



Combinatorial Biofilm-Releasing Agents with Colistin Reduces *Staphylococcus aureus* Biofilm Burden Below Clinical Threshold

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SUMMARY Biofilms are bacterial communities encased in an extracellular polymeric substance (EPS) that limits antibiotic penetration and promotes persistent infection. Biofilm formation contributes to antibiotic tolerance in pathogens such as *Staphylococcus aureus*, representing a major clinical challenge. We hypothesized that inducing biofilm dispersion would increase antibiotic susceptibility and improve biofilm clearance. Growth curve analysis demonstrated that glutamate, *cis*-2-decenoic acid (*cis*-DA), and amylase did not significantly affect *S. aureus* growth over 12 hours, whereas prolonged exposure to sodium nitroprusside (SNP) significantly reduced bacterial growth after 9 hours. Consequently, biofilm treatments were limited to 2 hours to minimize direct antimicrobial effects of SNP. Biofilm growth conditions were optimized, with mature biofilms generated after 4 days in 1:2 Brain Heart Infusion (BHI), shaking at 220 rpm. Biofilms were treated with releasing agents alone or in combination with 2x the minimum inhibitory concentration (MIC) of colistin (500 µg/mL). Although no statistically significant differences were observed between combination treatments and colistin alone, all combination treatments reduced biofilm cell counts. SNP and amylase reduced biofilm burden to near the clinical threshold (10⁵ CFU/mL), indicating a trend toward enhanced antibiotic efficacy and suggesting that dispersion-based adjunctive therapies may help reduce bacterial loads below levels associated with persistent biofilm infections.

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium frequently implicated in biofilm-associated nosocomial infections (1). Biofilms are microbial communities of bacterial cells encased in an extracellular polymeric substance (EPS) (2, 3) and are associated with approximately 80% of human bacterial infections (4). The EPS functions as a protective barrier that limits exposure to host immune defenses and antimicrobial agents (5). Novel strategies that promote biofilm dispersion without harming host tissues have gained attention (6). Dispersion represents the final stage of the biofilm lifecycle and involves the release of bacterial cells from the EPS (7). Dispersion may occur through either active or passive mechanisms, both of which compromise biofilm integrity and may increase susceptibility to antimicrobial treatment.

Active dispersion is triggered by environmental cues that initiate intracellular signaling pathways, frequently mediated by reductions in the secondary messenger c-di-GMP (6). Sodium nitroprusside (SNP) and *Cis*-2-decenoic acid (*cis*-DA), a fatty acid signaling molecule, both induce active dispersion by stimulating the production of EPS-degrading enzymes and recruiting phosphodiesterases (PDEs) that lower c-di-GMP levels (8). Reduced

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intracellular c-di-GMP promotes the transition from the sessile biofilm phenotype to the motile planktonic state by enhancing motility, thereby facilitating dispersion. Similarly, glutamate functions as a nutrient signal that triggers carbon flux, upregulates the dispersion regulator *bdlA*, and activates PDE activity(6).

In contrast, passive dispersion occurs independently of endogenous signaling pathways and is mediated by external physical or enzymatic forces. Amylase, a glycoside hydrolase, cleaves glycosidic linkages within polysaccharide components of the EPS matrix (9, 10). Amylase has demonstrated efficacy in disrupting *S. aureus* biofilms in *in vitro* and *in vivo* studies, without detectable host toxicity (11, 12).

Both active and passive dispersion strategies have been shown to enhance antibiotic susceptibility in biofilm-associated infections. Previous studies have demonstrated that dispersion induced by nitric oxide (NO) donors or EPS-degrading enzymes significantly reduces antibiotic tolerance in *S. aureus* biofilms when compared to antibiotic treatment (13-15). Colistin was selected because *S. aureus* exhibits intrinsic resistance to this antibiotic, allowing changes in biofilm susceptibility following dispersion to be evaluated independently of high baseline antimicrobial activity. This approach provides a stringent assessment of whether biofilm disruption alone can enhance antibiotic efficacy.

