NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

Disruption of Methicillin-resistant *Staphylococcus aureus* Biofilms with Enzymatic Therapeutics

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DECLARATION OF INTEREST

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ABBREVIATIONS

CFUs	Colony forming units
CLSM	Confocal laser scanning microscopy
CV	Crystal violet
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
EPS	Exopolysaccharide
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
OD	Optical density
PBS	Phosphate-buffered saline
SEM	Scanning electron microscopy
TSB	Tryptic soy broth

1. EXECUTIVE SUMMARY

Objective: Successful treatment of combat-related maxillofacial infections is significantly hindered by the formation of bacterial biofilms, which can be up to 5000 times less susceptible to conventional antimicrobials than planktonic cells. This profound drug tolerance has been linked, in part, to the extracellular matrix of the biofilm that protects the bacteria against antibiotics and the host immune system. Enzymatic debridement agents, which are used clinically to clear away necrotic tissue in chronic wounds to promote healing, have been tested against several bacterial wound pathogens. However, there remains a gap in knowledge of how these compounds impact clinically relevant bacterial biofilms. The purpose of this study was to establish an *in vitro Staphylococcus aureus* biofilm model that mimics wound-like conditions and employ this model to evaluate the anti-biofilm activity of four enzymatic compounds.

Methods: To establish the biofilm model, overnight cultures of methicillin-sensitive and methicillin-resistant *S. aureus* were diluted in tryptic soy broth supplemented with varying concentrations of human plasma from 0-50% and incubated statically in 96-well microplates for 24 hours at 37°C (n=3 to 4). Total biomass was quantified using the crystal violet assay to determine the plasma concentration that gave the most robust biofilms. To evaluate biofilm dispersal activity of the compounds, *S. aureus* biofilms were grown for 24 hours in broth with 10% plasma and then treated with varying concentrations of α-amylase, lysostaphin, bromelain, or papain for 2 or 24 hours (n=3 to 4). Biofilm biomass was quantified using the crystal violet assay, and the effects of the enzymes on the *S. aureus* exopolymeric matrix were visualized utilizing confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM).

Results: Supplementation with 10% human plasma resulted in the most robust and reproducible biofilm cultures. All four enzymes significantly reduced biofilm biomass compared to controls after 2 and 24 hours of treatment. Lysostaphin decreased biomass by up to 76% in the 6 bacterial strains, whereas the other agents reduced biomass by up to 94-98%. CLSM and SEM confirmed that the dispersal agents detached the polysaccharide matrix and bacteria from the growth surface. α-Amylase, bromelain, and papain caused removal of most of the polysaccharide matrix, but incomplete detachment of bacterial cells. Imaging also indicated that lysostaphin caused less degradation of the biofilm matrix than the other agents, but unlike the other enzymes, induced changes in cell morphology indicative of bacterial cell damage.

Conclusion: Use of enzymes may be an effective means of eradicating biofilms and a promising strategy to improve treatment of bacterial infections. Future studies will focus on the development of novel delivery systems for use of these agents in maxillofacial wounds.

2. INTRODUCTION

Head, neck, and maxillofacial areas are frequently susceptible to battlefield injuries, which can subsequently develop debilitating bacterial infections. *Staphylococcus aureus* is one of the leading causes of infections in deployed U.S. military personnel [1]. In a 2003-04 survey of infections treated at Combat Support Hospitals in Iraq, *S. aureus* accounted for 26% of the isolates from U.S. soldiers [2]. Battlefield associated *S. aureus* wound infections are especially difficult to treat because of an increased prevalence of multidrug resistant strains [3]. Indeed, the estimated annual cost of multidrug resistant *S. aureus* infections ranges from $15-36 million for the U.S. Army and ranks in the billions of dollars for the entire U.S. [4].

An additional factor that may significantly complicate the treatment of trauma related infections is formation of biofilms [5]. Microorganisms within biofilms form complex, often multi-species microbial communities that are enclosed in an exopolysaccharide (EPS) matrix and attached to a surface and each other [6, 7]. Bacterial biofilms are intrinsically less susceptible (500-5,000 fold) to antimicrobials than planktonic cells [8, 9] and are implicated in a vast array of chronic diseases including pulmonary infections, periodontitis, otitis media, and non-healing wounds [7, 10, 11]. The multidrug tolerance observed in biofilms is a transient, non-heritable phenotype [12] that differs from the mechanisms of conventional antibiotic resistance, such as upregulation of efflux pump expression and modification of antibiotic target sites, typically observed in planktonic cells [13]. Rather, the antimicrobial tolerance and phenotypic changes that occur in biofilms have been linked to the EPS matrix that surrounds and protects bacterial cells, similar to a wall garnering protection to a city [14]. Consequently, the EPS matrix has become a primary target for increasing bacterial biofilm susceptibility to antimicrobials and ultimately for biofilm removal.

Biofilm associated burn and wound infections are typically managed using debridement, which involves the removal of necrotic and infected tissue to promote healing [15]. Numerous debridement techniques are utilized clinically, including mechanical, surgical, osmotic, ultrasound, enzymatic, and maggot therapies [16]. Mechanical and surgical debridement are most commonly employed because of the rapidness and specificity of these techniques [17]. However, these procedures can cause pain, collateral tissue damage, extended hospital stays, and significant increases in healthcare costs [18]. Maggot therapy has shown promise in debriding non-healing chronic wounds, but was determined to be ineffective in treating wounds heavily colonized with certain wound pathogens [19]. Enzymatic debridement is an attractive alternative with potential effectiveness against a broad range of bacterial pathogens, is less painful, and like maggot therapy, can be applied at the bedside [20].

Although enzymatic debridement agents target necrotic tissue, they also degrade components of the bacterial biofilm EPS matrix, including polysaccharides, proteins, and bacterial/host DNA [21]. While these enzymes have been utilized clinically since the 1940s [20], there is a clear gap in knowledge regarding the optimal treatment times and working concentrations required to effectively degrade the EPS and their level of activity against biofilms cultured in the presence of host components. Recent studies have investigated the use of enzymes to disperse biofilms, but involved use of agents that may not easily translate to the clinic or lacked clear clinical relevance regarding the biofilm model. The main enzymes that have been tested on *S. aureus* biofilms in pre-clinical models include DNase I [22], dispersin B [22], lysostaphin [23], and proteinase K [22], while the most common and effective enzymatic agents for clinical use include papain/urea, bromelain, DNase I/fibrinolysin, krillase, sutilains, and collagenase [24]. In addition, many of the *in vitro S. aureus* biofilm models used to test dispersal agents lacked key host components that contribute to biofilm formation [22, 23, 25, 26]. Thus, the focus of this study was to evaluate the effect of dispersal agents against *S. aureus* biofilms using a more clinically relevant *in vitro* model and panel of enzymes. Specifically, this study utilized an *in vitro* human plasma biofilm model reported recently to form robust biofilms [27], clinical *S. aureus* strains, and enzymatic dispersal agents that have either been used clinically or can easily transition to the clinical setting. These enzymes included an anti-polysaccharide agent, α-amylase, an anti-peptidoglycan agent, lysostaphin, and two proteases, bromelain and papain.

