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Antibiofilm activity of bromelain from pineapple against *Staphylococcus aureus*

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ABSTRACT. Bromelain is a set of proteolytic enzymes usually obtained from pineapple (*Ananas comosus*). Although bromelain has distinguished therapeutic properties, little is known about its proteolytic potential against opportunistic pathogens related to wound healing complications, such as *Staphylococcus aureus*. This study aimed to investigate the antibiofilm and antibacterial activity of bromelain in 43 clinical strains of *S. aureus* isolated from chronic wounds and blood cultures. Bromelain's activity against *S. aureus* biofilm *in vitro* was assessed by analyzing biofilm formation in cultures grown in the presence of 1% bromelain and biofilm destruction after the addition of 1% bromelain to mature biofilms. Proteinase K and sodium metaperiodate were also added to mature biofilms in parallel to compare their activity with that of bromelain and, together with exopolysaccharide and protein production rate assays, to determine the chemical composition of the biofilm extracellular matrix of selected strains of *S. aureus*. Bromelain was also evaluated for its DNase activity and impact on cellular hydrophobicity and auto-aggregation. Mueller-Hinton agar dilution was used to determine bromelain minimal inhibitory concentration (MIC). Biofilm assays showed that 1% bromelain significantly inhibits *S. aureus* biofilm formation ($p = 0.0157$) by up to 4-fold and destroys its mature biofilms ($p < 0.0001$) by up to 6.4-fold, both compared to the control grown without bromelain. Biofilms of methicillin-resistant *S. aureus* strains isolated from chronic wounds were the most affected by bromelain treatment. No antibacterial activity was detected with bromelain MIC assays and the proteolytic activity of bromelain was identified as the main antibiofilm mechanism of the enzyme, though its DNase activity may also contribute. The epithelial therapeutic properties of bromelain combined with its antibiofilm activity against *S. aureus* make it a promising alternative to compose the therapeutic arsenal for the control of *S. aureus* biofilms in the context of wound care.

Keywords: bromelain; biofilm; *Staphylococcus aureus*; chronic wounds.

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Introduction

Staphylococcus aureus is an opportunist pathogen involved in different nosocomial infections such as pneumonia, meningitis, endocarditis, toxic shock syndrome, septicemia, and others (McGuinness, Malachowa, & DeLeo, 2017). *S. aureus* biofilm life form is associated with the development of these infections and with increased morbidity and mortality in patients (Khan, Shukla, Rizvi, Mansoor, & Sharma, 2011; Tacconelli et al., 2018). Biofilms are complex structures formed by microorganisms, and the extracellular matrix produced by them is mainly composed of extracellular polymeric substances (EPS) like exopolysaccharides, proteins, and extracellular DNA (Flemming et al., 2016; Hall-Stoodley et al., 2012). The extracellular matrix protects microorganisms from hostile conditions such as the host immune system and the effects of antibiotics (Eladawy, El-Mowafy, El-Sokkary, & Barwa, 2020; Munro, Highmore, Webb, & Faust, 2019). Thus, once the biofilm is established on a biotic or abiotic surface, its removing process becomes difficult.

S. aureus is one of the most abundant bacterial species in infected chronic wounds, mainly due to its ability to form biofilms (Kalan & Brennan, 2019; Wolcott et al., 2016). When biofilms are established on the surface of chronic lesions, the treatment becomes challenging, even more so if there are multidrug-resistant microorganisms associated with biofilms, such as methicillin-resistant *S. aureus* (MRSA). Numerous therapeutic alternatives have been developed for the care of epithelial lesions. However, there are still no

definitive therapeutic solutions for the management of chronic lesions. Thus, the study of new bioactive agents is important for the development of new strategies on the therapeutic arsenal.

Based on this relevance and demand, plant-derived proteolytic enzymes with potential activity against bacterial biofilms, such as bromelain, are important alternatives to aid in wound management. Bromelain is a set of proteolytic enzymes produced by members of the Bromeliaceae family, of which pineapple (*Ananas comosus* (L.) Merril) is the main representative (Lencastre Novaes et al., 2016). Although bromelain has known anti-inflammatory and necrotic tissue debridement properties (Ataide et al., 2017), little is known about the potential of this enzyme against opportunistic pathogens related to wound healing complications. Thus, this work aims to investigate bromelain's antibiofilm and antibacterial activity in *S. aureus* strains.

