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The Effects of *N*-acetyl Cysteine, Gentamicin, and Ciprofloxacin on Bacterial Biofilm *in vitro* in the Presence or Absence of High Oxidative Stress and High Glucose Levels

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ABSTRACT

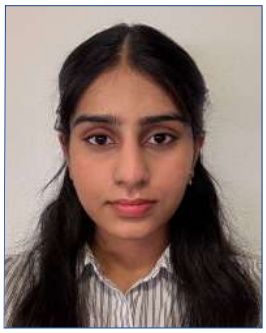
The purpose of this study was to determine if *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Staphylococcus xylosus* can form biofilm *in vitro*, and the effects of *N*-acetyl cysteine, ciprofloxacin, and gentamicin on biofilm growth in the presence or absence of oxidative stress and/or high glucose levels. These three bacterial strains were used as they are most commonly found in human chronic wounds. The goal of this study was to determine if *N*-acetyl cysteine can be used as a treatment for chronic wounds. The bacteria were cultured *in vitro* and formed biofilm within 24 hours. Treatments included *N*-acetyl cysteine, gentamicin, and ciprofloxacin, which were added to the cultured biofilm. For the oxidative stress condition, H_2O_2 was used, and for the high glucose levels, d(+)-Glucose was used, both of which were administered in a similar manner to the treatments. When used alone, *N*-acetyl cysteine was able to significantly dismantle the biofilm, even when the bacteria were combined. However, in the presence of oxidative stress or high glucose levels, *N*-acetyl cysteine was less effective at dismantling the biofilm, especially when the bacterial strains were combined. In conclusion, H_2O_2 and glucose diminish the efficacy of our treatments, which have a significantly detrimental effect on biofilm. While *N*-acetyl cysteine is not as effective at dismantling biofilm under conditions of high oxidative stress and high glucose levels, the addition of antibiotics becomes more helpful in these cases, making the treatment viable overall.

KEYWORDS: *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus xylosus*, Hyperglycemia, Microbial aggregates, Hydrogen peroxide

FACULTY MENTOR - Dr. Manuela Martins-Green; Department of Molecular, Cell and Systems Biology



Dr. Martins-Green is a Professor of Cell Biology at UCR. Originally from Portugal, she came to the U.S. on a Fulbright Fellowship and earned her Ph.D. from UC Davis. She has been on the UC Riverside faculty since 1993. She is a fellow of AAAS and founding Fellow of the Wound Healing Society (WHS). Her research focuses on the initiation and progression of chronic wounds, and she has won the 2026 Lifetime Achievement Award from WHS.



SAMYUKTHA VEDULA

Samyuktha is a researcher in Dr. Martins-Green's chronic wound laboratory. Samyuktha's research focuses on *in vitro* treatments for bacterial biofilms. She has presented at the 2024 Symposium on Advanced Wound Care, held in collaboration with the Wound Healing Society. Samyuktha graduated Summa Cum Laude with a B.S. in Biology and plans to apply to medical school.



SHAYLA NGUYEN

Shayla Nguyen is a fourth-year Biology major and researcher in Dr. Martins-Green's chronic wound laboratory, studying treatments of bacterial biofilms under hyperglycemic and oxidative stress conditions. She presented at the 2024 Symposium on Advanced Wound Care. Shayla received the Dr. Manuela & Mr. Randy Sosa Scholarship Award for her commitment to serving the Inland Empire. She will pursue an M.D. at the UCR School of Medicine.

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INTRODUCTION

Chronic wounds are associated with a significant impact on human health globally. Nearly 8.2 million Americans suffer from chronic wounds, with Medicare costs for treatment ranging from $28.1 billion to $96.8 billion [1]. Chronic wounds are a financial, clinical, and social burden on the healthcare system and on the individuals suffering from them. Chronic wounds cause pain, infection, and may even lead to amputation if the chronicity of the wounds is prolonged. Between 40.4% and 70% of lower extremity amputations result in death within five years post-amputation [2]. Several comorbidities, such as chronic disease, vascular insufficiency, diabetes, malnutrition, and age, can impact wound healing in these patients [3].

Acute wound healing is characterized by four stages: hemostasis, inflammation, proliferation, and remodeling [4,5]. However, chronic wounds do not follow the normal healing process. In these wounds, the healing and repair process is altered, resulting in chronic inflammation, excessive levels of reactive oxygen species (ROS), elevated levels of matrix proteases, and lack of angiogenesis [6,7]. Chronic wounds are colonized by pathogens, primarily bacteria from the skin microbiome. These pathogens result in infections that, in the presence of ROS, lead to development of biofilm [8]. The prevalence of biofilm-forming pathogens in chronic wounds is a key contributing factor to wound chronicity and impaired wound healing [4]. Current treatments with antibiotics are generally ineffective at killing biofilm-forming bacteria because the bacteria are embedded in extracellular polymeric substances (EPS), which provide protection from antibiotics. Furthermore, a "dormant" state is exhibited by these bacteria; therefore, the antibiotics, which affect protein synthesis, become ineffective [9,10].