3. MATERIALS AND METHODS

3.1 Bacterial strains

Six strains of *S. aureus* were used for this study. These included methicillin-sensitive *Staphylococcus aureus* (MSSA; ATCC # 29213), methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC # 33591) and 4 MRSA wound infection clinical strains, namely, IQ00070, SA5214, SA5123 and SA5120, obtained from the U.S. Army Institute of Surgical Research (JBSA-Fort Sam Houston, TX). Bacterial stocks were maintained at -80°C.

3.2 Enzymes

α-Amylase from *Bacillus subtilis* (Cat No. 10069), bromelain extracted from pineapple stem (Cat No. B4882), lysostaphin from *Staphylococcus simulans* (Cat No. L9043) and papain extracted from *carica papaya* (Cat No. 76220) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of lysostaphin were first prepared in 20 mM sodium acetate at pH 4.5 and

stored at -20°C. Working dilutions were then prepared using 20 mM Tris-HCl with 100 mM NaCl at pH 7.4. Working concentrations of the other enzymes were prepared daily from the powder form using 20 mM Tris-HCl and 100 mM NaCl at pH 7.4.

3.3 Biofilm growth and enzyme treatment

Overnight cultures of *S. aureus* were grown in tryptic soy broth (TSB; Cat No. 211825, Fisher Scientific, Hampton, NH) at 37°C with shaking at 250 rpm for 16-18 hours. Bacterial concentration was measured by reading the optical density at 600 nm (OD_{600}) using a Synergy HT microplate reader (BioTek®, Winooski, VT). To test the effect of human plasma on biofilm formation, the overnight cultures were diluted to obtain an OD_{600} of 0.1 (equivalent to 10^7 colony forming units (CFUs)/mL) using TSB supplemented with 0, 10, 20, or 50% human plasma (Cat No.130-11-08, Biological Specialty Corporation, Colmar, PA). Bacterial suspensions (100 µL) were then added to 96-well non-treated flat-bottom tissue culture polystyrene plates (Cat No. 12-566-202, Fisher Scientific) and incubated statically for 24 hours at 37°C to produce biofilms. The media was then removed by pipetting, and the biofilms in the wells were gently washed once with 1X phosphate-buffered saline (PBS; Cat No. 10010-023, Life Technologies, Carlsbad, CA) to remove non-adherent cells before use in the crystal violet (CV) assay described below.

To test the effect of the enzymatic dispersal agents, *S. aureus* overnight cultures were prepared as described above, diluted to an OD_{600} of 0.1 using TSB with 10% human plasma, seeded into 96-well plates, and incubated statically for 24 hours at 37°C for biofilm formation. The media was removed, and biofilms were washed once with 1X PBS. Various concentrations of the dispersal agents in a volume of 100 µL were added to the biofilms, and the plates were incubated for 2 or 24 hours at 37°C. Thereafter, the dispersal agent was aspirated off, and the biofilm was washed once with 1X PBS prior to use in the CV assay.

3.4 Crystal violet assay

A colorimetric CV assay for total biofilm biomass was used to evaluate the effect of human plasma and the dispersal agents on the *S. aureus* biofilms. In brief, 100 µL of 0.01% CV working solution (Cat No.V5265, Sigma-Aldrich) was added to the biofilms in each well and incubated for 30 minutes at room temperature. The CV solution was removed and the biofilms were washed once with 1X PBS. The plate was incubated at room temperature for 30 minutes to allow the wells to dry. After drying, 100 µL of 95% ethanol (Cat No. E7023, Sigma-Aldrich) was added to each well and incubated at room temperature for 30 minutes. CV absorbance was

measured at 595nm (OD_{595}) using a BioTek® Synergy HT microplate reader with Gen5™ v2.0 software.

3.5 Confocal laser scanning microscopy (CLSM)

To allow facile confocal microscopy imaging, biofilms were prepared on 5mm round glass coverslips (Cat No. 1217H19, Thomas Scientific, Swedesboro, NJ). The coverslips were placed in 96-well plates, coated with 100 µL of 1 mg/mL human fibrinogen (Cat No. F3879, Sigma-Aldrich) in PBS, and sterilized by UV light exposure for 15 minutes. After incubation for 24 hours at 4°C, the fibrinogen was removed from the coverslips and 100 µL of MRSA SA5120 suspended in TSB and 10% human plasma was added to each well. The coverslips were incubated statically at 37°C for 24 hours. Next, the media was removed from the wells and biofilms were washed once with 1X PBS then treated for 2 hours at 37°C with varying doses of dispersal enzymes. After treatment, the enzymes were removed from the wells and biofilms were washed once with 1X PBS. Polysaccharide and protein moieties in the biofilms were stained with 50 mM concanavalin A-Alexa Fluor® 488 conjugate (Cat No. C11252, Life Technologies) and 100 µL of FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (Cat No. F10318, Life Technologies), respectively, for 30 minutes. Thereafter, these two stains were aspirated from the wells, nucleic acids were stained for 5 minutes with 0.5 µg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Cat No. D1306, Life Technologies), and biofilms were washed once with 1X PBS. The coverslips were removed from the wells with needle-point tweezers, placed on a glass slide, and treated with 20 µL of *SlowFade*® Gold Antifade Mountant (Cat No. S36937, Life Technologies) to prevent loss of fluorescence. Square coverslips were mounted over the round coverslips on the microscope slides, and biofilms were imaged with an Eclipse C1 confocal laser scanning microscope (Nikon, Tokyo, Japan).