Material and methods

Bacterial strains

Staphylococcus aureus strains used in this study (Supplementary Table 1) belong to the culture collection of the *Laboratório de Controle Microbiológico* of the *Faculdade de Farmácia* at *Universidade Federal Fluminense* and were isolated from chronic wounds (n = 28) and blood cultures (n = 13) from patients at *Hospital Universitário Antônio Pedro* and *Hospital Santa Martha*, located in Niterói, Rio de Janeiro State, Brazil. The collection of samples from both chronic wounds (CAAE: 19468719.9.0000.5243) and blood (CAAE:0172.0.258.000.08) has been approved by the research ethics committee of the *Universidade Federal Fluminense*. Strains were grown in tryptic soy broth (TSB) supplemented with 1% glucose or tryptic soy agar (TSA) at 35ºC (± 2ºC) for 24 hours. *S. aureus* reference strains ATCC 29213 and ATCC 25923 were used as control when needed.

Bromelain solution

In this study, we used stem bromelain (Enzyme Commission Number: 3.4.22.32) obtained from commercial sources (Sigma-Aldrich, Saint Louis, MO, USA). Before each experiment, the enzymatic solution at the desired concentration was prepared from a 20% (w/v) stock solution of bromelain and sterile distilled water.

Determination of antibiofilm activity

To investigate bromelain's activity in inhibiting biofilm formation, 10 µL of bacterial cultures diluted and adjusted in TSB (supplemented with 1% glucose) to the McFarland scale of 0.5 (1.5×10^8 CFU mL^{-1}) were transferred to microtiter plate wells (Kasvi, Campina, Paraná State, Brazil) containing 190 µL of TSB (supplemented with 1% glucose) with or without 1% bromelain. After incubation at 35°C (± 2°C) for 24 hours, biofilm formation was measured according to previous work (Jung et al., 2017), with some adjustments. Briefly, wells were washed twice with 200 µL of saline solution (0.9%; w/v) to remove planktonic cells and the plates were allowed to dry at 35°C (± 2°C) for 2 hours, followed by biofilm staining with 100 µL of 0.1% crystal violet. After 30 minutes of incubation at room temperature, the wells were washed twice with 200 µL of saline solution (0.9%; w/v) and crystal violet was eluted with 100 µL of 95% ethanol. The optical density (OD) at 570 nm (OD_{570}) was then measured using SpectraMax M2e spectrophotometer (Molecular Devices, San Jose, CA, USA). The assays were carried out in triplicate.

To investigate bromelain's ability to destroy mature biofilms, 10 µL of bacterial cultures were added to microtiter plate wells (KASVI) containing 190 µL TSB supplemented with 1% glucose and without bromelain. The microtiter plate was incubated for 24 hours at 35°C (± 2°C) to allow the formation of mature biofilms. Afterward, the bacterial supernatant was replaced with 200 µL of saline solution (0.9%; w/v) supplemented with 1% bromelain, and the plate was incubated once more for 24 hours at 35°C (± 2°C). After incubation, biofilm staining with crystal violet was carried out as described above and the biofilm was measured at OD_{570} using SpectraMax M2e. The assays were carried out in triplicate.

Proteinase K and sodium metaperiodate assays

Analysis of the biofilm composition of selected *S. aureus* strains (ATCC 25923, ATCC 29213, 33.3, 34.3, 176, and 196) was carried out by treating mature biofilms with either proteinase K or sodium metaperiodate. Briefly, 10 µL of bacterial cultures were inoculated into microtiter plate wells (KASVI) containing 190 µL of TSB. Then, the plate was incubated for 24 hours at 35°C (± 2°C). Afterward, bacterial supernatants were removed, the wells were washed with 200 µL of saline solution (0.9%; w/v), and the mature biofilms were treated with either proteinase K or sodium metaperiodate. For the proteinase K treatment, 100 µL of proteinase K (Sigma-Aldrich) solution (1 mg/mL in 100

mM Tris-HCl, pH 8.0)(O'Neill et al., 2007) were added to the wells and the plates were incubated at 35°C (± 2°C) for 24 hours. For the sodium metaperiodate treatment, 180 µL of saline (0.9%; w/v) supplemented with 20 µL of 0.2 M sodium metaperiodate (Sigma-Aldrich) were added to the wells and the plates were incubated overnight at 5ºC (O'Neill et al., 2007). Both treatments were followed by crystal violet staining for biofilm quantification at OD_{570} as described above. The assays were carried out in triplicate.

