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Interference of sodium alginate and 2,2′-bipyridyl on bacterial growth and biofilm produced by *Staphylococcus aureus* isolates from bovine mastitis

Interferência do alginato de sódio e do 2,2′-bipiridil no crescimento bacteriano e no biofilme produzido por isolados de *Staphylococcus aureus* provenientes de mastite bovina

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Highlights

Sodium alginate and 2,2′-bipyridyl show antibacterial activity against *S. aureus.*
Sodium alginate and 2,2′-bipyridyl combat consolidated *S. aureus* biofilm.
Sodium alginate and 2,2′-bipyridyl interfere with the growth of *S. aureus.*

Abstract

S. aureus is considered the main etiologic agent of mastitis, standing out for its ability to produce biofilm, a structure of resistance against antibiotics and the host's immune system. Iron is an essential micronutrient in different biochemical pathways, being associated with the regulation of gene expression and biofilm production. In turn, iron chelators prevent the use of this nutrient by microorganisms, impairing growth. Therefore, the objective of this study was to evaluate the interference of iron chelators (sodium alginate and 2,2′-bipyridyl) on bacterial growth and on the biofilm produced by *S. aureus* from bovine mastitis. For this purpose, methodologies were developed based on the evaluation of the antimicrobial activity of the compounds, as well as the analysis of the interference with biofilm formation and bacterial

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growth under different conditions. In addition, an analysis of presence of resistance genes and the *icaA, icaD, sbnD* and *sfaD* genes was performed. Although 100% of them had important genes associated with the production of siderophores (*sbnD* and *sfaD*), the two chelators were able to interfere with the growth of the isolates. The isolates produced biofilm and had the *icaA* and *icaD* genes. Although there was no significant interference with biofilm formation, the two chelators interfered with mature biofilm. 2,2′-bipyridyl, in particular, harms the biofilm formed in 66.66% of isolates. Although the results showed strain-dependent performance, the study showed the potential of sodium alginate and 2,2′-bipyridyl in combating biofilm produced by *S. aureus,* which is promising in the treatment of mastitis.
Key words: Siderophore. Iron. Chelators. Gene. Staphyloferrin.

Resumo

S. aureus é considerado o principal agente etiológico da mastite, destacando-se pela capacidade de produzir biofilme, estrutura de resistência aos antibióticos e ao sistema imunológico do hospedeiro. O ferro é um micronutriente essencial em diferentes vias bioquímicas, estando associado à regulação da expressão gênica e à produção de biofilme. Por sua vez, os quelantes de ferro impedem a utilização desse nutriente pelos microrganismos, prejudicando o crescimento. Portanto, o objetivo deste estudo foi avaliar a interferência de quelantes de ferro (alginato de sódio e 2,2′-bipiridil) no crescimento bacteriano e no biofilme produzido por *S. aureus* de mastite bovina. Para tanto, foram desenvolvidas metodologias baseadas na avaliação da atividade antimicrobiana dos compostos, bem como na análise da interferência na formação de biofilme e no crescimento bacteriano sob diferentes condições. Além disso, foi realizada análise da presença de genes de resistência e dos genes *icaA, icaD, sbnD* e *sfaD.* Embora 100% deles apresentassem genes importantes associados à produção de sideróforos (*sbnD* e *sfaD*), os dois quelantes conseguiram interferir no crescimento dos isolados. Os isolados produziram biofilme e possuíam os genes *icaA* e *icaD*. Embora não tenha havido interferência significativa na formação do biofilme, os dois quelantes interferiram no biofilme maduro. O 2,2′-bipiridil, em particular, reduziu o biofilme formado de 66,66% dos isolados. Embora os resultados mostraram um desempenho cepa dependente, o estudo mostrou o potencial do alginato de sódio e do 2,2′-bipiridil no combate ao biofilme produzido por *S. aureus,* que é promissor no tratamento da mastite.
Palavras-chave: Sideróforo. Ferro. Quelante. Gene. Staphyloferrin.

Introduction

Mastitis is an inflammation of the mammary gland responsible for large economic losses. This disease is mainly caused by bacteria, with *Staphylococcus aureus* representing one of its main etiological agents (Langoni et al., 2015). This microorganism relies on important defense mechanisms, e.g., biofilm production, capable of conferring resistance to antimicrobial drugs, disinfectants, the host's immune system, and making the infection chronic (Tremblay et al., 2014). Infections caused by *S. aureus* with biofilm formation are rarely reversed by the host, and antibiotic therapy is usually unable to destroy this structure (Mashruwala et al., 2015). Therefore, the persistence of the infection caused by *S. aureus* highlights the importance of

discovering new therapeutical targets (Choby et al., 2016).