Although individual dispersion strategies have been investigated previously, direct comparisons between multiple active and passive releasing agents under identical experimental conditions, particularly in combination with antibiotic treatment, remain limited. Consequently, it is unclear which dispersion mechanism most effectively enhances antimicrobial activity and represents the greatest potential for clinical translation. Here, we directly compare three active dispersion-inducing agents (SNP, *cis*-DA, and glutamate) and one passive dispersion strategy (amylase), both alone and in combination with colistin to identify the most effective approach for reducing *S. aureus* biofilm burden. Our findings demonstrate that, while none of the combination treatments significantly improved biofilm clearance, SNP and amylase consistently reduced biofilm burden toward the clinically relevant threshold of 10^5 CFU/mL (11, 16, 17), highlighting these agents as promising candidates for future dispersion-based therapeutic strategies for clinical applications.

METHODS AND MATERIALS

Bacterial Culture Preparation

In this study, *Staphylococcus aureus* strain SA31 was used for the bacterial experiments. Planktonic overnight cultures of SA31 were prepared from a frozen stock in 10 mL of growth medium of Brain Heart Infusion (BHI) (Becton Dickinson; CAT #237500) or Lennox Broth (LB) (SigmaAldrich®; CAT #45-L3022). The overnight cultures were grown in 125 mL Erlenmeyer flasks, shaking at 37°C with shaking at 220 rpm for 16-18 hours.

Treatment preparation

To release cells from the biofilm, 310 nM *cis*-DA solution (Chemodex, Cat#DO249), 500 µM SNP (Sigma-Aldrich, Cat#71778), or 18 mM glutamate (Sigma-Aldrich, Cat#G1251) solutions were prepared in the appropriate assay media for immediate use. 500 U/mL amylase (from *Aspergillus oryzae*, Sigma-Aldrich, Cat#A9857) was prepared by dissolving lyophilized powder into the appropriate assay media and activated at 37°C for 30 minutes prior to use.

Growth curve

1:100 subcultures were grown in 250 mL Erlenmeyer flasks shaking at 220 rpm at 37°C for 12 hours in BHI with or without (media control) the releasing agents present. Every hour, absorbance values were measured at OD₆₀₀ for each subculture.

In vitro well-plate biofilm model optimization

1:100 subcultures were prepared in 10 mL of either 1:5 LB, 1:2 BHI, or 1:5 BHI in 125 mL Erlenmeyer flasks and incubated at 37°C shaking at 220 rpm for 4 hours. Following the *in vitro* 4-day biofilm 24-well plate model, biofilms were grown under static, 80 rpm, or 220 rpm shaking conditions in tissue-culture treated 24-well plates to ensure biofilm attachment. After 4-days of biofilm growth, spent media was discarded, attached biofilms were then

washed with 0.85% saline, and biofilm formation was determined by staining the attached biofilms with 1 mL of 0.1% crystal violet followed by incubation at 80 rpm for 15 minutes. Post incubation, the remaining crystal violet was discarded and each well was washed 3 times with 1 mL of deionized (DI) water. Plates were then dried overnight on the benchtop for 24 hours. Microscopy was completed using a VWR Basic Inverted Microscope and biofilms were imaged at 4X magnification. After taking microscopy images, the plates were eluted with 1 mL of 95% ethanol and incubated for 10 minutes at 37°C shaking at 80 rpm. The eluted crystal violet was transferred to a 96-well plate and read in the spectrophotometer at an absorbance of 570 nm. Absorbance was normalized to stained empty wells that contained only media.

Antibiotic preparation

10 mg/mL colistin (Research Products International; CAT # C70700) stock solution was prepared by dissolving the antibiotic into 0.85% saline and sterilized with a 0.22 µm filter (M2Sci; CAT #371-2215-OEM). Stock solutions were stored at -20°C.