DNaseI assay

To investigate whether bromelain exerts an effect on bacterial DNA, the activity of 1% bromelain on strain ATCC 25923 genomic DNA was compared with DNase I's activity (Promega, Madison, WI, USA). DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions. DNase I reactions used 12 µL of DNA, 4 µL of DNase I, 2 µL of buffer, and 2 µL of sterile ultrapure water, while treatment with 1% bromelain was performed using 12 µL of DNA, 1 µL of 20% bromelain, and 7 µL of water. Both reactions were incubated for one hour at 30ºC. After incubation, DNA treated with 1% bromelain was purified with Wizard® Genomic DNA Purification Kit (Promega) starting from the protein precipitation step. Ten microliters of genomic DNA from each reaction were submitted to 0.8% agarose gel electrophoresis for 1 hour at 80 volts. Then, the agarose gel was stained with ethidium bromide and exposed to UV light (L-PIX CHEMI-Loccus, Loccus Ltda, Cotia, SP, BR) to visualize DNA degradation.

Quantification of total exopolysaccharide and protein production rates

Total exopolysaccharide and protein production rates were quantified to determine the mechanisms involved in bromelain antibiofilm activity. Mature biofilms of selected *S. aureus* strains (ATCC 25923, ATCC 29213, 33.3, 34.3, 176, 196) grown in microtiter plate wells (KASVI) obtained as described above, were treated with 1% bromelain during 24 hours at 35°C (± 2°C), with mature biofilms not exposed to bromelain used as control. Ten replicates of each strain were used to collect the desired concentration of EPS. Saline solution (0.9%; w/v) and careful scraping with a micropipette tip were used to suspend mature biofilm adhered to the bottom of the wells. The bacterial suspensions were then centrifuged (5,600 g 10 $min.^{-1}$) and 0.5 mL of the supernatant was mixed with 2 mL of cold ethanol (Azeredo, Lazarova, & Oliveira, 1999). The samples were incubated overnight at 5°C. Subsequently, the samples were centrifuged (3,700 g 5 $min.^{-1}$ at 5°C) and the sedimented EPS contents were collected and resuspended in 500 µL of distilled water (Gong, Bolster, Benavides, & Walker, 2009; Rasamiravaka et al., 2015; Song et al., 2003).

For quantification of total exopolysaccharide production, the Phenol-Sulfuric Acid method was performed (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Gong et al., 2009). Fifty microliters of phenol (80%; w/v) (Sigma-Aldrich) and 2.5 mL of sulfuric acid (95%; w/v) (Sigma-Aldrich) were added to 500 µL of the suspended EPS solution. The tubes were incubated at room temperature for 1 hour. Finally, the tubes were submitted to spectrophotometry at OD_{480} (SPEC 20 MV, Coml. Indl. Dourado Ltda, Dourado, SP, BR) with glucose (1 mg mL^{-1}) as a standard.

The Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) was adopted for the quantification of the total protein production in biofilms. Firstly,1.5 mL of alkaline copper reagent (1 mL 2% $Na_2C_4H_4O_6$, 1 mL 1% $CuSO_4$*$5H_2O$, and 98 mL 2% $NaCO_3$ in 0.1 M NaOH) was added to 300 µL of suspended EPS solution, followed by the addition of 100 µL of Folin reagent (Sigma-Aldrich). The mixture was incubated at room temperature for 30 minutes and measured in the spectrometer at OD_{500} (SPEC 20 MV). Bovine serum albumin (BSA;1 mg mL^{-1}) (Promega) was used as standard.

The OD readings for each experiment were compared to standard curves for glucose and BSA to determine the concentration of exopolysaccharide and protein in each EPS solution, respectively.

Cell hydrophobicity and auto-aggregation

The hydrophobicity and auto-aggregation tests were performed according to a previous study (Song, Yu, Kim, Lee, & Paik, 2019), with some modifications. Colonies of selected *S. aureus* strains (ATCC 25923, ATCC 29213, 33.3, 34.3, 176, 196) were inoculated in tubes with 5.7 mL of TSB and 0.3 mL of 20% bromelain to a concentration equivalent to the McFarland scale of 0.5 (1.5×10^8 CFU mL^{-1}). The tubes were incubated overnight at 35ºC (± 2ºC). Cultures not treated with bromelain were used as control. Afterward, cultures were centrifuged (5.600 g 5 $min.^{-1}$) and the cell pellet obtained was washed in 500 µL of phosphate-buffered saline (PBS- pH 7.4). Each pellet was suspended to a final volume of 2 mL of PBS to an OD_{600} of 0.5 ± 0.05 (OD initial) (SPEC 20 MV). For hydrophobicity analysis, toluene (0.5 mL; Vetec, Duque de Caxias, Rio de Janeiro State,

Brazil) was added to each suspension, followed by vortexing for 2 minutes. After 15 minutes of incubation at room temperature, the OD_{600} of the lower aqueous layer was measured (OD treatment). For auto-aggregation analysis, bacterial suspensions were incubated for 4 hours at 37°C. Then, OD_{600} was measured for each suspension (OD treatment). Hydrophobicity and auto-aggregation percentages were calculated using the formula: (1 – OD treatment/OD initial) × 100.