We have previously shown, in a diabetic mouse model of chronic wounds, that wound healing was significantly improved and chronicity was reversed by *N*-acetyl cysteine (NAC) through the disappearance of the bacteria from the wound, resulting in the breakdown of the EPS [11,12]. EPS are composed of carbohydrates, proteins, extracellular DNA, and lipids, all of which establish biofilm structure [13]. Using an *in vitro* system, we showed that NAC dismantled Pseudomonas aeruginosa-made biofilm by killing the bacteria. At a pH below the pKa, NAC was able to penetrate bacteria and halt protein synthesis, leading to bacterial cell death [12].

Here, we further these studies by identifying the effects of NAC and the antibiotics Gentamicin (G) and Ciprofloxacin (C) on the biofilm formed by three bacterial strains, individually and in combination. *Enterobacter Cloacae (Ec), Staphylococcus Xylosus (Sx),* and *Pseudomonas Aeruginosa (Pa)* were used, as they are commonly found in human chronic wounds. We examined the amount of biofilm that formed *in vitro* after 24 hours of growth, and were able to show that all strains and combinations of bacteria formed biofilm after 24 hours of incubation, and all possessed unique morphologies. Significant dismantling of the established biofilm was observed following treatment with NAC, gentamicin, and ciprofloxacin (NAC+G+C). The addition of high oxidative stress, in the form of hydrogen peroxide, and high glucose levels to mimic hyperglycemia, diminished the effectiveness of our treatments, but they were still able to significantly reduce the amount of biofilm *in vitro.*

MATERIALS AND METHODS

Culturing bacteria *in vitro* to form biofilm: The three bacterial strains were cultured in sterile LB at 37°C for 16-18 h and then diluted to $1x10^6$ CFU/200 µL. 200 µL of the diluted solution was then pipetted into each well of a 96-well plate and incubated for 24 hours. Images were captured from an overhead view every 24 hours over the course of 72 hours.

Staining for biofilm: The contents of each well were transferred to an Eppendorf tube. The well was washed with 200 µL of double distilled water and transferred to the same microcentrifuge tube. The tubes were centrifuged at 21300 rpm for 5 minutes. The supernatants were discarded and the pellets were stained with 50 µL of 0.1% crystal violet stain for 5 minutes. The excess staining solution was then washed with 1000 µL of double-distilled water twice. The samples were then dried overnight or until completely dry.

Destaining of the biofilm: The dried samples were destained using 95% ethanol. 300 µL of 95% ethanol

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was added to each sample to destain the biofilm for 50 minutes. The samples were then centrifuged at 21300 rpm for 5 minutes. 200 µL of the supernatant was removed and analyzed using a spectrophotometer at OD590nm to determine the amount of biofilm present.

Treatment with NAC: *Pa, Ec,* and *Sx* were cultured, diluted, and plated using the method described above to form biofilm. The bacterial cultures were pipetted individually or in combination into the wells of a 96-well plate and incubated for 24 hours. NAC was dissolved in LB broth at 40-50°C. 20 µL of NAC at a concentration of 220 mg/mL was pipetted into each well, to ensure that the final concentration of NAC was 20 mg/mL per well. At the final concentration, the pH of NAC was 2.74. The plates were then incubated for 24 hours before biofilm quantification.

Other Treatments: The methods for all other treatments are the same as the general method described for NAC. Gentamicin, an antibiotic, was at a final concentration of 0.1 mg/ml, ciprofloxacin, another antibiotic, was at a final concentration of 0.2 mg/ml, hydrogen peroxide was at a final concentration of 500 µM, and d(+)-glucose was at a final concentration of 300 mg/dL.

Statistical Analysis: Statistical analysis was conducted using a student's t-test to determine the significance of the differences between the two samples of data. This form of statistical analysis was chosen for its efficiency and relative ease of implementation.

RESULTS

Distinct morphologies of biofilms developed by *Ec*, *Pa*, and *Sx* alone and in combination

To assess the biofilm-forming capabilities of the bacterial strains *Ec, Pa,* and *Sx*, the individual bacteria were cultured for 24 hours to form biofilm. Distinct morphologies were observed for all strains. The biofilm produced by *Ec* was characterized by growth primarily at the bottom of the well that was opaque and smooth. It remained relatively unchanged after culturing for 48 or 72 hours (**Fig. 1A**). The biofilm produced by *Sx* was characterized by growth at the bottom and sides of the well, and it was opaque and smooth. As time progressed, the biofilm increased in opacity and remained smooth (**Fig. 1A**). The biofilm produced by *Pa* formed at the top of the well, having a less smooth appearance than biofilm produced by *Ec* or