In order for the production of resistance structures and bacterial growth to occur, some pathogens require essential micronutrients for biochemical pathways. Iron is essential in several metabolic pathways, including bacterial growth, energy production, regulation of gene expression, and biofilm development (Cho et al., 2015). Iron is also crucial for the infectious life cycle of *S. aureus* (Choby et al., 2016). In situations of shortage of this nutrient, *S. aureus* produces two siderophores (Staphyloferrin A and B), small molecules with high affinity for iron (Kobylarz et al., 2016).

Molecules that act as chelating agents can serve as therapeutic alternatives to treat infections caused by *S. aureus*. These substances that interact with iron compete with invader microorganisms for the acquisition of the metallic ion (Kobylarz et al., 2016). Alginates, in that case, are biopolymers extracted from brown algae, with known iron-binding properties (Horniblow et al., 2015). In turn, 2,2'-bipyridyl, a chemical compound belonging to the class of nitrogenous heterocyclic compounds that can be synthesized by different methods, often involving the dimerization or closure of pyridine-based precursors (Yamada et al., 2019), is an iron chelator known by its capacity of decreasing biofilm formation in certain pathogens. This scenario is accompanied by the growing interest in using iron chelators as therapeutic agents (Mashruwala et al., 2015). From this perspective, this study aimed to evaluate the effect of sodium alginate and 2,2'-bipyridyl on the bacterial growth and biofilm formation of *S. aureus* isolated from bovine mastitis.

Materials and Methods

Bacterial strains and culture conditions

The experiment was performed using 12 *S. aureus* isolates obtained from bovine mastitis and two reference strains: ATCC 25923 methicillin-susceptible *S. aureus* (MSSA) and ATCC 33591 methicillin-resistant *S. aureus* (MRSA). The isolates were cultured in Brain Heart Infusion agar (BHI, Acumedia®, Lansing, USA) for 24h00 at 37 °C and kept under refrigeration (± 4 °C). The isolates were confirmed as S. aureus by conventional biochemical testing (DNAse, mannitol agar, Purple Agar Base, and coagulase) (Markey et al., 2013). An antimicrobial resistance confirmation was performed with an antibiogram for cephalexin (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), and tetracycline (30 µg) (Clinical and Laboratory Standards Institute [CLSI], 2019). The antimicrobials tested were chosen in order to compare the phenotypic and genotypic resistance profile.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC were determined using the M7-A9 protocol (CLSI, 2018). First, 200 µL of Müeller-Hinton (MH) broth (Kasvi®, São José dos Pinhais, Brazil) was added to all wells of 96-well microplates. The stock solutions alginic acid sodium salt from brown algae (Sodium alginate) (Sigma-Aldrich, San Luis, Missouri, USA) and 2,2'-bipyridyl (Sigma-Aldrich) were prepared using 10% ethanol (Neon, Suzano, São Paulo, Brazil). Then, these solutions were diluted (1:2) in the microplate, resulting in the concentrations of

500, 250, 125, 62.5, 31.2, 15.6, 7.8, and 3.9 µg mL^{-1}. Subsequently, a bacterial suspension at a concentration equivalent to the 0.5 tube of the McFarland standards ($1x10^8$ CFU mL^{-1}) was prepared in saline solution, which was adjusted to $1x10^6$ CFU mL^{-1} in MH broth to compose the inoculum. Finally, 10 µL of the inoculum was added to the microplate wells, including the bacterial viability control.

MH broth was used as a sterility control. After 24h00 of incubation at 37 °C, the content of each well was inoculated on a plate containing MH agar, which was then incubated at 37 °C for 24h00 to determine the MBC by visualization of bacterial growth. The MIC was determined by adding 20 µL of 1% 2,3,5-triphenyltetrazolium chloride (CTT) (Neon) into each microplate well. The MIC was read after 1 h of incubation at 37 °C by observing the change in color into a reddish hue.