3.6 Scanning electron microscopy (SEM)

MRSA SA5120 biofilms were grown on fibrinogen-coated glass coverslips using the aforementioned protocol, and then prepared for SEM as previously described [28]. Briefly, biofilm samples were fixed in 2.5% phosphate-buffered glutaraldehyde for 1 hour at 4°C. Samples were then washed thrice with 0.1 M phosphate buffer for 10 minutes at 4°C. Biofilms were dehydrated with serially increasing concentrations of ethanol in cold water (50%, 70%, 80%, 90%, 95%, and 100%) for 10 minutes each at 4°C. The samples were further dehydrated twice more with 100% ethanol for 10 minutes at 4°C and then treated with 50% ethanol/50% hexamethyldisilazane for 5 minutes with gentle rocking at room temperature. The final treatment

was 100% hexamethyldisilazane for 10 minutes with gentle rocking at room temperature. Excess liquid was removed from the wells and samples were left in a fume hood overnight to remove any remaining liquid. Coverslips were removed from the wells, mounted with carbon tape on specimen stubs, and sputter coated with gold using a Hummer 6.2 Sputter Coater (Anatech USA, Union City, CA). Imaging was performed with a SIGMA VP40 field emission scanning electron microscope (Carl Zeiss Inc., Jena, Germany).

3.7 Statistics

Data in the graphs are expressed as the mean and standard deviation of 3-4 independent experiments, in which six technical replicates were used for each experimental group. Statistical analysis was conducted with GraphPad Prism 6 (v. 6.04, GraphPad Software, Inc., La Jolla, CA). One-way ANOVA and Dunnett's multiple comparison tests were utilized to compare the control group means to the treated group means. *P*-values ≤0.05 were considered statistically significant.

4. RESULTS AND DISCUSSION

4.1 Supplementation of TSB media with human plasma enhances S. aureus biofilm formation

In the past decade, numerous studies have been conducted to develop more clinically relevant *in vitro S. aureus* biofilm models that better reflect *in vivo* wound conditions. To accomplish this, various types of media have been examined [29, 30], multiple microbial species have been grown together [29, 31-33], and host components have been supplemented to the media [27, 29-33]. One of the unifying aspects of these studies is the addition of plasma (or key components of plasma, namely, albumin [33]) to the media, which has been shown to promote cell attachment [34] and enhance *S. aureus* biofilm formation [27, 30]. Thus, in the current study, *S. aureus* biofilm formation over 24 hours was tested using TSB media supplemented with 0-50% human plasma, where the biofilm biomass was quantified with the CV assay. Similar to a recent study [27], results showed that the addition of as low as 1% plasma to TSB significantly increased biomass accumulation for 5 of the 6 strains cultured for 24 and 48 hours (Figure 1). It was also observed that supplementing the media with 10% plasma resulted in the highest bioaccumulation relative to cells grown in TSB alone for all of the MRSA strains. The antibiotic sensitive laboratory strain (ATCC 29213), in contrast, exhibited similar levels of biofilm formation for 1% and 10% plasma. Biofilms cultured with 50% plasma showed less attachment to the plate compared to cells supplemented with 10% plasma. In addition, cultures grown in 50% plasma appeared to form gelatinous clots in the media (data not shown), suggesting

bacteria formed a biofilm island in the media and primarily attached to each other rather than onto the plate. This is consistent with a recent study involving a *Pseudomonas aeruginosa* and *S. aureus* dual species biofilm model grown in 50% plasma that suggested *S. aureus* coagulates the plasma to form a “host derived matrix” to which the bacteria attach rather than to artificial surfaces [31]. Surprisingly, when *S. aureus* biofilms were grown for 48 hours, there were negligible differences in biomass compared to the 24-hour time point (Figure 1). A plasma concentration of 10% was selected for use in all subsequent experiments based upon these results.

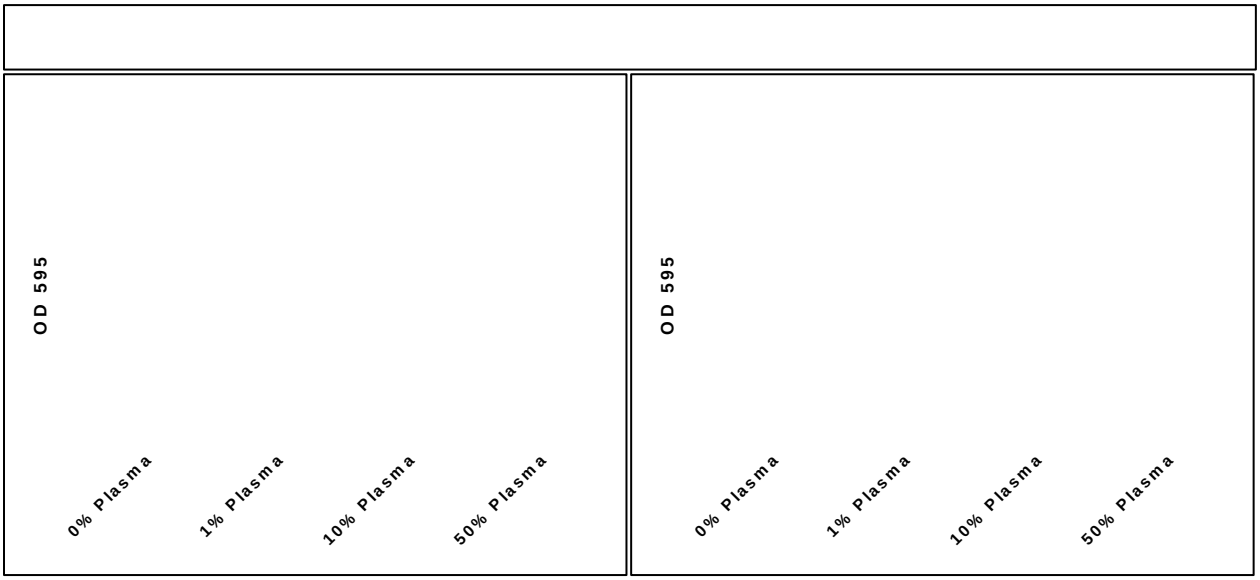

Figure 1. Effect of human plasma on *S. aureus* biofilm formation. *S. aureus* biofilms were cultured statically for 24 or 48 hours in TSB media supplemented with varying concentrations of human plasma. Biofilms were quantified using the crystal violet assay. Six strains of *S. aureus* were tested, and each treatment was performed in triplicate or quadruplicate with 6 technical replicates. Sample means were analyzed with one-way ANOVA followed by Dunnett's multiple comparisons tests. *p≤0.05 compared to the respective 0% plasma group mean.