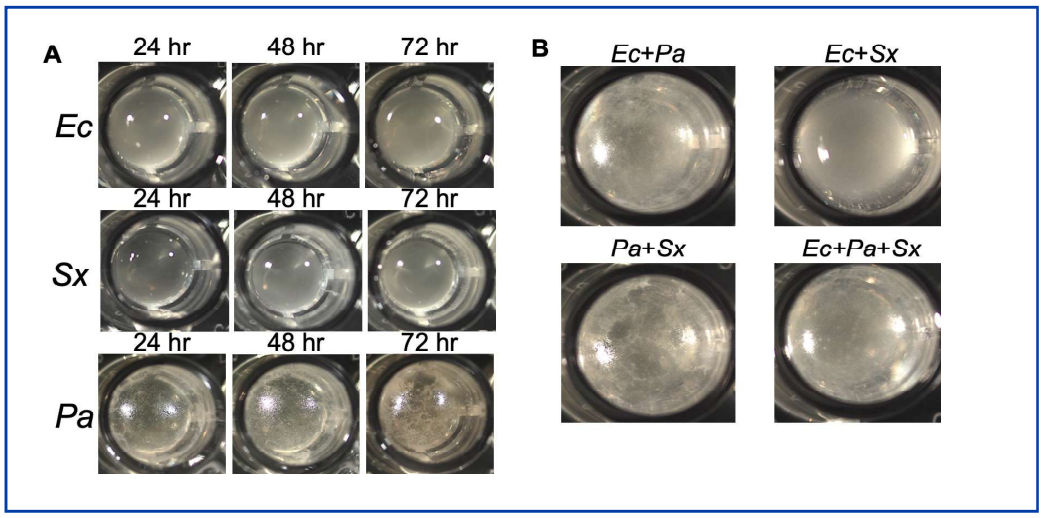


Figure 1. Visual representation of biofilm formation
Each well contained 200 µL of LB with the various cultures of bacteria. They were photographed from overhead. *Ec* is *Enterobacter cloacae*, *Pa* is *Pseudomonas aeruginosa*, and *Sx* is *Staphylococcus xylosus*. **(A)** Biofilms of the individual bacteria were photographed at 24 hour increments. **(B)** Biofilms of the various combinations of bacteria.

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Sx. After 72 hours of incubation, the biofilm took on an orangish hue, as opposed to its initial off-white color (**Fig. 1A**). The combinations of bacteria maintained the unique morphologies observed in the single strains of bacteria and were all able to produce biofilm within 24 hours post-incubation. The combination *Ec*+*Pa* had biofilm at both the top and the bottom of the well, with the biofilm at the top being less smooth than the biofilm at the bottom, which was also more opaque (**Fig. 1B**). The combination *Ec*+*Sx* had biofilm at the bottom and sides of the well, with the biofilm being more opaque at the bottom than at the sides (**Fig. 1B**). The combination *Pa*+*Sx* had biofilm at both the top, bottom, and sides of the well, with the biofilm at the top being less smooth than the biofilm at the bottom and sides, which was more opaque (**Fig. 1B**). The combination *Ec*+*Pa*+*Sx* had biofilm at both the top, bottom, and sides of the well, with the biofilm at the top being less smooth than the biofilm at the bottom and sides (**Fig. 1B**).

The effects of NAC, Gentamicin, Ciprofloxacin, and their combinations on established biofilm

Ec, Pa, Sx, and their combinations, were cultured for 24 hours to develop biofilm. These biofilms were then treated with NAC, NAC+G, NAC+C, and NAC+G+C. They were incubated for another 24 hours before being stained with 0.1% crystal violet and destained with 95% ethanol. The supernatants were analyzed by measuring OD590 nm. For *Ec*, all four treatments had a statistically significant impact on dismantling the established biofilm ($p<0.001$ for NAC, NAC+G, NAC+G+C; $p<0.01$ for NAC+C). The effects of NAC alone or NAC+C were indistinguishable and were significantly better than NAC+G or NAC+G+C. G seems to significantly decrease the effects of NAC, while C does not seem to have a drastic impact on the effectiveness of NAC. The effects of NAC+G+C are like NAC+G, with the addition of C not having a drastic impact on G's inhibitory effect on NAC (**Fig. 2A**). For *Pa*, all four treatments had a statistically significant impact on dismantling the established biofilm ($p<0.001$ for NAC, NAC+G, NAC+G+C; $p<0.01$ for NAC+C). The effect of NAC alone was better than NAC in combination with G and with both antibiotics combined. However, C was highly effective in reversing the effectiveness of NAC alone. (**Fig. 2B**). For *Sx*, NAC was able to significantly dismantle the established biofilm, performing better alone than in combination with the antibiotics ($p<0.001$). The addition of C to NAC did not have a major impact on NAC's effects. However, when G was added to NAC, the effects of NAC on dismantling biofilm were reversed (n.s.). When both antibiotics are combined with NAC, the addition of C appears to combat G's inhibitory effect on NAC (**Fig. 2C**). Overall, NAC alone can significantly dismantle the established biofilm in all three