Minimum Inhibitory Concentration (MIC)

A minimum inhibitory concentration assay was performed to determine the optimal concentration of colistin required to inhibit the growth of planktonic SA31 cells. The MIC of colistin was determined using a 96-well plate with 1:2 serial dilutions down the plate resulting in a 1,000-1.95 mg/mL range of colistin. Each well was inoculated with 20 µL of SA31 overnight culture adjusted to an OD₆₀₀ of 0.5. One column contained only LB to serve as a negative control, and another column contained LB and inoculant without colistin to serve as a positive control. The plate was then incubated at 37°C shaking at 220 rpm for 24 hours. Cell growth was measured at an absorbance of 600 nm using the spectrophotometer (SpectraMax® San Jose, CA, USA; Molecular Devices). MIC was determined by the lowest concentration of colistin in which the absorbance was equivalent to the negative control.

In vitro well-plate biofilm model (4-day)

Cell viability was determined using an *in vitro* 4-day biofilm model to measure the remaining bacterial load in the biofilm following treatment. Tissue-culture-treated 24-well plates (Cole-Palmer; CAT #229123) were inoculated with 500 µL of subculture grown in 1:2 BHI at 37°C shaking at 220 rpm with 12-hour media changes. Treatments were carefully prepared as previously stated with and without the respective 2 x MIC of colistin (1,000 µg/ml). Treatments included 1:2 BHI media control, releasing agent alone, or releasing agent with 2 x MIC colistin. Prior to treatment, unattached cells were removed, and biofilms were rinsed with 0.85%. Biofilms were treated for 2 hours in the same growing conditions of 37°C shaking at 220 rpm. Following treatment, the supernatant was discarded, and the remaining biofilm was rinsed with 0.85% saline. Biofilms were collected by physical disruption into fresh saline then homogenized for 10 seconds before being serially diluted and drop-plated on full-strength LB 1.5% agar plates. Drop plates were incubated for 48 hours at 37°C and CFU/mL were enumerated.

Statistical analysis

All statistical analyses were performed utilizing GraphPad Software, Inc. Multivariate analyses were performed by either unpaired student t-test, one-way, or two-way analysis of variance (ANOVA) by Tukey's multiple comparison test as noted in the figure legends.

RESULTS

SNP slows *S. aureus* bacterial growth. A growth curve was completed to determine whether the use of exogenous releasing agents impacts *S. aureus* growth. Bacterial subcultures were grown for 12 hours in 25 mL of media containing either a media control or releasing agent. Absorbance was measured at OD₆₀₀ hourly using a spectrophotometer.

No significant difference in bacterial growth was observed over a 12-hour period in the presence of glutamate, *cis*-DA, or amylase compared to the LB control (Figure 1), suggesting that these releasing agents do not impact *S. aureus* bacterial growth or exhibit cell toxicity. Interestingly, SNP exposure resulted in a significant reduction in bacterial growth between 9

and 12 hours of growth ($p < 0.0001$, $p = 0.0025$, $p = 0.0009$, and $p = 0.0006$, respectively). Consequently, biofilm treatment times were limited to 2 hours which is well below the 9-hour time point at which SNP began to significantly inhibit bacterial growth. Therefore, the observed effects on biofilm clearance are unlikely to be attributable to SNP-mediated growth inhibition.