4.2 Enzymatic dispersion of S. aureus biofilms over 2 hours

After establishing the *in vitro* biofilm model, the ability of dispersal enzymes to degrade mature MSSA and MRSA biofilms was evaluated. Six strains of *S. aureus in vitro* biofilms grown over 24 hours were treated with varying concentrations of α-amylase, lysostaphin, bromelain, or papain for 2 hours at 37°C. Following the 2-hour treatments, all *S. aureus* biofilm strains displayed a dose-dependent response to α-amylase and bromelain and were markedly sensitive to the 3 highest papain concentrations (Figure 2). In contrast, the dispersal effect of lysostaphin appeared to reach a maximum level at 50 µg/mL for all 6 bacterial strains and did not increase

substantially with increasing doses up to 200 µg/mL. In addition, all of the strains were less sensitive to dispersal by lysostaphin compared to the other enzymes. The highest enzyme concentrations tested caused the following decreases in biomass compared to the vehicle controls for the 6 *S. aureus* strains: 94-96% for 10 mg/mL of α-amylase, 32-61% for 200 µg/mL of lysostaphin, 83-94% for 100 µg/mL of bromelain, and 85-94% for 100 µg/mL of papain.

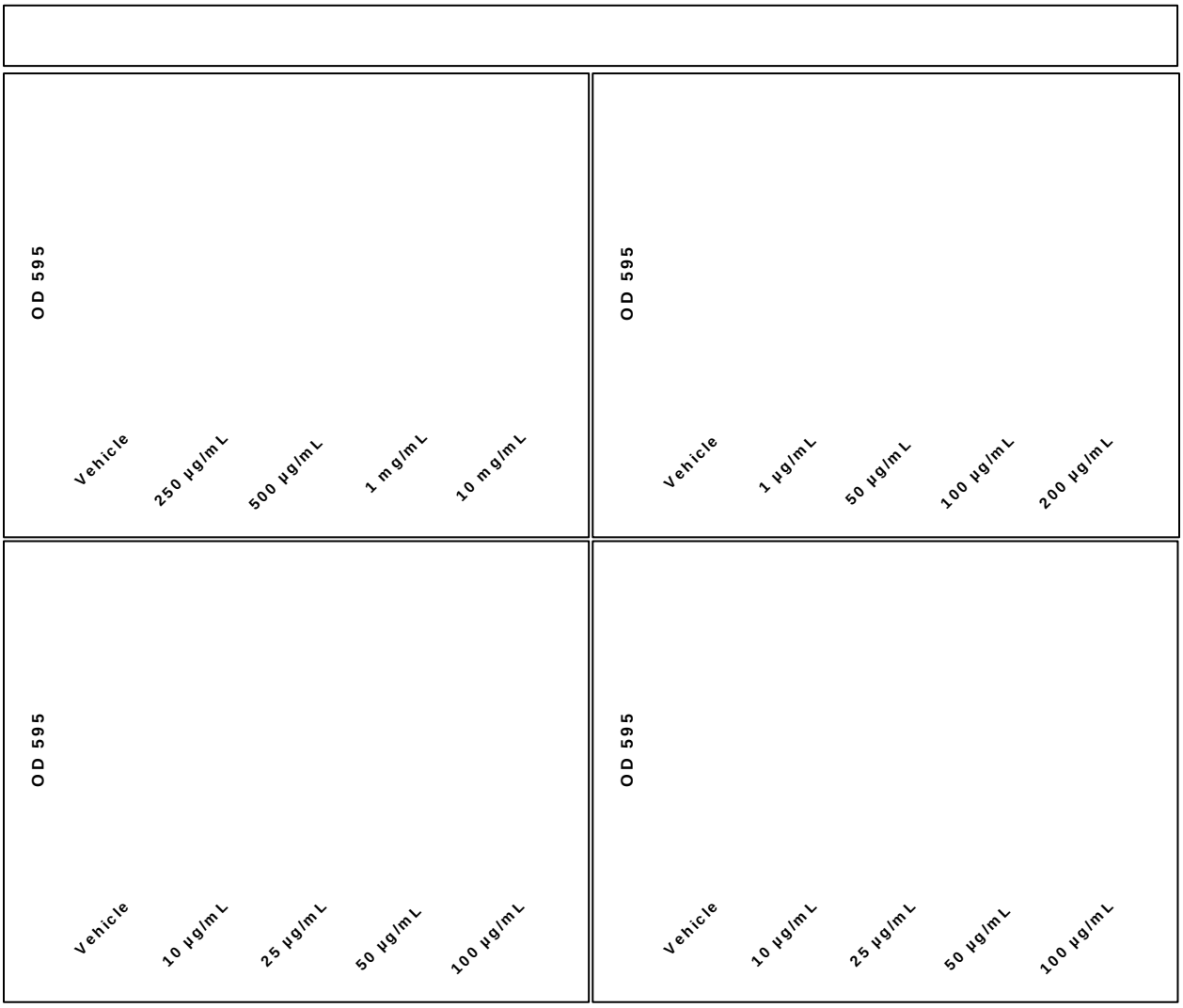

Figure 2. Dispersion of *S. aureus* biofilms with 2-hour enzymatic treatments. *S. aureus* biofilms were cultured in 96-well microplates for 24 hours then treated for 2 hours with α-amylase, lysostaphin, bromelain, or papain. The crystal violet assay was used to determine the total biomass remaining after the 2-hour treatment. Six strains of *S. aureus* were tested and each treatment was done in triplicate with 6 technical replicates. Sample means were analyzed with one-way ANOVA followed by Dunnett's multiple comparisons tests. *p≤0.05 compared to the respective vehicle control group mean.

The data also indicate that the relative sensitivities of the individual *S. aureus* strains were the most variable in response to lysostaphin treatment. One MRSA strain, SA5214, did not show significant decreases in biomass in response to lysostaphin at any of the tested concentrations, indicating strain dependent sensitivity to this agent. In contrast to lysostaphin treatment, all *S. aureus* strains showed significant and much more robust responses to the 3 highest concentrations of α-amylase, bromelain, and papain. Overall, no consistent pattern in the relative level of response of the antibiotic sensitive strain (ATCC 29213) versus the 5 MRSA strains to the four enzymes was noted, though testing of additional MSSA and MRSA strains is needed to confirm this finding.