Minimum inhibitory concentration (MIC) of bromelain

The Mueller-Hinton agar dilution test was performed to determine the minimum inhibitory concentration (MIC) of bromelain (CLSI, 2018). In this experiment, 21 selected strains of *S. aureus* from chronic wounds and blood cultures were evaluated. Initially, strains were cultured in TSA and incubated at 35ºC (± 2ºC) for 24 hours. Isolated colonies were suspended in 2 mL of sterile saline solution (0.9%; w/v) until reaching the 0.5 standard McFarland. Using the Steers applicator, approximately 2µL of the bacterial suspension was inoculated onto the experimental plates containing Mueller-Hinton agar (HiMedia, LBS Marg, Mumbai, India) with increasing dilutions of bromelain (0.25, 0.5, 1.0, 2.0, and 4.0%). Cultured plates without bromelain were used as control. Plates were incubated at 35ºC (± 2ºC) for 24 hours. Bacterial growth was visualized with the naked eye.

Statistical analysis

Statistical analysis was performed using the software BioEstat (version 5.4) and GraphPad Prism (version 9.3.0). Mann-Whitney nonparametric test was used to evaluate differences in biofilm OD_{570} between control and bromelain-treated (biofilm inhibition and destruction) groups. Kruskal-Wallis test, followed by Dunn's multiple comparisons test, was used to analyze the differences in anti-biofilm activity (measured by biofilm OD_{570}) that treatment with bromelain, proteinase K or sodium metaperiodate had on mature biofilms. The significance level adopted was $p < 0.05$.

Results

Bromelain showed no antibacterial activity against *S. aureus* strains. Bacterial growth was observed on all bromelain concentrations (MIC > 4%) tested (data not shown). Based on these results, we decided to use the median value of bromelain concentration tested (1%) in subsequent experiments.

When in the presence of 1% bromelain, biofilm formation was significantly impaired compared to the control ($p = 0.0157$) (Figure 1A). Strain-level analysis showed that 28 of the 43 *S. aureus* isolates showed a reduction of up to 4-fold in biofilm formation in the presence of 1% of bromelain when compared to control without bromelain (Figure 1B). Destruction of mature biofilm with bromelain was even more prominent than biofilm inhibition. Mature biofilms were significantly destroyed when exposed to 1% bromelain ($p < 0.0001$) (Figure 1A). Biofilm destruction was observed in the majority of strains, with a reduction in biofilm OD of up to 6.4-fold when compared to control without bromelain (Figure 1C).

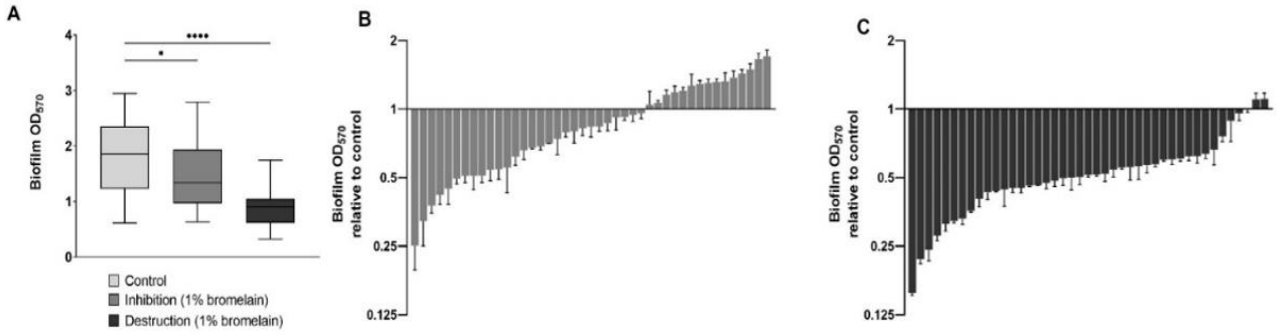

Figure 1. Activity of 1% bromelain in biofilm formation and destruction of mature biofilms. (A) Optical density at 570 nm (OD_{570}) of biofilms from reference, chronic wound, and blood culture strains of *Staphylococcus aureus* (n = 43). Control: biofilms formed without bromelain. Inhibition (1% bromelain): biofilms formed in the presence of 1% bromelain. Destruction (1% bromelain): mature biofilms exposed to 1% bromelain for 24 hours. Statistically significant difference related to the control group * $p = 0.0157$ and **** $p < 0.0001$. (B) Biofilm formation at strain level in the presence of 1% bromelain. (C) Mature biofilm destruction at strain level upon exposure to 1% bromelain. The columns represent the OD_{570} values of biofilm formation (B) and mature biofilm (C) from *S. aureus* strains (n = 43) treated with 1% bromelain treatment compared to the untreated control group.

