature among the anti-biofilm EPS previously described for *Bacillus* strains. The anti-biofilm EPS produced by *B. licheniformis* T14 showing anti-biofilm activity against *S. aureus* and other pathogens had a molecular weight of 1,000 kDa and is composed mainly of fructose/fucose/glucose (Spano *et al.* 2013). Another study revealed a simple EPS structure of 1,800 kDa containing monomeric units of a-D-galactopyranosyl-glycerol-phosphate, which showed anti-biofilm activity against several pathogenic and non-pathogenic strains, including *S. aureus* (Sayem *et al.* 2011). Finally, the FT-IR analyzes demonstrated the presence of specific functional groups in the EPS purified from *Bacillus* supernatants, such as pyranose ring, beta-type glycosidic linkage, and alkynes.

In summary, this study reveals new EPS produced by *B. subtilis* and *B. velezensis* with similar structures, which have anti-biofilm activity against *S. aureus* isolated from bovine mastitis. These findings expand the possibility of using these naturally produced and abundant compounds as adjuvant therapy to control prevalent infections of the bovine mammary gland caused by *S. aureus*. However, future experiments are warranted to evaluate the safety of using EPS as an intramammary infusion and its efficacy *in vivo* to prevent or treat bovine mastitis caused by *S. aureus*.