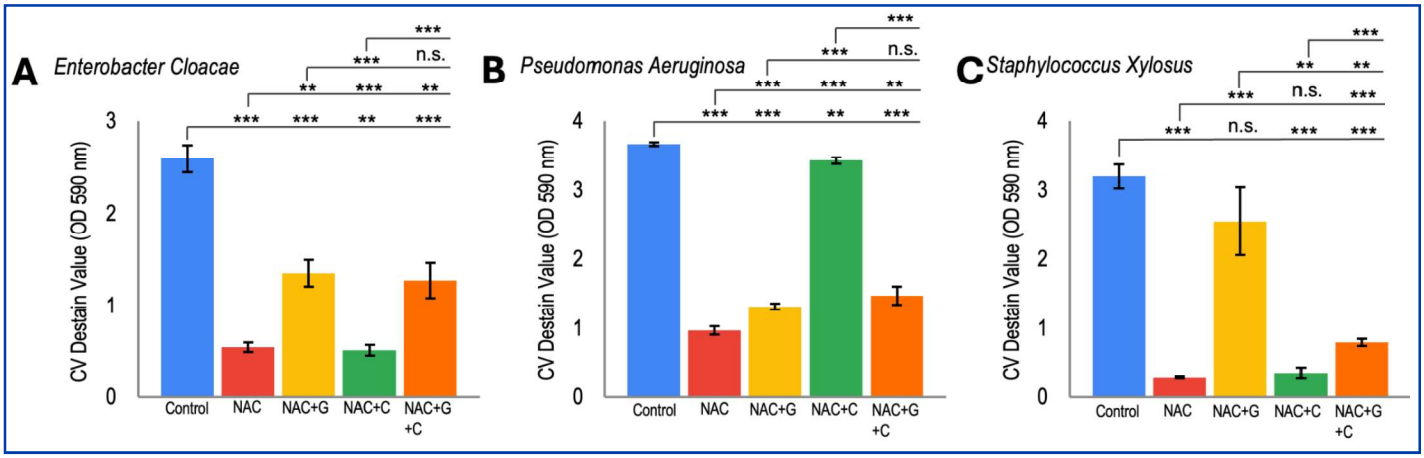


Figure 2. The effects of N-acetyl cysteine (NAC), N-acetyl cysteine+Gentamicin (NAC+G), and N-acetyl cysteine+Ciprofloxacin (NAC+C), and N-acetyl cysteine+Gentamicin+Ciprofloxacin (NAC+G+C) on individual strains of bacteria.

The graphs show the amount of biofilm formed 24 hours post-treatment, stained with crystal violet and quantified at OD590 nm.

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individual strains of bacteria. The effects of NAC by itself were almost always better than NAC in combination with the antibiotics. (**Fig. 2**).

When the bacteria were combined, the established biofilm was dismantled by all of the treatments, but in general, no significant differences were observed between the treatments (**Fig. 3**). For the combination *Ec*+*Pa*, all treatments had a statistically significant impact on the established biofilm, dismantling it significantly ($p<0.001$ for NAC, NAC+C, NAC+G+C; $p<0.01$ for NAC+G). There was no significant difference between the effects of NAC alone and NAC with one or more of the antibiotics (**Fig. 3A**). For the combination *Ec*+*Sx*, a similar pattern was observed. All treatments resulted in a significant dismantling of the established biofilm with no statistically significant difference NAC and NAC plus the antibiotics ($p<0.001$ for NAC+G and NAC+G+C; $p<0.01$ for NAC and NAC+C) (**Fig. 3B**). For the combination of *Pa*+*Sx*, all treatments were similar in that they dismantled the established biofilm even though no statistically significant difference between the effects of NAC and NAC plus the antibiotics were observed ($p<0.001$ for NAC, NAC+C, NAC+G+C; $p<0.05$ for NAC+G) (**Fig. 3C**). For the combination *Ec*+*Pa*+*Sx*, all treatments were effective at dismantling biofilm ($p<0.001$). The combinations of NAC+G and NAC+G+C are significantly better at dismantling established biofilm than NAC alone, although the magnitude of this difference is not dramatic. The addition of G seems to improve NAC's effectiveness, whereas the addition of C does not have a drastic impact (**Fig. 3D**). Overall, the biofilms established by the combined bacteria were all effectively dismantled by all treatments. NAC alone was usually as effective as NAC in combination with the antibiotics (**Fig. 3**). The primary result was that NAC, by itself, was usually almost as good, if not better, than NAC with the antibiotics (**Fig. 2, 3**).

The effects of oxidative stress on the efficacy of NAC and NAC with Gentamicin and Ciprofloxacin in dismantling established biofilm

The bacterial strains *Ec, Pa, Sx,* and their combinations were cultured for 24 hours to form biofilm. To analyze how oxidative stress impacts the efficacy of NAC and NAC plus antibiotics, hydrogen peroxide (H_2O_2) was added to the treatments as a source of oxidative stress. In a dose-dependent experiment, no relevant difference in biofilm development was found when comparing the effects of