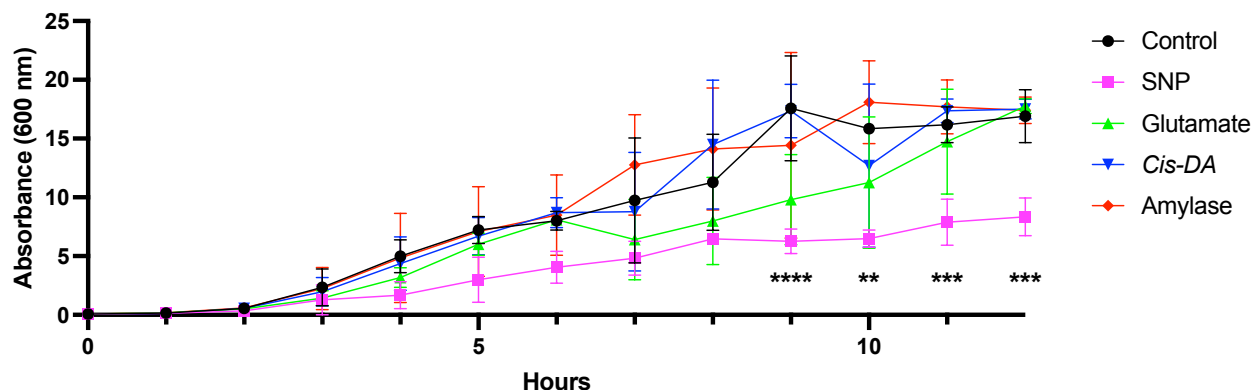


FIG. 1 SNP Slows *Staphylococcus aureus* Growth. SA31 subcultures were grown for 12 hours in either LB (control) or LB containing a releasing agent of 10 nM *cis*-DA, 500 μ M SNP, 18 mM glutamate or 500 U/mL amylase. Absorbance at 600 nm was measured every hour for 12 hours. Statistical significance was determined by two-way repeated-measures ANOVA to compare each treatment to the LB control at each time point. Mean $\pm$ standard deviation is shown. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). $n = 2$ -3/group.

Optimization of a 4-day *S. aureus* *in vitro* biofilm growth model under 220 rpm shaking conditions and 1:2 BHI. To determine optimal biofilm growth conditions for *S. aureus*, biofilms were grown shaking at either 0 rpm, 80 rpm, or 220 rpm in 1:5 LB, 1:5 BHI, or 1:2 BHI media. Following incubation, biofilms were stained with crystal violet and imaged before being eluted with ethanol to measure absorbance at OD570 nm.

The greatest biofilm biomass, as measured by crystal violet absorbance, was observed in 1:2 BHI at 0 rpm (0.10), followed by 80 rpm (0.088), and then 220 rpm (0.084) (Figure 2). Although the 0 rpm condition produced the highest average absorbance, it also exhibited the greatest variability among replicates, as reflected by the large error bars. In contrast, biofilms grown at 220 rpm demonstrated more consistent biomass while maintaining comparable absorbance values. Representative microscopy images of crystal violet-stained biofilms supported these findings and demonstrated more uniform biofilm formation under 220 rpm shaking conditions (Figure 3).

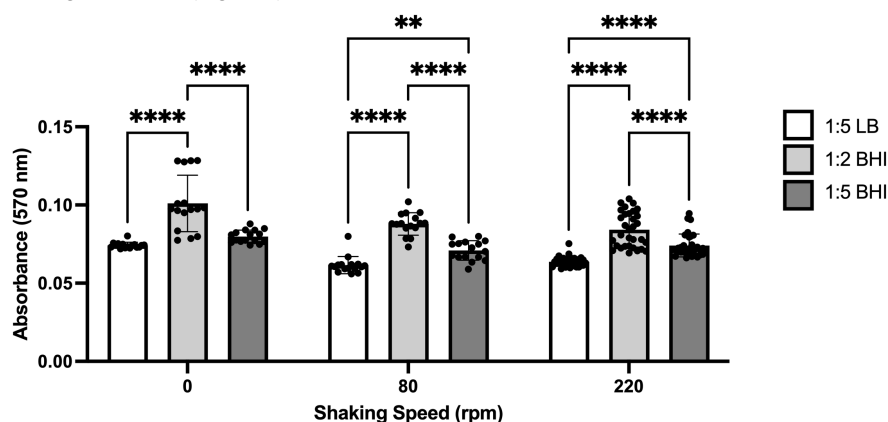


FIG. 2 *S. aureus* biofilms optimized under various shaking speeds and media conditions. 4-day biofilms were grown for 0 rpm, 80 rpm, or 220 rpm and in 1:5 LB, 1:2 BHI, or 1:5 BHI concentrations. On the final day, biofilms were stained with 1 mL of 0.1% crystal violet for 10 minutes at 80 rpm and 37 °C. Biofilms were then rinsed three times with 1 mL of 0.85% saline. Biofilms were eluted with 1 mL of 95% ethanol to collect absorbance using a spectrophotometer at OD570. Statistical significance was determined using a two-way ANOVA test. Mean $\pm$ standard deviation is shown. $n = 16$ -32.