Molecular characterization

The extraction of genomic DNA from the S. aureus isolates was performed according to Aldous et al. (2005) and Ausubel et al. (2003). Subsequently, polymerase chain reaction (PCR) was used to search for the genes referring to biofilm production (icaA (accession No. AF086783) and icaD (accession No. AF086783)) (Vasudevan et al., 2003), siderophores (sbnD (accession No. AY251022.1) (Beasley et al., 2009) and sfaD (accession No. AP009351) (Rosa et al., 2022), in addition to genes related to identification (nuc) (Kateete et al., 2010) and resistance (blaZ (accession numbers M15526, M25252, M25253, M25254, M25257, U58139, X04121, XI6471, and X25734) (Vesterholm-Nielsen et al., 1999) and mecA (accession No. CP102575.1) (Mo & Wang, 1997)). In all reactions, ATCC 33591 was used as a positive control. The same reaction conditions presented by Rosa et al. (2022) were used in this experiment.

Biofilm

Quantification of biofilm production

The microplate adhesion methodology proposed by Merino et al. (2009) was used to evaluate whether the Staphylococcus spp. isolates produced biofilm. Each isolate was inoculated in 3 mL of Trypticase Soy Broth with 0.25% glucose (TSBg) and incubated at 37 ºC for 24h00. Then, 5 µL of the bacterial inoculum was added to the wells of the sterile 96-well microplate containing 195 µL of TSBg. Next, the microplate was incubated at 37 ºC for 24h00, washed three times with 200 µL of sterile distilled water, and stained with 100 µl of 0.25% gentian violet (Proquímios, Rio de Janeiro, Brazil) for 5 min.

Subsequently, the wells were washed again and received 200 µL of ethanol-acetone [99.8% ethyl alcohol P.A. (NEON, São Paulo, Brazil) with 99.5% acetone P.A. (Fmaia, Belo Horizonte, Brazil) (80:20)]. The absorbance reading was performed in an EXPERT PLUS-UV microplate reader (ASYS, Cambridge, United Kingdom) at a wavelength of 620 nm. The negative control consisted of the TSBg medium. The assays were performed in triplicate, and the isolates were classified for their biofilm production according the optical density values (DO), following Stepanović et al. (2000).

Interference on biofilm formation

For the interference assay during biofilm formation, the colonies isolated from the culture of *S. aureus* were inoculated in 3 mL of the TSBg broth and incubated at 37 °C for 24h00. Next, 100 µL of this suspension was inoculated in the wells of 96-well microplates, which then received 100 µL of the iron chelators [2,2'-bipyridyl at the equivalent MBC of each isolate (final concentration of 1/2 MBC in the well) and 3% sodium alginate], separately. Next, the microplate was incubated at 37 °C for 24h00, washed three times with 200 µL of sterile distilled water in each well, and dried at ambient temperature. After drying, the wells were stained, washed, and read according to item 2.4.1 (Merino et al., 2009).

Interference on mature biofilm

The evaluation of the interference of sodium alginate and 2,2'-bipyridyl on the mature biofilm of *S. aureus* was performed according to Merino et al. (2009) and Johnson et al. (2002). For that purpose, the isolates were incubated at 37 °C for 24h00 in TSBg broth (Acumedia®) and, from this inoculum, 100 µL was added to the wells of the 96-well microplates, which were then incubated at 37 °C for 24h00. Next, the wells were washed three times with 200 µL of sterile distilled water in each well, after which 200 µL of 2,2'-bipyridyl at 1/2 MBC of each isolate and 3% sodium alginate were also added. The absorbance reading was performed in a microplate reader at the wavelength of 620 nm after applying the substances (reading at 0 h) and after 24h00 of incubation at 37 °C (reading at 24h00). The samples were analyzed in triplicate.

Growth of S. aureus in the presence of an exogenous iron source

The MH agar received 3% sodium alginate or the corresponding MBC value of 2,2'-Bipyridyl for the isolate in Petri dishes. An aliquot of the bacterial suspension in saline solution at the equivalent concentration of the 0.5 tube of the McFarland standards (~$1x10^8$ CFU mL^{-1}) was swabbed on a plate containing MH agar. Next, 5-mm wide wells were made in the agar, receiving liquid MH agar in order to form a fine layer in each well. After the medium solidified, 15 µL of Iron (III) chloride ($FeCl_3$) at the concentration of 4 µM was added to the well. Sterile distilled water was added to a well to serve as a negative control. The assay was conducted in biological and technical triplicate.