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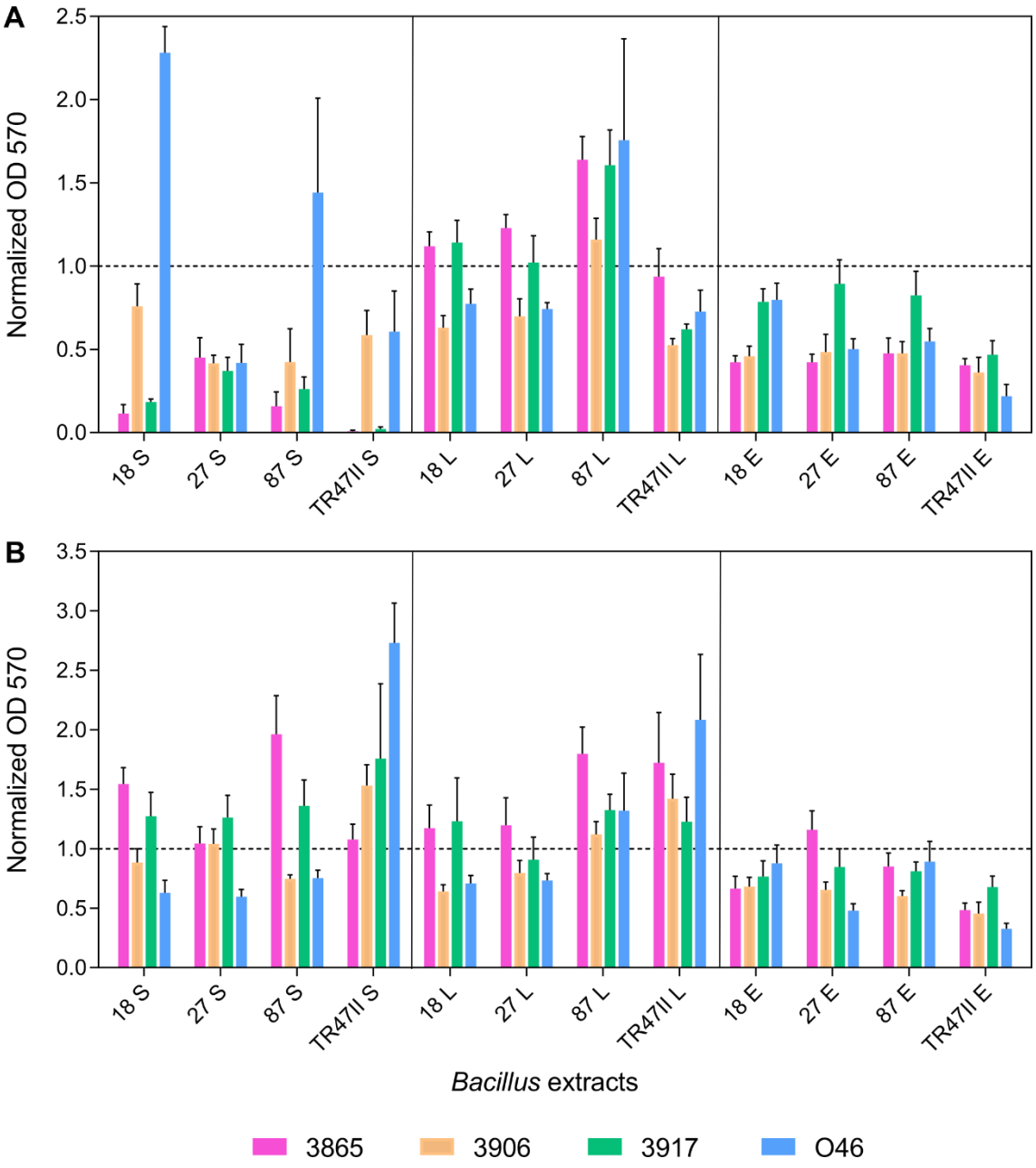
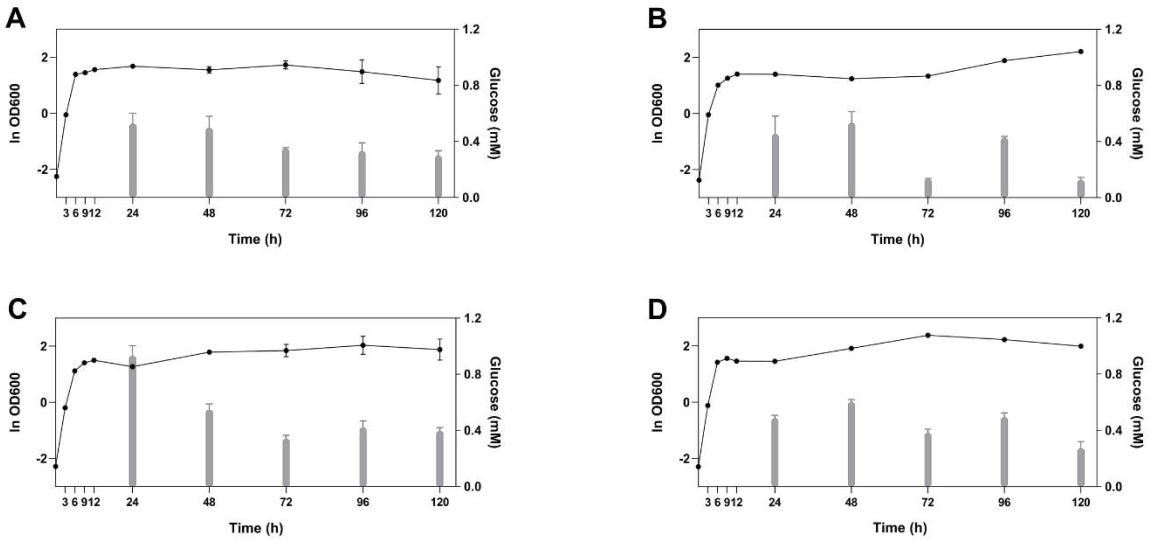
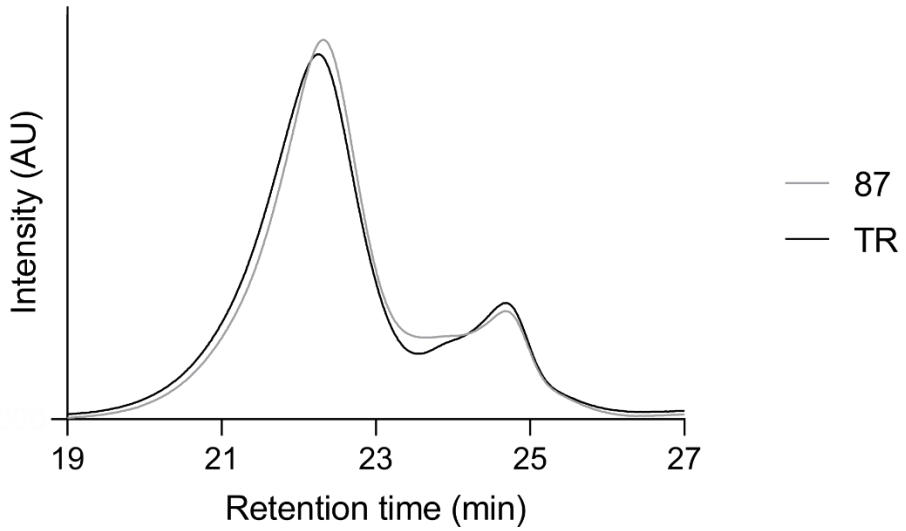
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Effect of *Bacillus* cell-free supernatants (S), lipopeptides (L), and exopolysaccharides (E) in the biofilm formation of *S. aureus*. Bars with different colors represent *S. aureus* strains as shown in the legend. Panels A and B show the activity of filtered and autoclaved extracts, respectively. The biofilm was measured at OD 570 using the crystal violet assay. For comparison, the biofilm OD of the controls (*S. aureus* only) was normalized to 1.0. Bars represent the standard error of the mean.