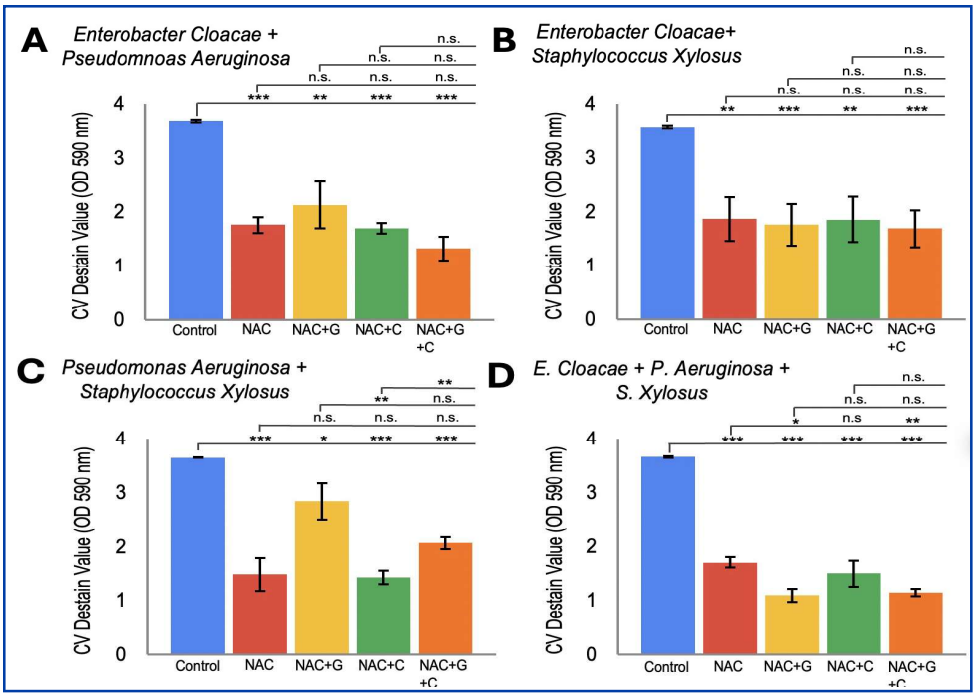


Figure 3. The effects of *N*-acetyl cysteine (NAC), *N*-acetyl cysteine+Gentamicin (NAC+G), and *N*-acetyl cysteine+Ciprofloxacin (NAC+C), and *N*-acetyl ysteine+Gentamicin+Ciprofloxacin (NAC+G+C) on the combined strains of bacteria.

The graphs show the amount of biofilm formed 24 hours post-treatment, stained with crystal violet and quantified at OD590 nm.

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250 μM and 500 μM of H_2O_2 (not shown). Therefore, 500 μM was used. For all three bacteria, the addition of H_2O_2 to increase oxidative stress had no significant impact on the established biofilm (**Fig. 4**). For *Ec*, the addition of H_2O_2 significantly decreased the efficacy of both NAC and NAC+G+C. However, under oxidative stress conditions, NAC and NAC+G+C were still able to significantly dismantle the established biofilm, with no significant difference between their effects ($p<0.001$ for NAC+H_2O_2; $p<0.01$ for NAC+G+C+H_2O_2) (**Fig. 4A**). For *Pa*, the addition of H_2O_2 significantly decreased the efficacy of both NAC and NAC+G+C. While NAC+H_2O_2 became ineffective at dismantling the established biofilm (n.s), NAC+G+C+H_2O_2 was still able to significantly dismantle the established biofilm ($p<0.001$) (**Fig. 4B**). For *Sx*, oxidative stress significantly diminished the effectiveness of both NAC and NAC+G+C. NAC and NAC+G+C, under oxidative stress conditions, were still able to significantly dismantle the established biofilm ($p<0.001$ for NAC+H_2O_2; $p<0.01$ for NAC+G+C+H_2O_2), with no significant difference between the two treatments (**Fig. 4C**).

When we combined the bacteria, the effects of H_2O_2 alone on established biofilms were minimal (**Fig. 5**). For the combination of *Ec*+*Pa*, the ability of NAC to dismantle the biofilm was significantly diminished upon the addition of H_2O_2, but it was still significant when compared to the control with no treatment ($p<0.05$), but not the H_2O_2 treatment (n.s.). The effects of NAC+G+C on biofilm dismantling are similarly diminished upon the addition of H_2O_2, but they were still a significantly effective treatment ($p<0.001$) (**Fig. 5A**). For *Ec*+*Sx*, the effects of NAC were not significantly impacted by the addition of H_2O_2, as the difference between the effects of NAC alone and NAC+H_2O_2 was not significant, and therefore it remains an effective treatment to dismantle the established biofilm ($p<0.001$). The effects of NAC+G+C ($p<0.001$) were also not significantly impacted by the addition of H_2O_2, as the difference between the effects of NAC+G+C alone and NAC+G+C+H_2O_2 is not significant (**Fig. 5B**). With *Pa*+*Sx*, the ability of NAC to dismantle the biofilm was significantly diminished upon the addition of H_2O_2. The effects of NAC+G+C were similarly diminished upon the addition of H_2O_2, but it was still effective at dismantling the biofilm (**Fig. 5C**). For *Ec*+*Pa*+*Sx*, the effects of NAC are significantly diminished upon the addition of H_2O_2. However, it remains an effective treatment for dismantling