Biofilm formation analysis also allowed the identification of relevant findings of bromelain action in *S. aureus* biofilms. On bromelain exposure, the ability of wound isolates to form biofilm was significantly more affected than blood isolates (p = 0.0010) (Figure 2A). Mature biofilms from chronic wound isolates were also significantly more destroyed than those from blood isolates (p < 0.0001) (Figure 2B).

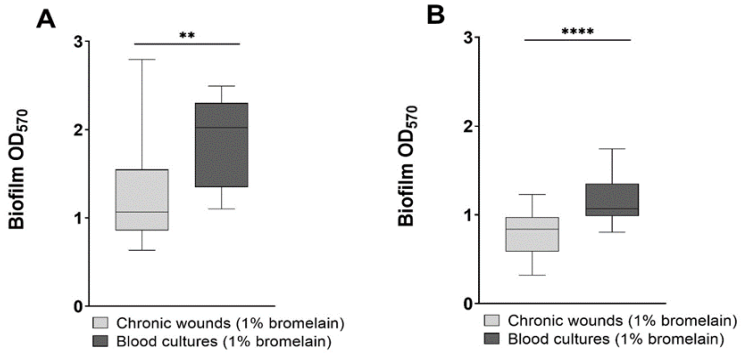

Figure 2. Antibiofilm activity of 1% bromelain among *Staphylococcus aureus* strains isolated from chronic wounds (n = 28) and blood cultures (n = 13). (A)Optical density at 570 nm (OD_{570}) of biofilms grown in the presence of 1% bromelain. (B) OD_{570} of mature biofilms treated with 1% bromelain. ** p = 0.0010. **** p < 0.0001.

It was also observed that mature biofilms from MRSA strains were more susceptible to bromelain activity than biofilms formed by methicillin-susceptible *S. aureus* strains (MSSA) (p = 0.0469) (data not shown). Taken together, these results indicate that 1% bromelain acts more effectively on *S. aureus* biofilms of strains isolated from chronic wounds and resistant to methicillin.

Mature biofilms of *S. aureus* strains were more susceptible to treatments with proteinase K (p = 0.0023) and 1% bromelain (p = 0.0374) than to treatment with sodium metaperiodate that degrades carbohydrates (p > 0.9999) (Figure 3A). Destruction of mature *S. aureus* biofilms by 1% bromelain and proteinase K treatments lead to reductions in OD measurement of up to 6.9-fold and 12.5-fold, respectively, when compared to untreated control (Figure 3B). In the study, these results indicate that proteins were more prominently involved in biofilm formation than exopolysaccharides.

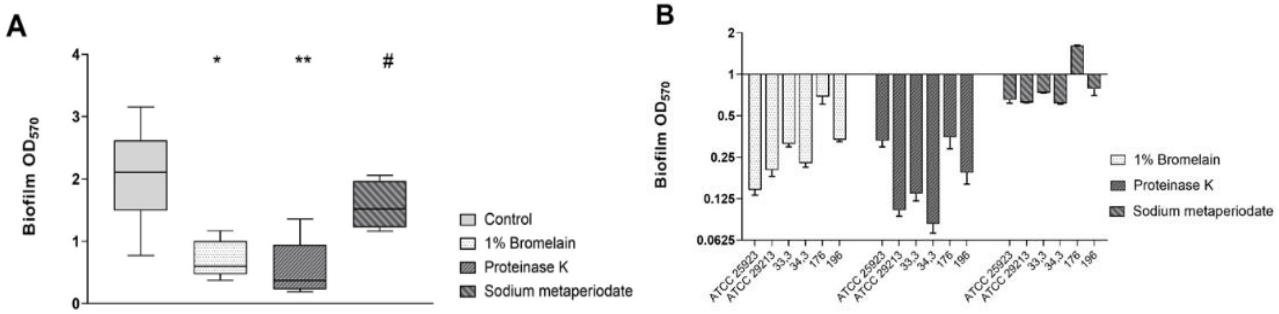

Figure 3. (A) Optical density at 570 nm (OD_{570}) of *Staphylococcus aureus* mature biofilms treated with 1% bromelain, proteinase K, and sodium metaperiodate. *S. aureus* strains: ATCC 25923, ATCC 29213 (reference), 33.3, 34.3 (chronic wound) 176, and 196 (blood culture). Control: biofilms not exposed to test substances. There was a significant difference between treatments in *S. aureus* (Kruskal-Wallis p = 0.0006). Significant statistical difference in relation to the control group *p = 0.0374 and **p = 0.0023. # Significant statistical difference in relation to proteinase K treatment (Dunn's *post hoc*). (B) Biofilm destruction OD_{570} of *S. aureus* strains after treatments with 1% bromelain, proteinase K, and sodium metaperiodate compared to the untreated control group.