The number of prior studies investigating the dispersal of *S. aureus* biofilms using similar exposure times and concentrations for the 4 enzymatic agents tested in the current study is limited. One previous study showed that treatment of biofilms with 10 mg/mL of α-amylase for 3 hours caused an estimated 86% dispersion [35], which is similar to that seen in the current investigation. Another prior study revealed that a 2-hour treatment of biofilms (grown without plasma) with 6.25 µg/mL of lysostaphin caused 57% dispersion [36], an effect similar to that observed for two of the strains in this study at higher concentrations of 50-200 µg/mL of lysostaphin. This disparity may be due to multiple factors, including differences in the biofilm model and the type of *S. aureus* strains utilized in the two investigations. No previous reports of similar studies using bromelain and papain are available.

Confocal laser scanning microscopy imaging was performed using MRSA SA5120 biofilms to confirm the dispersing efficacies of the 4 agents. Biofilms were exposed to the enzymes for 2 hours then stained with the fluorescent dyes concanavalin A-Alexa Fluor® 488, SYPRO® Ruby, and DAPI for visualization. The captured images show decreased amounts of the biofilm EPS matrix (green) in the enzyme treated samples compared to the vehicle control sample (Figure 3). These results agree with the data from the CV assays described above in that the images clearly indicate that α-amylase, bromelain, and papain caused greater levels of biofilm removal than lysostaphin. Though the mechanisms of action differ significantly, the anti-polysaccharide compound, α-amylase, and the 2 proteinases, bromelain and papain appeared to have similar dispersal activities against the polysaccharide rich matrix of the biofilms.

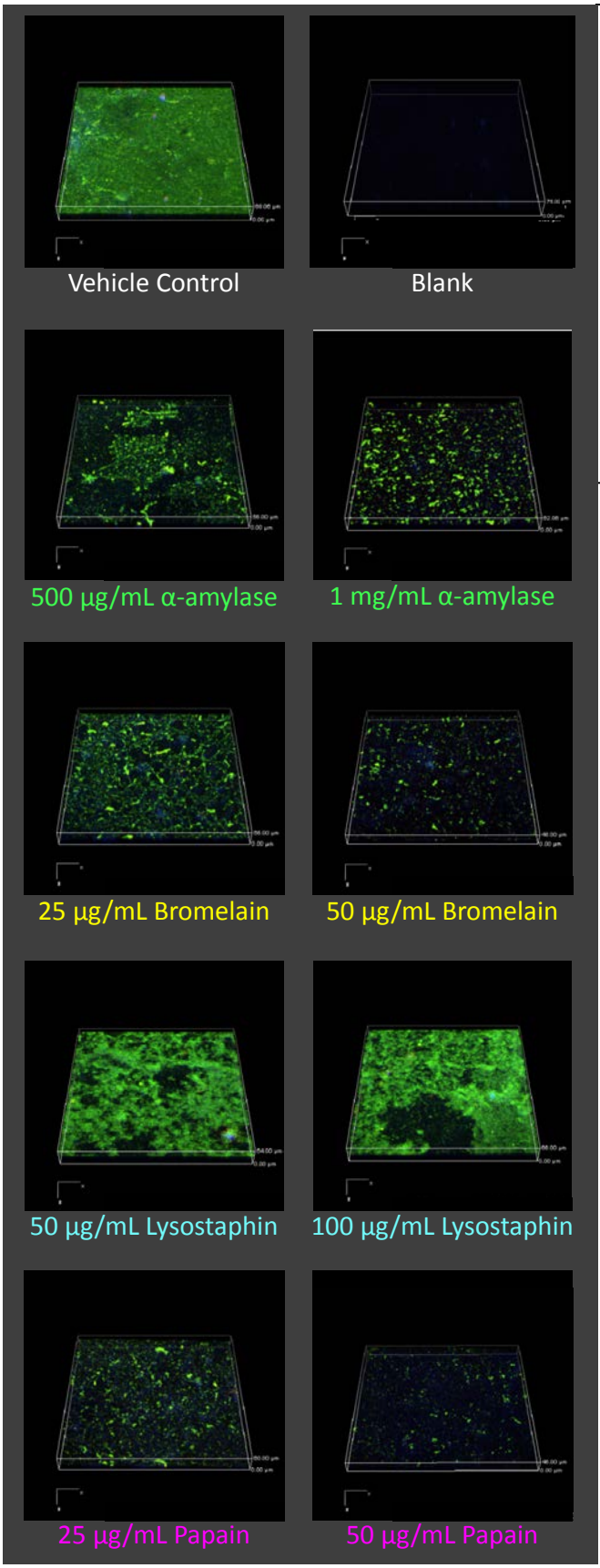

Figure 3. Characterization of MRSA SA5120 biofilms with CLSM after a 2-hour treatment with dispersal agents. MRSA SA5120 biofilms were cultured on glass coverslips for 24 hours then treated with α-amylase, bromelain, lysostaphin, or papain for 2 hours. The biofilms were stained with the fluorescent dyes concanavalin A-Alexa Fluor® 488 (green), Sypro® Ruby (red), and DAPI (blue) and visualized using a Nikon Eclipse C1 confocal laser scanning microscope with a 20X lens.

Visualization of enzyme treated MRSA SA5120 biofilms was also performed using SEM, which confirmed that α-amylase, bromelain, and papain degraded the EPS matrix and caused bacterial detachment (Figure 4). Lysostaphin appeared to induce structural changes in the biofilm, but in agreement with the CV assay and CLSM imaging results, caused less degradation of the matrix compared to the other 3 agents. Imaging with SEM at higher magnifications revealed that lysostaphin altered the cell morphology of *S. aureus* without much effect on the EPS, which remained largely intact (Figure 5C). In contrast, treatment with α-amylase, bromelain, and papain did not appear to affect cell morphology (Figure 5B, D, and E). This result is consistent with a previous study where up to 200mg/mL of α-amylase had no effect on the viability of planktonic *S. aureus* over 24 hours [35]. The data are also consistent with the known mechanism of action of lysostaphin, which is a well-characterized bactericidal agent that targets the cross-linking pentaglycine bridges of the *S. aureus* bacterial cell wall and can lyse planktonic cells within 10 minutes at concentrations as low as 2-5 µg/mL [23, 37]. Though the bactericidal activities of bromelain and papain against *S. aureus* have not been clearly elucidated, papain showed no antibacterial effect on 138 isolates of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* after 24 hours of treatment [38].