The plates were incubated at 37 °C for 24h00 and evaluated for the formation of the bacterial growth halo around the wells.

S. aureus growth curves

For the preparation of the pre-inoculum, the samples of *S. aureus* were incubated in BHI broth at 37 °C for 24h00. The initial D.O. of all cultures was standardized at approximately 0.03 nm. The entire test was performed in biological triplicate. It should be noted that the isolates 1, 3, 5, and 12 were tested along with ATCCs 25923 and 33591 to evaluate all MIC profiles found for sodium alginate and 2,2'-Bipyridyl. Next, these cultures were stirred at 180 rpm for 10 h. During this period, aliquots were withdrawn every two hours (2, 4, 6, 8, and 10 h) to measure the optical density. All isolates were subjected to three treatments: BHI broth with the addition of 2,2'-Bipyridyl at the corresponding MIC of each isolate; BHI with

the addition of sodium alginate (3%), and BHI broth without the addition of chelating agents, as a control. The entire test was performed in biological triplicate.

It should be noted that, for this test, it was necessary to perform growth curve assay to standardize the test concentration of sodium alginate since a result referring to the MIC and MBC was not obtained. Growing sodium alginate concentrations were tested for this standardization assay (0.5, 1, 1.5, 2, 2.5, and 3%) during the growth of ATCC 25923 and 33591.

Statistical analysis

The results of S. aureus growth curves were analyzed by the multiple comparisons parametric test and two-way ANOVA, with Tukey's post-test. The statistical analyses were performed in the software GraphPad Prism 8®, and the results were plotted with the mean ± standard deviation. All tests used the significance level of $p<0.05$.

Results

All 12 isolates tested were confirmed as S. aureus by biochemical tests and confirmation of the presence of the nuc gene. The genes blaZ and mecA were not present in any isolate. In the phenotypic analyses, all isolates were sensitive to gentamicin, ciprofloxacin, and cephalexin, with 25% (3) being resistant to tetracycline. There was an intermediate sensitivity profile to erythromycin in 66.66% (8) of the isolates (Table 1).

Table 1
Sensitivity profile of the Staphylococcus aureus isolates

Isolates	GEN	TET	CIP	ERI	CFX	IRMA
1	S	R	S	S	S	0.2
2	S	S	S	S	S	0.0
3	S	S	S	I	S	0.0
4	S	S	S	S	S	0.0
5	S	S	S	I	S	0.0
6	S	S	S	I	S	0.0
7	S	R	S	I	S	0.2
8	S	S	S	I	S	0.0
9	S	S	S	I	S	0.0
10	S	R	S	S	S	0.2
11	S	S	S	I	S	0.0
12	S	S	S	I	S	0.0
ATCC 25923	S	S	S	S	S	0.0
ATCC 33591	R	R	R	I	S	0.6

CIP: ciprofloxacin; CFX: cephalexin; ERI: erythromycin; GEN: gentamicin; TET: tetracycline; IRMA: multiple antibiotic resistance; S: sensitive; I: intermediate; R: resistant. ATCC. American Type Culture Collection.

With regard to antibacterial activity, sodium alginate showed no inhibitory and/or bactericidal activity against the *S. aureus* isolates at any concentration. The MIC of 2,2'-Bipyridyl ranged from 7.8 to 62.5 µg mL^{-1}, whereas the MBC ranged from 15.6 to 250 µg mL^{-1}, as seen in Table 2.

Table 2
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 2,2'-Bipyridyl against *Staphylococcus aureus* isolated from bovine mastitis

Isolates	2,2'-Bipyridyl	
	MBC (µg mL^{-1})	MIC (µg mL^{-1})
1	15.6	7.8
2	15.6	7.8
3	125	31.2
4	250	7.8
5	125	15.6
6	250	15.6
7	125	15.6
8	250	15.6
9	250	7.8
10	62.5	7.8
11	250	31.2
12	250	7.8
ATCC 25923	250	15.6
ATCC 33591	250	62.5

ATCC: American Type Culture Collection.