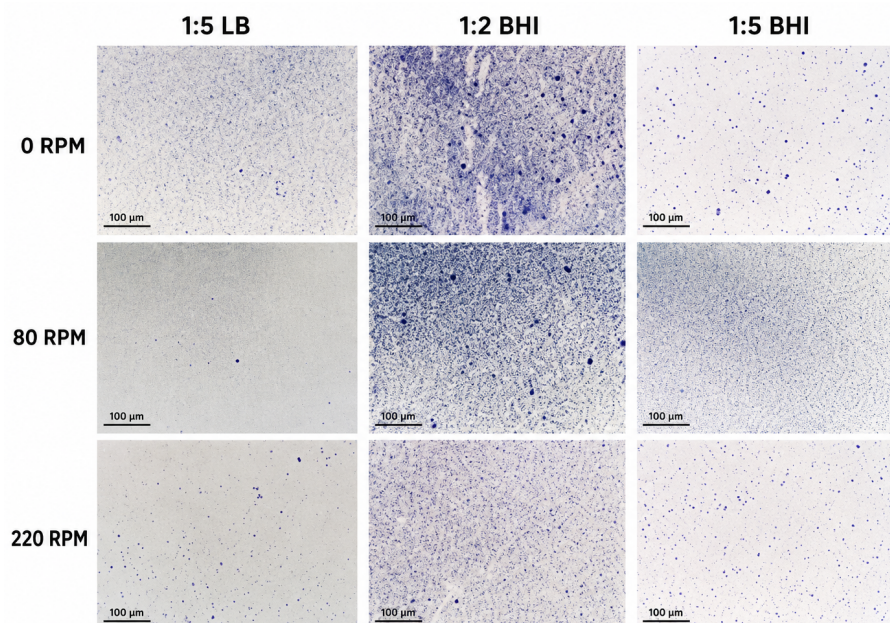


FIG. 3 Representative *S. aureus* biofilms imaged after 4 days of growth. 4-day biofilms were grown for 0 rpm, 80 rpm, or 220 rpm and in 1:5 LB, 1:2 BHI, or 1:5 BHI concentrations. On the final day, biofilms were stained with 1 mL of 0.1% crystal violet for 10 minutes at 80 rpm and 37 °C. Microscopy was completed at 4X magnification to visualize biofilm mass. n= 16-32.

Selected combination treatments reduce biofilm load toward clinical threshold. To determine whether releasing agents enhanced the antibiotic efficacy of colistin on *S. aureus* biofilms, 4-day biofilms were treated with media alone (control), 2 x MIC colistin (1,000 μg/mL), releasing agent alone, or a combination of releasing agent and 2 x MIC colistin. The colistin concentration was established using a minimum inhibitory concentration (MIC) assay (Supplemental Figure S1). Following treatment, biofilms were collected, serially diluted, and drop-plated to enumerate viable cells as CFU/mL (Figure 4).

Overall, combination treatments did not result in statistically significant reductions in biofilm burden compared to colistin alone (Figure 4A, B). However, all combination treatments exhibited reductions in biofilm-associated CFU/mL relative to colistin alone (2.64×10^6 CFU/mL). Moreover, combination treatments with SNP ($p > 0.999$ compared to SNP alone) and amylase ($p = 0.9960$ compared to amylase alone) resulted in more pronounced reductions in viable biofilm cells with CFU/mL values approaching the clinical threshold of 10^5 CFU/mL of 2.24×10^5 CFU/mL and 2.11×10^5 CFU/mL, respectively (Figure 4B). Although these reductions did not reach statistical significance, the observed decreases represent biologically meaningful improvements in biofilm clearance. Collectively, these results suggest that releasing agents, particularly SNP and amylase, may enhance colistin-mediated disruption of *S. aureus* biofilms.