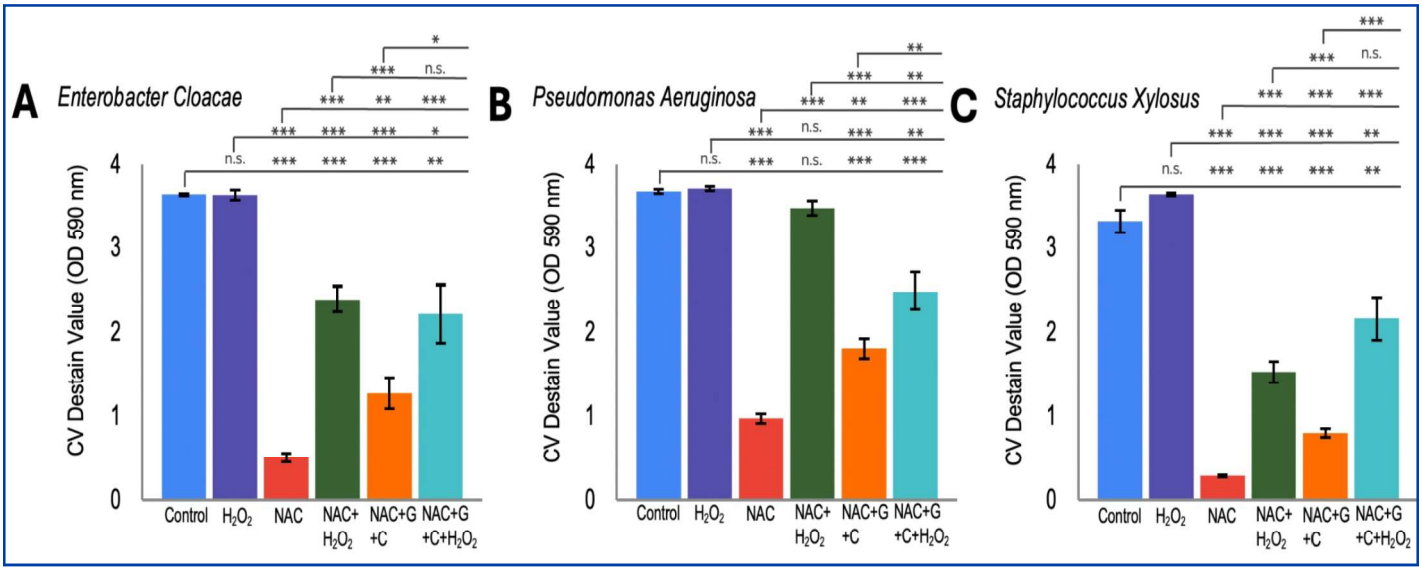


Figure 4. The effects of N-acetyl cysteine (NAC), Gentamicin (G), and Ciprofloxacin (C) on individual bacteria in the presence or absence of oxidative stress.

Hydrogen peroxide was used as a source of oxidative stress. The graphs show the amount of biofilm formed 24 hours post-treatment, stained with crystal violet and quantified at OD590 nm.

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established biofilm. The effects of NAC+G+C are similarly diminished upon the addition of H_2O_2, though it, too, remains a significantly effective treatment against the established biofilm (**Fig. 5D**).

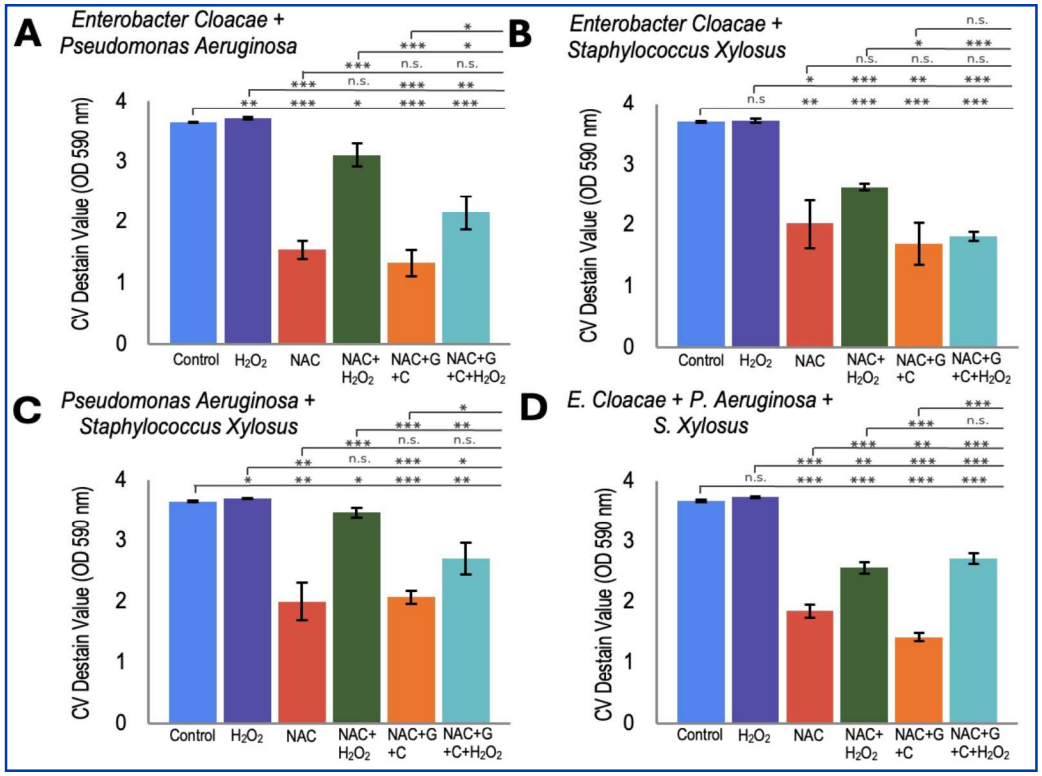


Figure 5. The effects of N-acetyl cysteine (NAC), Gentamicin (G), and Ciprofloxacin (C) on combinations of bacteria in the presence or absence of oxidative stress.