Supplementary Figure 2. Bacterial growth and exopolysaccharide (EPS) production by *Bacillus velezensis* 18 (A), *Bacillus altitudinis* 27 (B), *Bacillus velezensis* 87 (C) and *Bacillus subtilis* TR47II (D). The amount of EPS estimated as glucose equivalents by the phenol-sulfuric assay is shown in gray bars. Bacterial growth (OD 600nm) is represented by a line connecting filled circles. The experiment was performed with three biological replicates and the error bars represent the standard error of the mean.



Supplementary Figure 3. HPLC-SEC chromatograms of EPS purified from *Bacillus velezensis* 87 and *Bacillus subtilis* TR47II.

SUPPLEMENTARY TABLES

Supplementary Table 1. *Bacillus* strains used in this study

Bacteria	Method of identification	Source	State/Country	Reference (DOI)
B. velezensis 48	rRNA 16S and rpoB sequencing	Potato field	MG/Brazil	-
B. velezensis 78	rRNA 16S and rpoB sequencing	Mango roots	MG/Brazil	-
B. velezensis 83	rRNA 16S and rpoB sequencing	Mango roots	MG/Brazil	-
B. velezensis 87	rRNA 16S and rpoB sequencing	Washing of coffee planting soil	MG/Brazil	-
B. cereus/thuringiensis 32	rRNA 16S and rpoB sequencing	Forest of the coffee nursery	MG/Brazil	-
B. cereus/thuringiensis 55	rRNA 16S and rpoB sequencing	Potato field	MG/Brazil	-
B. cereus/thuringiensis 90	rRNA 16S and rpoB sequencing	Washing of coffee planting soil	MG/Brazil	-
B. cereus/thuringiensis 94	rRNA 16S and rpoB sequencing	Leaf litter of the coffee nursery	MG/Brazil	-
B. toyonensis 21	rRNA 16S and rpoB sequencing	Forest of the coffee nursery	MG/Brazil	-
B. toyonensis 86	rRNA 16S and rpoB sequencing	Washing of coffee planting soil	MG/Brazil	-
B. altitudinis 27	rRNA 16S and rpoB sequencing	Forest of the coffee nursery	MG/Brazil	-
Bacillus cereus 12	Fatty acid methyl esters (FAME) analysis	Bovine mastitis	MT/Brazil	-

Bacillus cereus 13	Fatty acid methyl esters (FAME) analysis	Bovine mastitis	MT/Brazil	-
Bacillus cereus/thurigiensis 14	Fatty acid methyl esters (FAME) analysis	Bovine mastitis	MT/Brazil	-
B. subtilis LBBMA RI4914	Fatty acid methyl esters (FAME) analysis	Production water of oil exploration field	ES/Brazil	10.1016/j.fuel.2016.04.080
B. subtilis LBBMA 111A	Fatty acid methyl esters (FAME) analysis	Mangrove region contaminated with oil	RJ/Brazil	-
B. subtilis LBBMA AP01	Fatty acid methyl esters (FAME) analysis	Contamination of an agar plate used for isolation of phytopathogenic fungi	MG/Brazil	-
B. subtilis TR47II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis LBBMA 155	Fatty acid methyl esters (FAME) analysis	Mangrove region contaminated with oil	RJ/Brazil	-
B. subtilis TR10	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR12	rRNA 16S sequencing and fatty acid methyl	Soil	ES/Brazil	10.1007/s00792-015-0740-7

	esters (FAME) analysis			
B. subtilis TR22	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR27II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR35II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. subtilis TR59II	rRNA 16S sequencing and fatty acid methyl esters (FAME) analysis	Soil	ES/Brazil	10.1007/s00792-015-0740-7
B. velezensis 18	rRNA 16S sequencing	*Hevea brasiliensis* stalk	AM/Brazil	-
B. wiedmannii 93	rRNA 16S sequencing	*Hevea brasiliensis* leaf	AM/Brazil	-
B. subtilis 140	rRNA 16S sequencing	*Hevea brasiliensis* leaf	AM/Brazil	-
B. tequilensis 174	rRNA 16S sequencing	*Hevea brasiliensis* root	AM/Brazil	-
B. cereus 201	rRNA 16S sequencing	*Hevea brasiliensis* root	AC/Brazil	-

Bacillus sp. 204	rRNA 16S sequencing	*Hevea brasiliensis* root	AC/Brazil	-
Bacillus sp. 210	rRNA 16S sequencing	*Hevea brasiliensis* root	AC/Brazil	-
B. thuringiensis 221	rRNA 16S sequencing	*Hevea brasiliensis* leaf	AM/Brazil	-