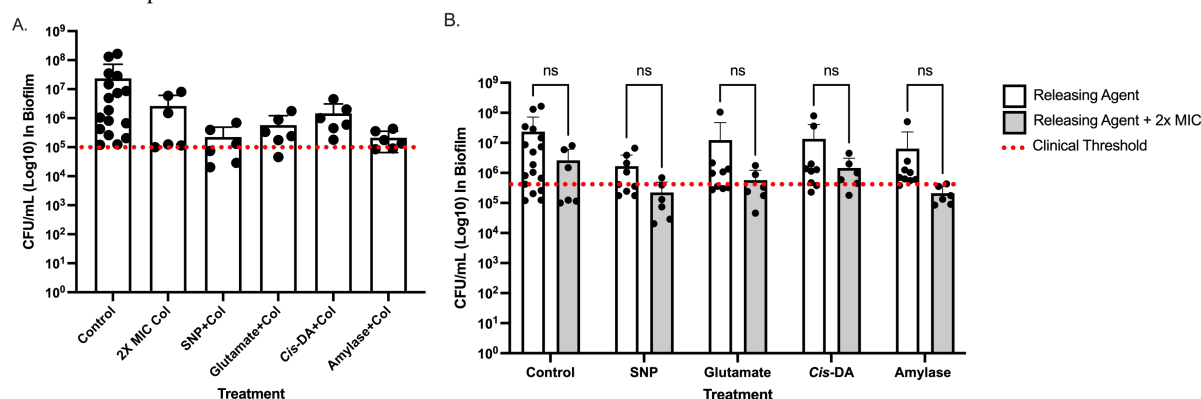


FIG. 4 Combinational treatments reduced biofilm load to clinical threshold. 4-day SA31 biofilms were treated for a period of two hours with 1:2 BHI (control), 2X MIC value of 500 μg/mL colistin alone (control), RTs alone (B), or the RTs with 2X MIC of 500 μg/mL colistin alone (A, B). After treatment, supernatant was removed, and biofilms were rinsed and harvested with 1 mL of 0.85% saline. Biofilms were homogenized then serially diluted and drop-plated to determine CFU/mL. Statistical significance was determined via One-way analysis of variance (ANOVA) compared to media control (A) or two-way ANOVA (B). Mean±standard deviation is shown. n=6-17.

DISCUSSION

The induction of biofilm dispersion is a promising new avenue to intrinsically increase the susceptibility of the biofilms without causing harm to the host. While released cells from the biofilm following dispersion may lead to new colonization in isolated treatment conditions, quick and efficient antibiotic exposure may serve to target the vulnerable released cells before re-infection. The biofilm left behind is vulnerable and generally susceptible to antibiotic exposure as well. The implementation of exogenous releasing agents in combination with antibiotic treatment enables greater susceptibility to antibiotic exposure and improves the probability of full clearance of the biofilm.

This study was carried out to assess the efficacy of exogenous releasing agents on *S. aureus* biofilms as an isolated treatment and in combination with antibiotics to improve clearance of biofilms. Growth curve analysis demonstrated that prolonged exposure to SNP reduced the growth of *S. aureus* compared with unexposed cultures. This effect was most pronounced after 9 hours of continuous exposure. These results suggest that SNP has a potential cytotoxic effect on *S. aureus* cells. This can be attributed to the release of NO from SNP, which at sufficiently high concentrations has been shown to exert bactericidal activity against *S. aureus* (18). Because biofilms in this study were exposed to SNP for only 2 hours, well before growth inhibition was observed, it is unlikely that the reduction in biofilm burden resulted from direct bactericidal activity of SNP. Rather, the shorter exposure period was intended to promote biofilm dispersion while minimizing the effects of prolonged NO exposure on bacterial viability. Nevertheless, the potential influence of SNP on bacterial growth represents a limitation of this study and should be considered when interpreting the results. Future studies should further optimize SNP concentration and exposure time to maximize biofilm dispersion while minimizing direct antimicrobial effects.