The genes *icaA* and *icaD* were observed in all *S. aureus* isolates. Furthermore, in the phenotypic evaluation, the isolates showed different levels of biofilm production capacity, ranging from weak (41.66%), to moderate (50%) and strong (8.33%) (Table 3). In biofilm formation, sodium alginate did not interfere with the biofilm of six *S. aureus* isolates (50%) tested, in addition to ATCC 33591, but was capable of reducing biofilm formation in ATCC 25923 and two isolates (16.67%), which went from moderate to weak biofilm producers. For four other isolates (33.33%), there was an increase in the biofilm production capacity, changing from weak to moderate (N= 3, 25%) and from moderate to strong (N=1, 8.33%) in the presence of sodium alginate.

Table 3
Interference of sodium alginate and 2,2'Bipyridyl on biofilm formation by Staphylococcus aureus

Isolates	Biofilm quantification		Biofilm formation				Mature biofilm	
	Mean OD	Classification	Sodium alginate		2,2'-bipyridyl		Sodium alginate (%)	2,2'-Bipyridyl (%)
			Mean OD	Classification	Mean OD	Classification		
1	0.123	Weak	0.176	Moderate	0.173	Moderate	92.06	105.95
2	0.187	Moderate	0.256	Moderate	0.336	Forte	94.55	103.60
3	0.248	Moderate	0.099	Weak	0.110	Weak	98.26	106.02
4	0.188	Moderate	0.331	Forte	0.158	Moderate	97.26	114.29
5	0.113	Weak	0.121	Weak	0.241	Moderate	99.27	94.74
6	0.119	Weak	0.224	Moderate	0.183	Moderate	100.00	93.92
7	0.251	Moderate	0.120	Weak	0.109	Weak	99.64	94.33
8	0.132	Moderate	0.165	Moderate	0.140	Moderate	95.39	101.16
9	0.106	Weak	0.178	Moderate	0.090	Weak	93.86	119.23
10	0.083	Weak	0.105	Weak	0.150	Moderate	109.25	71.72
11	0.194	Moderate	0.186	Moderate	0.097	Weak	98.64	109.29
12	0.068	Weak	0.095	Weak	0.099	Weak	105.43	107.38
ATCC 25923	0.221	Moderate	0.088	Weak	0.107	Weak	105.97	104.05
ATCC 33591	0.064	Weak	0.119	Weak	0.113	Weak	87.75	108.33

OD: optical density. ATCC: American Type Culture Collection.

2,2'-Bipyridyl showed no interference with biofilm formation in four isolates (33.34%), including ATCC 33591. However, there was a reduction in the intensity of biofilm production of strain ATCC 25923 and in three other isolates (25%), which changed from moderate to weak producers. The five other isolates (41.67%) increased their biofilm production in the presence of the chelating agent, from weak to moderate (N= 33.33%) and from moderate to strong (N=1, 8.33).

The two chelating agents tested showed action against mature biofilm. Sodium alginate interfered with the biofilm produced by 9 isolates (75%), and 2,2'-Bipyridyl interfered with 4 biofilm-producing isolates (33.33%). The biofilm produced by ATCC 25923 was affected by the presence of both chelating agents. The results obtained suggest that the chelators tested present a performance dependent on the challenged strain.

With regard to the potential production of siderophores, all isolates tested had the genes *sfaD* and *sbnD.* However, the addition of an exogenous iron source (4 µM) was not sufficient to favor the growth of *S. aureus* associated with both chelating agents tested (sodium alginate and 2,2'-bipyridyl). A reduction was observed in the growth of the isolates when these were subjected to incubation with iron chelators compared to the control condition (Figure 1). In general, after 4 hours, there was a statistical difference between 2,2'-bipyridyl and sodium alginate in relation to the control group, as well as between the two chelating agents. Although interference was observed in the growth pattern of the isolates submitted to the medium with the two iron chelators, it was noted that 2,2'-Bipyridyl showed better results, as it caused the stationary phase to be reached more quickly in all the isolates. The data individually shown by the isolates can be seen in Figure 1S-6S of the supplementary material.