Hydrogen peroxide was used as a source of oxidative stress. The graphs show the amount of biofilm formed 24 hours post-treatment, stained with crystal violet and quantified at OD590 nm.

The effects of D(+)-Glucose on the efficacy of NAC and NAC with Gentamicin and Ciprofloxacin

The bacterial strains *Ec, Pa, Sx,* and their combinations were cultured for 24 hours to form biofilm. This study used d(+)-glucose to emulate the heightened blood glucose levels of a diabetic patient, as chronic wounds are prevalent in people with Type II diabetes. To analyze how high glucose levels impact the efficacy of NAC and NAC+G+C, d(+)-glucose was added to the culture media. In a dose-dependent experiment, we found that there was no relevant difference in biofilm development when comparing the effects of 150, 200, 250, and 300 mg/dL of d(+)-glucose (not shown). Therefore, 300 mg/dL of glucose was used. Specifically, 300 mg/dL is the blood glucose concentration of an untreated diabetic patient. For all three bacteria, the established biofilm was not significantly impacted by the addition of glucose to mimic hyperglycemia (**Fig. 6**). For *Ec*, the efficacy of NAC was significantly diminished upon the addition of glucose. However, it remains a significantly effective treatment at dismantling established biofilm ($p<0.001$). The effects of NAC+G+C were not significantly impacted by the addition of glucose, as there was no significant difference between NAC+G+C and NAC+G+C+Glucose; the established biofilm was effectively dismantled by both treatments ($p<0.001$) (**Fig. 6A**). For *Pa*, the addition of glucose significantly diminishes the effectiveness of NAC ($p<0.05$). The addition of glucose also significantly diminished the effectiveness of NAC+G+C ($p<0.01$). However, in this case, it remains a significantly effective treatment at dismantling established biofilm (Fig. 6B). For *Sx*, the addition of glucose significantly diminishes the effectiveness of NAC. However, its effects remain significant at dismantling the established biofilm ($p<0.001$). The effects of NAC+G+C were also significantly inhibited by the addition of glucose. Nevertheless, it also remains an effective treatment at dismantling the established biofilm ($p<0.001$) (**Fig. 6C**).

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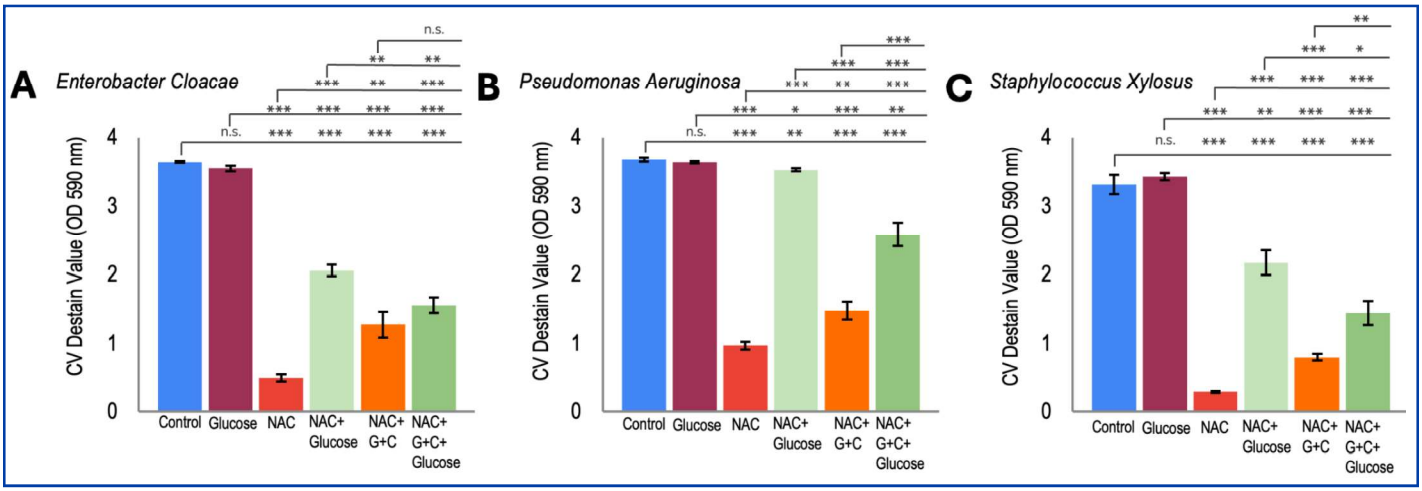


Figure 6. The effects of N-acetyl cysteine (NAC), Gentamicin (G), and Ciprofloxacin (C) on singular strains of bacteria in the presence or absence of heightened glucose levels.

D(+)-glucose was used to emulate heightened glucose levels characteristic of diabetes. The graphs show the amount of biofilm formed 24 hours post-treatment, stained with crystal violet and quantified at OD590 nm.