Bromelain was able to reduce total protein production rates and protein concentrations in *S. aureus* biofilms (Figure 4). In the presence of 1% bromelain, the reduction of total protein production rate among *S. aureus* mature biofilms ranged from 5.5-fold to 101.2-fold when compared to the untreated control. The mature biofilm of wound isolates was strongly affected by 1% bromelain treatment. These biofilms were close to eradication when submitted to treatment with 1% bromelain and had the lowest protein production rates (35.8-fold and 101.2-fold reduction) among tested strains. Since protein concentrations in mature biofilms were significantly reduced after 1% bromelain treatment, these results corroborate the proteolytic activity of bromelain in *S. aureus* biofilms. On the other hand, treatment with 1% bromelain had little impact on exopolysaccharide quantification, with no more than a 1.05-fold reduction in exopolysaccharide production when compared to the untreated control (data not shown).

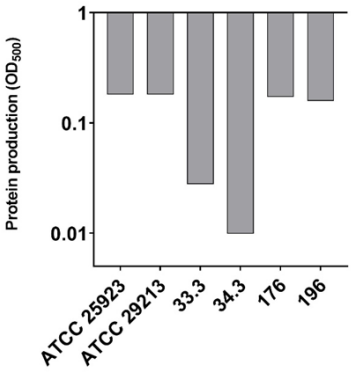

Figure 4. Bromelain's effects on protein production. Total protein content of *Staphylococcus aureus* mature biofilms treated with 1% bromelain was measured by optical density at 500 nm (OD_{500}). *S. aureus* strains: ATCC 25923, ATCC 29213 (reference), 33.3, 34.3 (chronic wound) 176, and 196 (blood culture).

The hydrophobicity assay showed that 1% bromelain exerts robust effects on cell surface hydrophobicity of *S. aureus* strains (Table 1). All *S. aureus* strains showed reduced hydrophobicity rates upon exposure to 1% bromelain, reaching reductions of up to 45% as observed in the ATCC 29213 strain. Regarding the auto-aggregation, some isolates treated with 1% bromelain, compared to the control, showed higher auto-aggregation percentages and others lower, showing that auto-aggregation, as well as hydrophobicity, can vary from strain to strain.

Table 1. Bromelain's effects on cellular hydrophobicity and auto-aggregation of *Staphylococcus aureus*

	Strains					
	ATCC 25923	ATCC 29213	33.3	34.3	176	196
Hydrophobicity (%)						
Control	27.02	37.5	27.45	20.41	17.64	21.57
Treated	6.0	-7.69	4.1	-17.31	-4.88	-6.38
Auto-aggregation (%)						
Control	24.4	10.64	11.8	14.3	10.0	19.23
Treated	11.8	21.57	4.0	17.65	14.63	22.45

Note: Control= no exposition to 1% bromelain. Treated= treatment with 1% bromelain.

The DNase assay revealed that 1% bromelain degraded the genomic DNA of *S. aureus* ATCC 25923 similarly to the DNase I treatment (supplementary Figure 1).

Discussion

Few studies to date have investigated bromelain activity against bacterial biofilms. It has been reported that bromelain at a concentration of 1% shows a significant effect against *Streptococcus mutans* biofilms, being identified as a promising alternative in the combat of secondary caries (Orem, Martins, Andrade, Capillé, & Portela, 2017). In addition, bromelain can be more efficient in eradicating mature biofilms of *Klebsiella pneumoniae* than alpha-amylase and beta-amylase enzymes (Mohamed, Mohamed, Khalil, Azmy, & Mabrouk, 2018). Here, we show that 1% bromelain has a promising activity against *S. aureus* biofilms associated with chronic wounds and bloodstream infections.