Supplementary Table 2. Primers used in the RT-qPCR assay to evaluate the expression of biofilm-related genes

Gene	Direction	Sequence	Product length (bp)
icaA	F	5'- AAG TGC AGT TGT CGA TGT TG -3'	116
	R	5'- ACA TGG CAA GCG GTT CAT AC -3'	
icaC	F	5'- ATG GAG ACT ATT GGA ACG TTA CC -3'	91
	R	5'- TGC GTG CAA ATA CCC AAG AT -3'	
clfB	F	5'- CGG AAG TGC TGA TGG TGA TTC -3'	101
	R	5'- GGA TCT GGT TCT GGG CTT G -3'	
fnbA	F	5'- GTT TCA GAA GTT AAA GGC ACA GAT G -3'	109
	R	5'- CGA CAC GTT GAC CAG CAT G -3'	
ebps	F	5'- AGA GAA TAC GGA GCA ACA GTT TC -3'	139
	R	5'- TGT GCC AGC CTC ATT TTG AAC -3'	
eno	F	5'- AGA ATC ATT ACG TTG GGG TAC TG -3'	95
	R	5'- ACC ACC TTC GTC ACC TAC TG -3'	
nuc	F	5'- CAC AAA CAG ATA ACG GCG TAA ATA G -3'	124
	R	5'- ACC GTA TCA CCA TCA ATC GCT -3'	
aur	F	5'- ACA CAA GAG ACT GCG AAC TTA G -3'	136
	R	5'- CCT CTT TTC CAG GTG TGT AGA C -3'	
psm	F	5'- ATA ATG ACG GCG CAA AAT TAG G -3'	69
	R	5'- TAC CTA GTA AAC CTA CGC CAT TTT C -3'	

FINAL CONSIDERATIONS

Overall, the current study revealed the potential of compounds produced by *Bacillus* spp. in decreasing the virulence of *S. aureus* without affecting its growth. Our results showed that exopolysaccharides produced by *B. subtilis* and *B. velezensis* were able to inhibit biofilm formation by *S. aureus* and that lipopeptides produced by these strains were capable of decreasing the hemolytic activity of this important dairy cattle pathogen. This is the first study to characterize an anti-biofilm effect of exopolysaccharides against bovine mastitis pathogens and also the first study to reveal an anti-hemolytic effect of lipopeptides. We also characterized the active compounds and provided information about their mechanisms of action. The capacity of different compounds present in the supernatant of *Bacillus* strains to inhibit biofilm formation and hemolysin production by *S. aureus* isolated from bovine mastitis demonstrates the potential use of bioactive molecules produced by *Bacillus* spp. in the prevention/treatment of bovine mastitis. New studies are needed to assess *in vivo* the safety and efficacy of the selected compounds to control bovine mastitis with a view to future applications.

APPENDIX A

Other academic-scientific activities during the PhD (2019-2023)

Peer-reviewed papers published

1. **Sabino, Y.N.V.**, Cotter, P.D., Mantovani, H.C. Anti-virulence compounds against *Staphylococcus aureus* associated with bovine mastitis: a new therapeutic option? Submitted to: *Microbiological Research* **271**, 127345 (2023). https://doi.org/10.1016/j.micres.2023.127345

2. do V. Barroso, M., da Silva, J.S., Moreira, S.M., **Sabino, Y.N.S.**, Rocha, G.C., Moreira, M.A., Bazzolli, D.M.S., Mantovani, H.C. Selection of multidrug-resistant enterobacteria in weaned pigs and its association with in-feed subtherapeutic combination of colistin and tylosin. *Curr Microbiol* **79**, 349 (2022). https://doi.org/10.1007/s00284-022-03053-7

3. Oliveira, L.B.A., **Sabino, Y.N.V.**, Barroso, M. do V., Ferreira, R.K., Lima, J.F., Arcuri, P.B., Carneiro, J. da C., Mendonça, R.J. de, Ribeiro, J.B., Ferreira-Machado, A.B. and Paiva, A.D. 2021. Inhibition of *Listeria monocytogenes* by bacteriocin-producing *Bacillus velezensis* isolated from silage. *Res, Soc Dev* **10**, e2610917783 (2021). https://doi.org/10.33448/rsd-v10i9.17783

4. **Sabino Y.N.V**., de Araújo K.C, de Assis F.G.D.V, Moreira S.M, Lopes T.D.S, Mendes T.A.O., Huws S.A, Mantovani H.C. *In silico* screening unveil the great potential of ruminal bacteria synthesizing lasso peptides. *Front Microbiol* **11**, 576738 (2020). https://doi.org/10.3389/fmicb.2020.576738