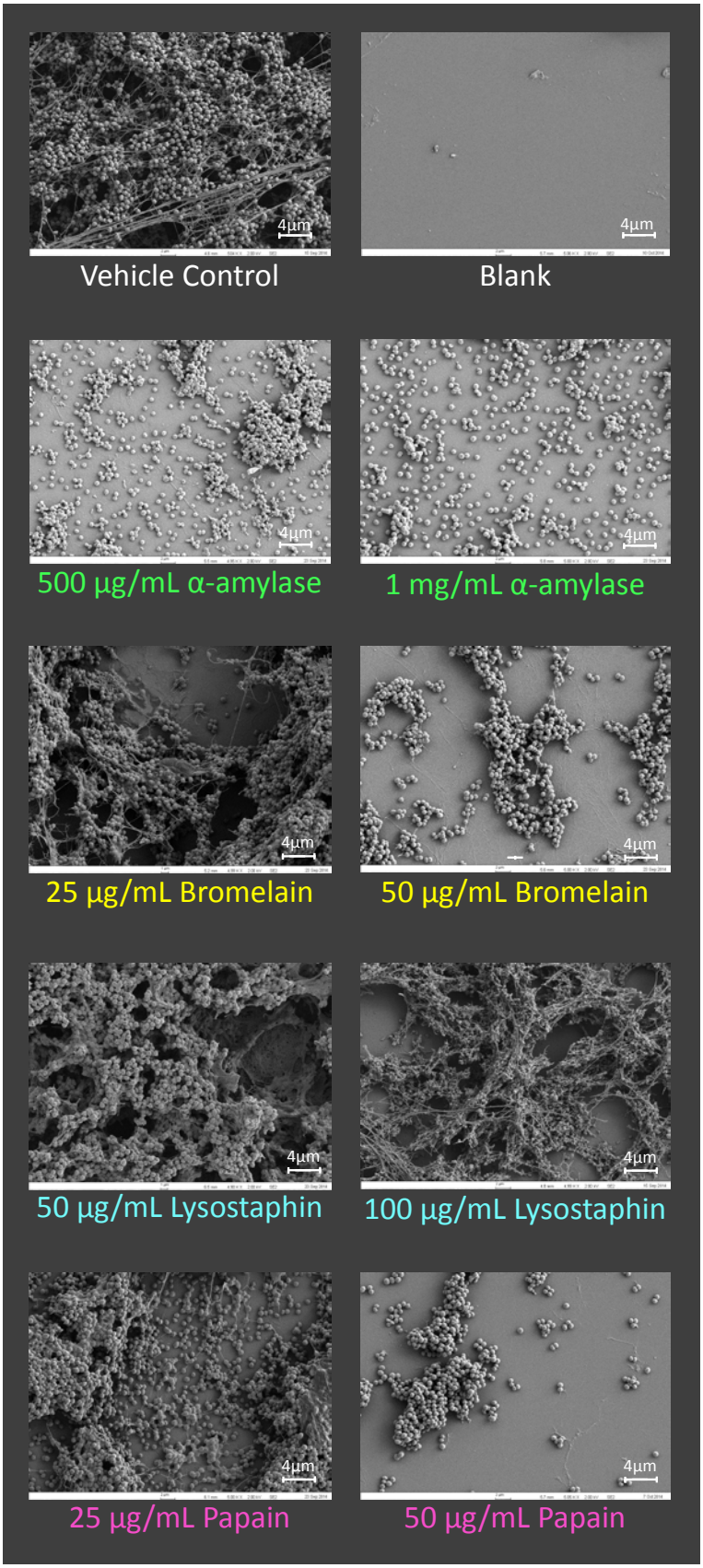

Figure 4. SEM images of MRSA SA5120 biofilms treated with dispersal agents. MRSA SA5120 biofilms were cultured on glass coverslips for 24 hours then treated with α-amylase, bromelain, lysostaphin, or papain for 2 hours. Biofilms were fixed, dehydrated, sputter coated with gold, and imaged using a Sigma-VP40 field emission scanning electron microscope (Carl Zeiss). Images were captured at 5,000X magnification. Scale bars were set at 4 µm.