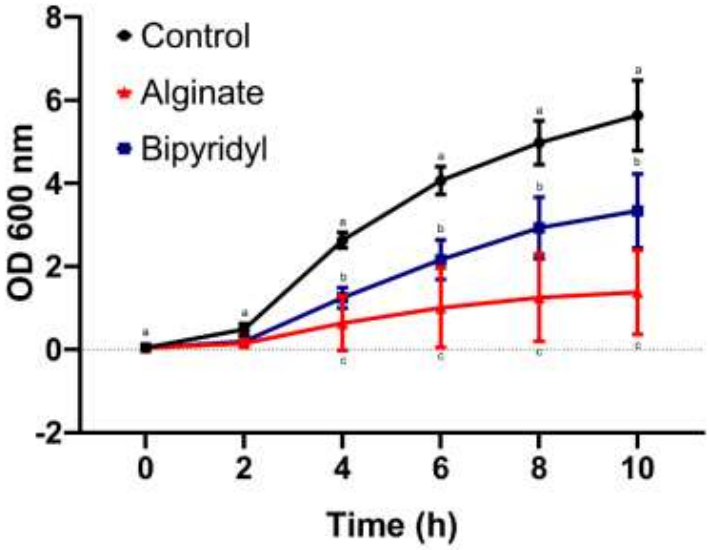

Figure 1. Mean of bacterial growth expressed in O.D. of the isolates subjected to growth under three conditions (control, 2,2-bipyridyl [MIC], and sodium alginate [3%]), for 10 h. The letters indicate statistically equal means, at each time, by Tukey's post-test with a p-value <0.05.

Discussion

β-lactams are antimicrobials from the Penicillin class commonly used in infections caused by *Staphylococcus* spp. The enzyme β-lactamase, codified by the *blaZ* gene, hydrolyzes the β-lactam ring. In addition, the *blaZ* gene is related to the production of the low-affinity penicillin-binding protein, providing the microorganism with antimicrobial resistance (Marques et al., 2017). The presence of the *mec*C gene in MRSA isolates represents a potential problem for diagnosis since it is not detected in standard PCR assays for mecA (Blair et al., 2014), and the intermediate sensitivity profile to erythromycin can be worrying. According to Rao et al. (2022), erythromycin sensitivity is high in *S. aureus* isolates obtained from dairy herds in the USA compared to other animal species, including humans.

Although the isolates of the present study showed high sensitivity to the antimicrobials tested, it is known that the use of sub-doses, the poor application of the medication, and the excessive time of treatment could be directly related to the acquisition of resistance by the microorganisms involved in the pathogenesis of mastitis (Lopes et al., 2013; Mota et al., 2012). Therefore, several studies have been conducted to search for alternative therapies, aiming to optimize/reduce the use of conventional antimicrobials in dairy production (Cheng & Han, 2020; Krömker & Leimbach, 2017).

The results obtained show that the chelating agent 2,2′-bipyridyl presented bactericidal action against strains of *S. aureus* isolated from bovine mastitis, which produce biofilm and are resistant to beta-lactams. The concentration of 250 μg mL^{-1} was able to inhibit the multidrug-resistant strain ATCC MRSA 33591 and also ATCC MSSA 25923, highlighting its effectiveness against MSSA and MRSA isolates. In addition to having an affinity for iron, this chelating agent stands out for having antimicrobial, antifungal, anti-inflammatory, antioxidant and antitumorigenic properties (Rishi et al., 2017), suggesting its efficiency also in the treatment of mastitis caused by MSRA isolates. *S. aureus* is often associated with cases of antimicrobial resistance and subsequent flaws in the treatment of bovine mastitis (Mota et al., 2012; Awad et al., 2017; Krewer et al., 2013; Suleiman et al., 2017). Therefore, the use of 2,2′-bipyridyl as a new therapy is promising to assist in solving the problem of antimicrobial resistance.

In the present study, the iron chelators sodium alginate and 2,2′-bipyridyl were not efficient against the formation of biofilm produced by *S. aureus* isolates. Biofilm is a great obstacle in the treatment of mastitis and several infections since, with the protection provided to the pathogen, there is a difficulty in effectively eliminating the agent, with a consequent evolution of the infection into a chronic state (Schönborn et al., 2017). Therefore, the biofilm-producing S. aureus isolates are considered much more resistant to antibiotic therapy than free-living cells (Snel et al., 2014). In the study conducted by (Cho et al., 2015), it was also possible to observe the resistance of ATCC 25923 in biofilm formation when exposed to growing concentrations of 2,2′-bipyridyl (312 μM to 2.500 μM). These results suggest that, although biofilm formation by *S. aureus* involves the capture of iron, the synthesis of an extracellular polysaccharide substance

can be a more important factor in this process for *S. aureus* (Lin et al., 2012) since all isolates had the genes *ica*A and *ica*D, considered essential for the production of Polysaccharide intercellular adhesin (PIA) (Arciola et al., 2012).