5. **Sabino, Y.N.V.**, Santana, M.F., Oyama, L.B. *et al.* Characterization of antibiotic resistance genes in the species of the rumen microbiota. *Nat Commun* **10**, 5252 (2019). https://doi.org/10.1038/s41467-019-13118-0

6. **Sabino, Y.N.V.**, Fochat, R.C., Lima, J.C.F. *et al.* Antibacterial activity and lantibiotic post-translational modification genes in *Streptococcus* spp. isolated from ruminal fluid. *Ann Microbiol* **69**, 131–138 (2019). https://doi.org/10.1007/s13213-018-1407-2

Manuscript in preparation

1. **Sabino, Y.N.V.**, Melo, M.D., Silva, G.C., Mantovani, H.C. Antimicrobial resistance in plasmids of Enterobacteriaceae in the One Health Context.

Abstracts and oral presentations in scientific events

1. **Sabino, Y. N. V**. From harm to benefits - from resistance to therapy: the potential of the bacterial world. Speaker at the Congresso online de Microbiologia (COMICBIO), 2021.

2. Araujo, K.C.; **Sabino, Y. N. V.**; Oliveira, L. L.; Silva, J.S.; Santos, M. H.; Mantovani, H. C. Combinatorial therapy based on antimicrobial peptides to inhibit bovine mastitis pathogen In: V International Symposium of Microbiology and Biotechnology, 2021, Viçosa.

3. **Sabino, Y. N. V.**; Araujo, K.C.; Totola, M. R.; Queiroz, M. V.; Abreu, L. M.; Mendes, T.O.A.; Mantovani, H. C. Exopolysaccharides produced by *Bacillus* spp. inhibit the biofilm formation of *S. aureus* isolated from bovine mastitis In: V International Symposium of Microbiology and Biotechnology, 2021, Viçosa.

4. **Sabino, Y. N. V.**; Santos, F. G.; Moreira, A. J. S.; Oyama, L.; Huws, S.A.; Santana, M. F.; Mantovani, H. C. Antibiotic resistance genes in species of ruminal microbes In: 30º Brazilian Congress of Microbiology, 2019, Maceió.

5. Silva, J.S.; Barroso, M.V.; Magalhães, S.M; Moreira, A. J. S.; **Sabino, Y. N. V.**; Rocha, G.C.; Moreira, M.A.S.; Mantovani, H. C. Antibiotic resistance profile of *Enterobacteriaceae* isolates from swine feces after weaning In: International Symposium of Veterinary Science, 2019, Viçosa.

6. Teixeira, R.C.; **Sabino, Y. N. V.**; Araujo, K.C.; Barroso, M.V.; Silva, J.S.; Mantovani, H. C. Antimicrobial activity of Gram-negative bacteria isolated from swine feces In: International Symposium of Veterinary Science, 2019, Viçosa.

7. Melo, M.D.; **Sabino, Y.N.V.**; Mantovani, H.C. Conservation analysis of tetracycline resistance genes among bacteria from the gastrointestinal tract of ruminants and humans In: International Symposium of Veterinary Science, 2019, Viçosa.

8. Barroso, M.V.; Magalhães, S.M; Silva, J.S.; **Sabino, Y.N.V.**; Braz, L.C.; Rocha, G.C.; Bazzolli, D.M.S.; Moreira, M.A.S.; Mantovani, H.C. Genetic determinants for beta-lactams, tetracycline and colistin resistance in multidrug-resistant enterobacteria isolated from weaned piglets In: International Simposium of Veterinary Science, 2019, Viçosa.

9. **Sabino, Y.N.V.**; Santana, M.F.; Moreira, A.J.S.; Santos, F.G.; Oyama, L.B.; Huws, S.A.; MANTOVANI, H. C. Genomic, transcriptomic and in vitro analyses of acquired antibiotic resistance genes in the rumen microbiome In: 30º Brazilian Congress of Microbiology, 2019, Maceió.

10. **Sabino, Y.N.V.**; Santos, F.G.; Araujo, K.C.; Santana, M.F.; Mantovani, H.C. Prevalence and expression of antibiotic resistance genes in the gastrointestinal tract microbiome of ruminants and humans In: International Symposium of Veterinary Science, 2019, Viçosa.

Prizes and awards

1. Best Oral Presentation (Category: One Health) with the abstract entitled: Prevalence and expression of antibiotic resistance genes in the gastrointestinal tract microbiome of ruminants and humans, International Symposium of Veterinary Science, 2019.