Staphylococcal biofilms can be classified into polysaccharide-dependent biofilms, whose extracellular matrices have an abundance of Polysaccharide Intercellular Adhesin (PIA) (Arciola, Campoccia, Ravaioli, & Montanaro, 2015), or polysaccharide-independent biofilms, which are composed mainly of proteins (O'Neill et al., 2007; Schilcher & Horswill, 2020). The extracellular matrices of *S. aureus* strains in our study were predominantly made of proteins. Other studies have also found that *S. aureus* biofilms may not significantly rely on exopolysaccharides (Izano, Amarante, Kher, & Kaplan, 2008; Waryah et al., 2017). Proteins are common components in *S. aureus* biofilms, representing most of *S. aureus* adhesins. These adhesins are involved in surface and intercellular adhesion and can even be found in the extracellular matrix. Examples of *S. aureus* adhesins are the proteins covalently anchored to the cell wall, like the surface protein SasG. The protein SasG plays an important role in the formation of *S. aureus* biofilms through auto-polymerization and producing fibrils that promote intercellular adhesion (Achek et al., 2020; Geoghegan et al., 2010; Otto, 2018).

The high amount of proteins in extracellular matrices of *S. aureus* biofilms allows an efficient antibiofilm activity of proteolytic enzymes in these biofilms, which explains why biofilms from different strains of *S. aureus* have been significantly affected by treatment with proteases. O'Neill and colleagues (O'Neill et al., 2007), for example, observed that proteinase K was able to significantly destroy the biofilms of MRSA strains, while sodium metaperiodate did not exhibit such activity. Recently, proteinase K was identified as one of the effective antibiofilm agents for the treatment of MRSA biofilms (Abd El-Hamid et al., 2020). In our study, bromelain, which is a cysteine endopeptidase with a broad range of protein substrates (Corzo, Waliszewski, & Welti-Chanes, 2012), had an antibiofilm activity similar to that of proteinase K. Although bromelain exact mechanism of action still needs to be determined, its known that proteolytic activity seems to support the ability of bromelain to inhibit and destroy *S. aureus* biofilms, as observed in this study.

In addition to exopolysaccharides and proteins, eDNA is an important component of the extracellular matrix of biofilms. The eDNA molecule shows negative charge and potential electrostatic activity that contribute to cell adhesion to surfaces, host factors, and other cells (Lister & Horswill, 2014; Rabin et al., 2015), even favoring *in vitro* biofilm formation (Otto, 2018; Sarkar, 2020; Whitchurch, Tolker-Nielsen, Ragas, & Mattick, 2002). Previous studies observed that *S. aureus* biofilms were susceptible to DNase I treatment, indicating the relevance of eDNA in those biofilms (Izano et al., 2008; Waryah et al., 2017). Thus, bromelain DNase activity might be an additional antibiofilm mechanism used by these enzymes, although further studies are still necessary to evaluate this activity on biofilms.

Hydrophobicity and auto-aggregation are properties related to increased cell adhesion, which is associated with biofilm formation (Choi, Bae, & Lee, 2015). Thus, impairment of these cellular properties by bromelain might be linked to its proteolytic and DNase activity on protein adhesins and eDNA molecules presented on the cell surface.

Furthermore, it was observed that 1% bromelain acts more effectively on *S. aureus* biofilms of strains isolated from chronic wounds and resistant to methicillin. Biofilm formation in MRSA relies on the release of eDNA molecules and the presence of cell surface-associated proteins. While biofilms of MSSA strains are mostly dependent on PIA (McCarthy et al., 2015; O'Neill et al., 2007). Faced with these facts, it's understandable that biofilms of chronic wound strains in this study have been more affected by 1% bromelain given its proteolytic and DNase activity.

Infected wounds can increase the risk of limb loss and mortality. Biofilms are found in more than 90% of chronic wounds, and *S. aureus* is one of the most abundant species in these wounds (Attinger & Wolcott, 2012). The presence of biofilms on wound surfaces makes their treatment even more challenging if the microorganisms present in the biofilms are multi-drug resistant (Kalan et al., 2019; Munro et al., 2019). Thus, the greater activity of bromelain in *S. aureus* biofilms from chronic wounds and MRSA strains may correspond to a highly promising alternative for the control of *S. aureus* biofilms in chronic wounds, contributing to the healing of skin injuries.

Bromelain did not show antibacterial activity in this study. Similarly, this protease did not show antibacterial activity in a study that investigated the role of essential oils and enzymes in *K. pneumoniae* strains (Mohamed et al., 2018). A plant protease close to bromelain, papain, did not exhibit antibacterial activity in previous study either (de Oliveira et al., 2014). Papain was not able to prevent the growth of methicillin-resistant strains of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* isolated from clinical bacteremia (de Oliveira et al., 2014). However, the use of bromelain conjugated to bacterial nanocellulose membrane appears to be a promising strategy in the development of the antibacterial activity of this enzyme (Lencastre Novaes et al., 2016).