On the other hand, the ability to disaggregate mature biofilm observed in the present study is an important result, as it reveals that sodium alginate and 2,2'-bipyridyl constitutes a promising alternative to be used in this problem, contributing to a more effective treatment of mastitis in cases when biofilm is already mature. This structure can block or reduce the activity of the antimicrobials used for treatment, significantly decreasing the efficacy of antibiotic therapy. Furthermore, the biofilm produced by *S. aureus* is commonly associated with antimicrobial resistance in cases of mastitis (Krewer et al., 2013; Goetz et al., 2017). The reduced efficacy of antimicrobial drugs in the presence of biofilm is related to pores inside the matrix, which are small enough to block the passage of several molecules, including antibiotics (Patel, 2005). This may justify, when evaluating the interference of the consolidated biofilm, the greater effectiveness of 2,2'-Bipyridyl, with its small chemical structure, compared to sodium alginate, which in turn has a larger and more complex molecule.

The cases of mastitis caused by *S. aureus* are considered hard to treat and prone to resurgence due to the survival mechanisms adopted by the pathogen in the mammary gland, e.g., biofilm formation. This chronicity is associated with the tridimensional structure of biofilm, which facilitates the distribution of oxygen and nutrients to the cells, in addition to facilitating the colonization of these cells in other places, enabling the promotion of new infection sites and biofilm formation (Gomes et al., 2016). The results of the interference of iron chelators obtained in this study are important because the microorganisms present in the biofilm are considerably more resistant to the inflammatory cells of the host's mammary gland and antibiotics due to the reduction in antibiotic diffusion within the matrix (Cho et al., 2015; Aslantaş & Demir, 2016). Furthermore, the use of antibiotic therapy normally reverses only clinical signs, without biofilm destruction (Chagas et al., 2015).

The siderophores produced by *S. aureus* are small molecules of low molecular weight, with high affinity for iron and binding proteins of the host (Cho et al., 2015). The presence of the *sfa*D and *sbn*D genes in the isolates tested affirms the high survival potential in environments with iron shortage. However, iron absorption through siderophores can be discontinued with the introduction of selective chelating agents with high affinity for this metal (Zhou et al., 2015). The molecules that act as iron chelators compete with invading microorganisms for the acquisition of the iron molecule, culminating in low concentrations of free iron in body fluids (Kobylarz et al., 2016). This can explain the results found in the *S. aureus* growth assays in the presence of iron chelators. The interference of iron chelators (sodium alginate and 2,2'-bipyridyl) on the growth pattern of *S. aureus* isolates stresses the importance of this nutrient for the good development of almost all pathogenic microorganisms.

The compounds tested in this study highlight their potential for use in combined therapies, which is interesting for the

eradication of *S. aureus* biofilms (Kranjec et al., 2021). The combat against biofilms extremely important in order to prevent the chronicity of mastitis, which can easily lead to the emergence of bacterial resistance (Gomes et al., 2016; Zaatout et al., 2020).

The selective performance of 2,2′-bipyridyl and sodium alginate in relation to different challenged strains highlights the need for more detailed studies on molecular mechanisms that govern this selective action and the possibility of exploring synergies between iron chelators and antimicrobial agents traditional methods to strengthen resistant strains, since understanding variations in sensitivity between species can open paths for personalized applications, further increasing their effectiveness.

There is a diversity of studies in the literature on the possible toxic effects of 2,2′-bipyridyl derivatives, however there are no recent publications in this field for the chelator without any complex linked to it. It would be interesting if there were research that addressed a safety margin for the use of this chemical compound.

Conclusion

Sodium alginate and 2,2′-bipyridyl present promising characteristics for the control of *S. aureus* in bovine mastitis, in addition to having the ability to interfere with the structure of the mature biofilm, an important factor associated with cases of persistent mastitis. However, the variable performance of the chelators tested suggests strain-dependent action, highlighting their potential use in combined therapies.

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Authors' Contributions

ACBN is joint first author on this work. All authors contributed to the study's conception and design. Material preparation and data collection were performed by ACBN, DSR, RFSS, NBF, AWCF, GVG, HCB, MMC. Data analysis was performed by ACBN, DSR, RFSS, NBF, AWCF, GVG, HCB, and MMC. The first draft of the manuscript was written by ACBN, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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