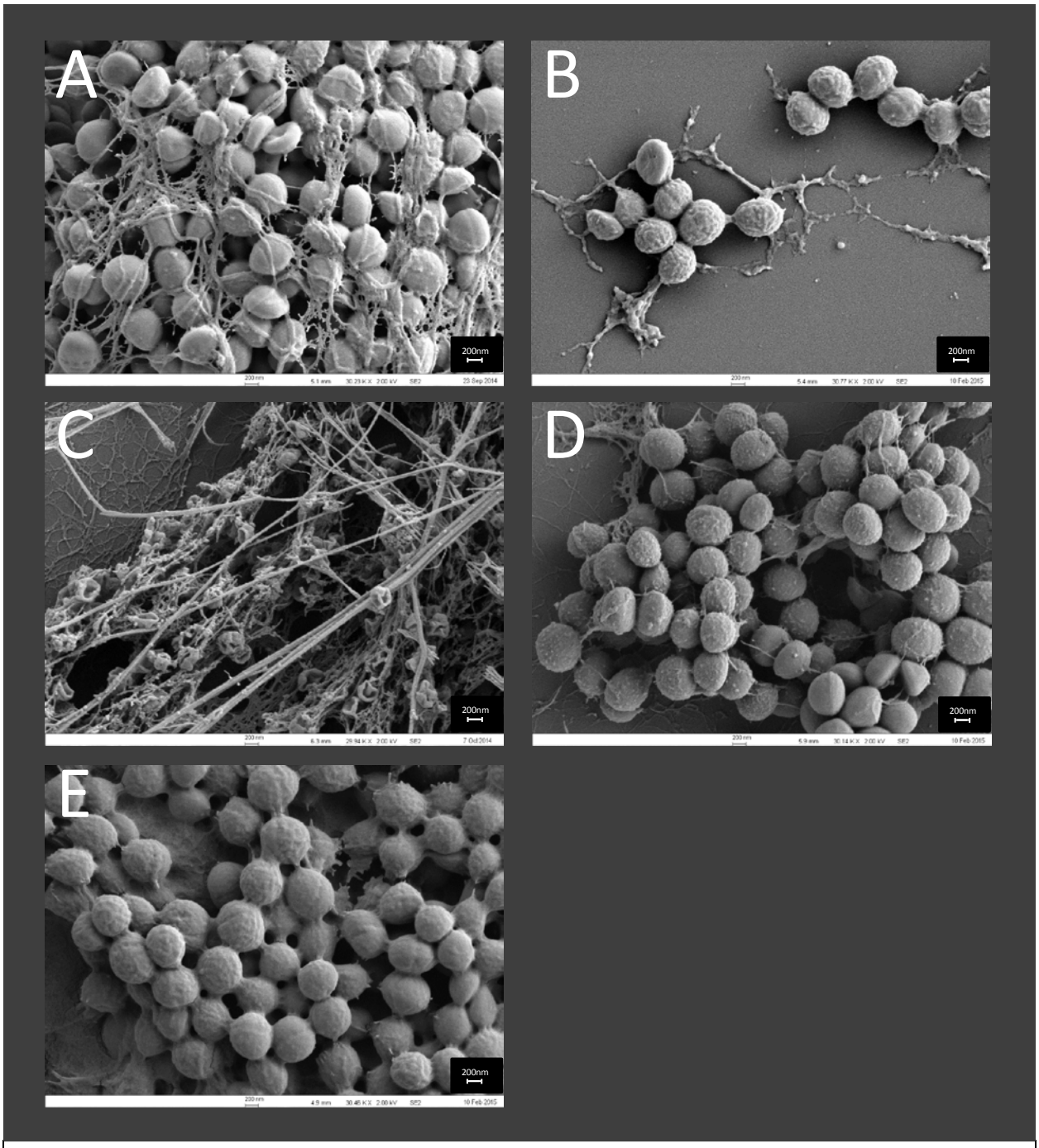

Figure 5. SEM images of the effect of dispersal agents on MRSA SA5120 cell morphology. MRSA SA5120 biofilms were cultured for 24 hours on glass coverslips then treated for 2 hours with A) vehicle, B) 1000 µg/mL α-amylase, C) 100 µg/mL lysostaphin, D) 50 µg/mL bromelain, or E) 50 µg/mL papain. Biofilms were fixed, dehydrated, sputter coated with gold, and imaged using a Sigma-VP40 field emission scanning electron microscope (Carl Zeiss). Images were captured at 30,000X. Scale bars were set at 200 nm.

4.3 Enzymatic dispersion of S. aureus biofilms over 24 hours

After establishing the enzymatic activities of the four agents for a 2-hour treatment, the biofilm degradation efficacies were assessed using a longer duration of treatment and reduced dispersal agent concentrations. *S. aureus* biofilms were cultured over 24 hours with 10% plasma then treated for 24 hours at 37°C with α-amylase, lysostaphin, bromelain, or papain (Figure 6). At the 24 hour treatment timepoint, the highest enzyme concentrations utilized caused the following decreases in biofilm biomass compared to the vehicle controls in the 6 *S. aureus* strains: 93-97% for 250 µg/mL of α-amylase, 95-97% for 25 µg/mL of bromelain, and 94-98% for 25 µg/mL of papain. Thus, it was observed that increasing the length of enzymatic treatment from 2 to 24 hours for these 3 agents allowed reduction of the enzyme concentration required to effectively degrade the biofilms. For lysostaphin, the greatest reduction in biofilm biomass for all 6 bacterial strains was achieved at 50 µg/mL and ranged from 49-76%. The highest dose of lysostaphin tested, namely 250 µg/mL, was less effective at dispersing some of the MRSA strains than the 50 µg/mL dose and caused only 6-35% dispersal of the biofilms. The reason for this pattern of response to lysostaphin is unknown, though one possible explanation is a more rapid lysis of bacteria and release of inhibitory cellular components that diminish enzymatic activity at the higher dose [39].

Several previous studies involving 24-hour treatment of *S. aureus* biofilms (cultured without plasma) with the 4 enzymes used in the current investigation have been reported [35, 36, 40]. For example, one prior study showed that only 6.25 µg/mL of lysostaphin in PBS were needed to cause a 7 fold reduction in *S. aureus* biomass [36]. Only a 1-2 fold decrease in biomass for most of the *S. aureus* strains at 8-16 times that concentration was observed in the current study. In a prior investigation of papain, only 5 µg/mL suspended in a similar Tris-HCl vehicle were required to completely disperse *S. aureus* biofilms [40]. This papain concentration is 2-5 fold lower than that needed to thoroughly disrupt biofilms in the current investigation. A previous study of α-amylase applied to *S. aureus* biofilms showed that 20-100 mg/mL were required to achieve maximal dispersion [35]. This concentration is 80-1000 times higher than the dose of α-amylase shown to elicit maximal biofilm dispersion in the current study. However, there were notable differences in several of the study parameters used in the current work and the aforementioned previous studies that may account for disparities in the results. These include the enzyme source and purity, enzyme vehicle, strains of *S. aureus*, and composition of the biofilm growth medium. At this time, no relevant previous studies have been conducted concerning the impact of a 24-hour treatment with bromelain on *S. aureus.*