However, the absence of antibacterial activity of bromelain does not detract from the antibiofilm activity of this enzyme. The antibiofilm approaches are alternatives that evade the high potential development of bacterial resistance, as they are aimed at combating EPS from the extracellular matrices of biofilms. Furthermore, they promote greater efficiency in the treatment of biofilms, as they act as dispersive agents, facilitating the exposure of bacterial cells to antibiotics (Kranjec et al., 2021). In this context, bromelain is safely delineated as a potential alternative for antibiofilm control in *S. aureus*.

Conclusion

This study aimed to investigate the antibiofilm and antibacterial activity of bromelain in a variety of *S. aureus* strains from chronic wounds and blood cultures. It was observed a promising antibiofilm activity of bromelain, mainly in the biofilms of chronic wound strains that showed resistance to methicillin, with the

proteolytic activity of bromelain being the main antibiofilm mechanism of this enzyme. Its DNase activity may have been relevant in the inhibition and destruction of biofilms, but further studies are necessary to evaluate this activity on biofilms. Bromelain did not exhibit antibacterial activity against *S. aureus* strains. However, the strong activity of bromelain in *S. aureus* biofilms from chronic wounds strains, its efficiency at a concentration of 1%, and the therapeutic properties of this enzyme make bromelain a promising alternative to the therapeutic arsenal to control *S. aureus* in wounds.

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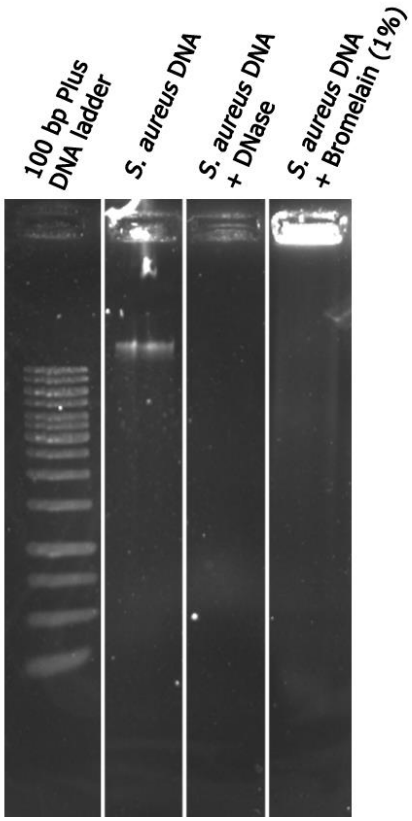
Supplementary Table 1. *Staphylococcus aureus* strains used in the study.

ID #	Strain	Origin	Methicillin susceptibility
1	ATCC 25923	Reference	Sensitive
2	ATCC 29213	Reference	Sensitive
3	16*MM	Chronic wound	Resistant
4	33MM	Chronic wound	Sensitive
5	11MM	Chronic wound	Resistant
6	29*MM	Chronic wound	Resistant
7	41MM	Chronic wound	Sensitive
8	25.2	Chronic wound	Sensitive
9	26.1	Chronic wound	Sensitive
10	30.3	Chronic wound	Resistant
11	33.3	Chronic wound	Sensitive
12	34.3	Chronic wound	Sensitive
13	24.1	Chronic wound	Sensitive
14	27.1	Chronic wound	Sensitive
15	28.1	Chronic wound	Resistant
16	1	Chronic wound	Resistant
17	3	Chronic wound	Resistant
18	4	Chronic wound	Sensitive
19	5	Chronic wound	Sensitive
20	7	Chronic wound	Resistant
21	9	Chronic wound	Sensitive
22	10	Chronic wound	Resistant
23	13	Chronic wound	Resistant
24	14	Chronic wound	Resistant
25	15	Chronic wound	Sensitive
26	16	Chronic wound	Sensitive
27	19	Chronic wound	Resistant
28	21	Chronic wound	Sensitive
29	24	Chronic wound	Resistant
30	26	Chronic wound	Sensitive
31	CM 160	Blood culture	Sensitive
32	176	Blood culture	Sensitive
33	196	Blood culture	Sensitive
34	283	Blood culture	Sensitive
35	363	Blood culture	Resistant
36	459	Blood culture	Resistant
37	475	Blood culture	Sensitive
38	479	Blood culture	Resistant
39	489	Blood culture	Sensitive
40	492	Blood culture	Resistant
41	499	Blood culture	Sensitive
42	504	Blood culture	Sensitive
43	510	Blood culture	Sensitive



Supplementary Figure 1. DNase activity of bromelain. Untreated *S. aureus* genomic DNA, DNA treated with DNase I, and DNA treated with 1% bromelain were submitted to 0.8% agarose gel electrophoresis, stained with ethidium bromide and exposed to UV light for visualization of DNA degradation.