It was imperative to identify optimal conditions for biofilm formation to ensure reproducible development of mature *S. aureus* biofilms. Previous studies have shown that *S. aureus* cultures sediment easily, and therefore optimal biofilm formation is commonly performed under shaking conditions (19, 20). A range of nutrient media, including LB and BHI, have both previously been used to establish *S. aureus* biofilms (19, 21). However, there is currently limited information directly comparing media formulations for maximizing biofilm biomass over a defined incubation period. Our optimization experiments demonstrated that 1:2 BHI produced the greatest biofilm biomass, while microscopy indicated that 220 rpm shaking conditions generated the most consistent and uniformly distributed mature biofilms. We therefore selected growth conditions of 37 °C shaking at 220 rpm for 4 days to generate mature *S. aureus* biofilms.

Biofilms were then treated with releasing agents in the presence or absence of colistin. Colistin was selected because of its limited intrinsic activity against Gram-positive bacteria, providing a stringent model for evaluating whether biofilm dispersion enhances antibiotic susceptibility beyond the effects of the antibiotic alone. By disrupting the extracellular polymeric substance (EPS), releasing agents were expected to improve antibiotic penetration and increase bacterial susceptibility to treatment. Combination treatments of SNP and amylase produced the greatest reductions in viable biofilm-associated bacteria compared to colistin alone. These reductions indicate that SNP and amylase enhanced the disruption of the biofilm, resulting in fewer surviving bacterial cells following antibiotic treatment. However, these reductions were not statistically significant and should therefore be interpreted as preliminary trends rather than definitive evidence of improved therapeutic efficacy. Although these differences were not statistically significant, the reductions in bacterial burden approached the clinical relevance threshold of 10^5 CFU/mL (11, 16, 17). Additional studies with increased statistical power and validation in more clinically relevant biofilm models are needed before conclusions regarding clinical use can be drawn.

The lack of statistical significance may reflect several factors. First, the *in vitro* biofilm model used in this study does not fully recapitulate the complexity of biofilms encountered during human infection, where host immune responses, nutrient gradients, fluid shear, and polymicrobial interactions contribute to biofilm persistence (19, 22). Second, only a single 2-hour exposure to each releasing agent was evaluated. Finally, variability inherent to biofilm formation may have limited the statistical power to detect modest but biologically meaningful reductions in bacterial burden.

Although a single 2-hour treatment did not produce statistically significant reductions in biofilm burden, clinically, biofilm-associated infections are rarely treated with a single antimicrobial exposure. Instead, patients typically receive multiple rounds of antibiotic therapy with or without debridement over the course of several days and, in some instances, several years (23). Therefore, future studies should investigate whether repeated cycles of dispersion treatment followed by antibiotic exposure further reduce biofilm burden and ultimately achieve statistically significant improvements in bacterial clearance. Such studies would better mimic clinical treatment regimens and may reveal cumulative benefits of repeated dispersion-based therapy.

This study aimed to assess and document the application of releasing agents as a means of inducing dispersion and improving biofilm clearance of *S. aureus* during antibiotic treatment. While all combination treatments reduced bacterial burden relative to colistin alone, these differences did not reach statistical significance. Consequently, the findings should be considered proof-of-concept rather than evidence of clinical efficacy. Nevertheless, the consistent trend observed with SNP and amylase suggests that both active and passive dispersion strategies warrant further investigation as adjunctive approaches for enhancing antibiotic treatment of biofilm-associated infections.

Future research should also consider that certain releasing agents may reduce efficacy or even suppress functionality of certain antibiotics. Amylase has previously been found to sequester certain antibiotics within *P. aeruginosa* biofilms (24), suggesting that interactions between releasing agents and antimicrobials may influence treatment outcomes. Future studies should investigate whether administering releasing agents as a pre-treatment before antibiotic exposure enhances biofilm clearance compared with simultaneous administration. Additionally, evaluating these therapies using clinically relevant infection models, larger sample sizes, and multiple treatment cycles will be important to determine whether the promising trends observed in this study translate into meaningful therapeutic benefit.

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