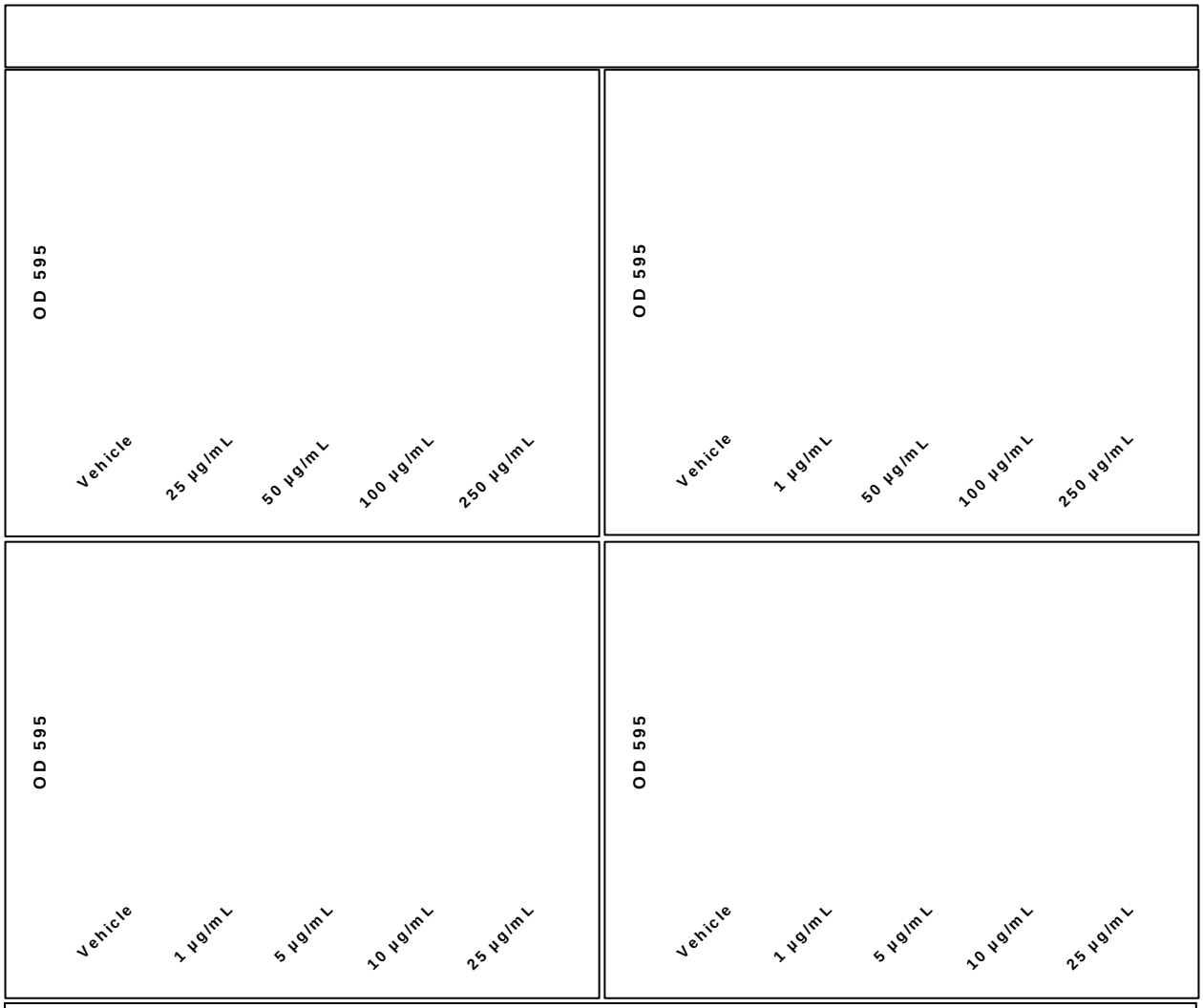

Figure 6. Degradation of *S. aureus* biofilms after 24-hour treatment with dispersal agents. *S. aureus* biofilms were cultured in 96-well microplates for 24 hours then treated for 24 hours with α-amylase, lysostaphin, bromelain, or papain. The crystal violet assay was used to determine the total biomass remaining after the 24-hour treatment. Six strains of *S. aureus* were tested and each treatment was done in quadruplicate with 6 technical replicates. Sample means were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons tests. *p≤0.05 compared to the respective vehicle control group mean.

4.4 Conclusions

Overall, it was observed that human plasma enhanced the *in vitro* biofilm formation of *S. aureus*, and that the clinical MRSA strains required greater plasma concentrations for maximal biofilm formation versus the antibiotic sensitive laboratory strain (ATCC 29213). Utilizing the human plasma biofilm model, 4 dispersing enzymes (α-amylase, lysostaphin, bromelain, and papain) were tested on *S. aureus* biofilms at 2 and 24 hours, and optimal treatment

concentrations were determined. Compared to prior studies of biofilms cultured without plasma, these findings suggest that biofilms established in growth media supplemented with 10% human plasma are more recalcitrant to dispersal by 2- and 24-hour treatments with lysostaphin and 24-hour treatment with papain. However, as human plasma significantly increases biofilm biomass, it is difficult to directly compare these data to the efficacy of dispersing enzymes on biofilms cultured with little or no plasma. The decreased dispersal observed in the current study may simply be due to the presence of more starting substrate that the enzyme must break down. Supplementation of growth media with 10% human plasma may also protect *S. aureus* biofilms from vancomycin in a similar fashion by increasing the number of bacteria in the starting biofilm cultures [27]. In addition to increasing biofilm biomass, human plasma may also upregulate expression of cell surface molecules that facilitate bacterial adhesion to surfaces, increase cell wall thickness, and change the composition of the extracellular matrix of the biofilm [27]. All of these factors could influence susceptibility of biofilms to enzymatic dispersal.

This study focused on examining biofilm dispersing enzymes *in vitro*, while maintaining similarities to clinical wounds through the addition of plasma and examination of clinical isolates, and using clinically relevant enzymatic agents. Future studies will focus on examining activity of dispersal enzymes against other species of bacteria commonly isolated from wound infections, use of enzyme combinations to maximize biofilm dispersion, and characterization of dispersal agent toxicity on host cells.

5. MILITARY SIGNIFICANCE

Bacterial wound infections are problematic for U.S. military members around the globe, and biofilms are recognized as a major factor contributing to treatment failure and chronic wounds in injured personnel. Development of novel strategies to better deal with biofilm infections is critical for addressing this healthcare challenge and maintaining the operational readiness of our warfighters. Use of enzymatic biofilm dispersal agents is an attractive approach because it has the potential to be effective against a broad range of bacterial pathogens, including multidrug resistant strains; can be combined with antibiotics to enhance current therapeutic regimens; the treatment can be administered at the bedside; and most of the tested agents induced marked biofilm removal after only a 2-hour duration of exposure. The current study provides the framework for future development of products that incorporate the dispersal agents into field deployable wound dressings, which could be utilized to both prevent and treat biofilm infections.

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14. ABSTRACT

The purpose of this study was to establish an *in vitro Staphylococcus aureus* biofilm model that mimics wound-like conditions and employ this model to evaluate the anti-biofilm activity of four enzymatic compounds. Varying concentrations of human plasma from 0-50% were supplemented into growth media to evaluate biomass accumulation in 2 laboratory and 4 clinical multi-drug resistant *S. aureus* strains. Supplementation with 10% human plasma resulted in the most robust and reproducible biofilms in all 6 strains. The enzymes papain, bromelain, α-amylase, and lysostaphin were then tested on the 6 *S. aureus* strains in the 10% human plasma biofilm model, and biofilm biomass was quantified using the crystal violet assay. All four enzymes significantly reduced biomass compared to controls in all strains after 2 and 24 hours of treatment. Lysostaphin decreased biomass by up to 76%, whereas the other agents reduced biomass by up to 94-98% in the 6 bacterial strains. Visualization of the biofilms with confocal laser scanning microscopy and scanning electron microscopy confirmed that the dispersal agents detached the biofilm matrix and bacteria from the growth surface, and lysostaphin, but not the other enzymes, induced changes in cell morphology indicative of bacterial cell damage. Overall, our results indicate use of enzymes may be an effective means of eradicating biofilms and a promising strategy to improve treatment of multi-drug resistant bacterial infections.

15. SUBJECT TERMS
Biofilm, Infection, Methicillin-resistant *Staphylococcus aureus*, Enzymes, α-amylase, Lysostaphin, Papain, Bromelain

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