For all bacteria combinations, the addition of glucose to emulate hyperglycemia had no significant impact on the established biofilm (**Fig. 7**). For *Ec*+*Pa*, the addition of glucose significantly diminished the effectiveness of NAC (n.s.) at dismantling the biofilm. The same is true for the effects of NAC+G+C on biofilm (n.s.); they too are significantly decreased by the addition of glucose. There is no significant difference between the effects of NAC and NAC+G+C when glucose is added (**Fig. 7A**). For *Ec*+*Sx*, the effects of NAC were not significantly impacted by the addition of glucose ($p<0.01$); there was no significant difference between the effects of NAC alone and NAC+Glucose. The effects of NAC+G+C were also not significantly impacted by the addition of glucose ($p<0.001$) as the difference between the effects of NAC+G+C alone and NAC+G+C+Glucose were also not significant. It, too, remains an effective treatment for dismantling the established biofilm (**Fig. 7B**). For *Pa*+*Sx*, the addition of glucose significantly diminished the effectiveness of NAC at dismantling the biofilm. Although its effects remain significant when compared to the non-treated control, it becomes insignificant when compared to the biofilm solely treated with glucose. The effects of NAC+G+C are also significantly inhibited by the addition of glucose (n.s.). There is no significant difference between the effects of NAC and NAC+G+C when glucose is added (**Fig. 7C**). For *Ec*+*Pa*+*Sx,* the effects of NAC are significantly diminished upon the addition of glucose. The effects of NAC+G+C are similarly diminished upon the addition of glucose, though it, too, remains a significantly effective treatment against the established biofilm. While both are effective ($p<0.05$ for NAC; $p<0.001$ for NAC+G+C), NAC+G+C is a significantly better treatment than NAC alone when glucose is added (**Fig. 7D**).

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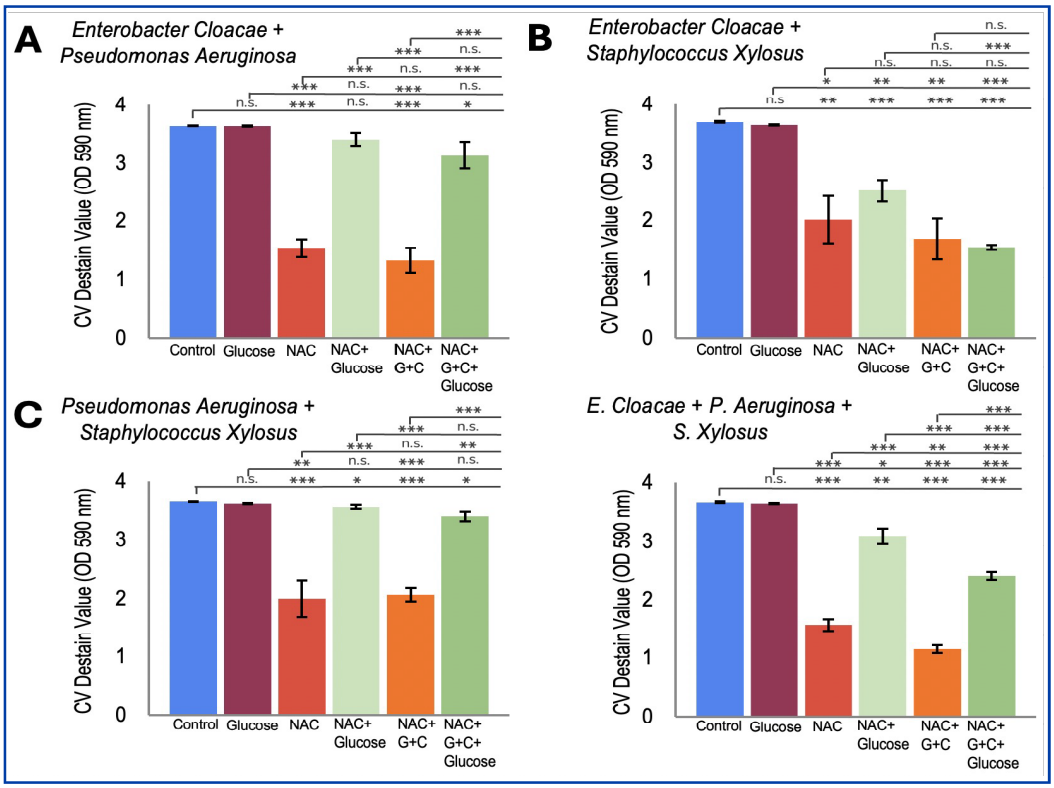


Figure 7. The effects of *N*-acetyl cysteine (NAC), Gentamicin (G), and Ciprofloxacin (C) on combinations of bacteria in the presence or absence of heightened glucose levels.

D(+)-glucose was used to emulate heightened glucose levels characteristic of diabetes. The graphs show the amount of biofilm formed 24 hours post-treatment, stained with crystal violet and quantified at OD590 nm.

DISCUSSION

In this study, *in vitro* biofilm formation by the bacterial strains *Ec, Pa,* and *Sx* was shown within 24 hours of incubation, individually and in combination. The treatments, under varying conditions, significantly dismantled the established biofilm. Specifically, NAC was generally very effective at dismantling established biofilms. However, under conditions of high oxidative stress and hyperglycemia, NAC's efficacy was decreased, as was the efficacy of the other treatments for nearly all the bacteria, though they are not rendered ineffectual. Overall, the treatments remain effective at dismantling biofilm.

Macroscopic visualization of the biofilms showed that the bacteria can develop unique biofilms. The integrity of the EPS, which surrounds microbial communities, and the three-dimensional structure of the matrix determine biofilm morphology. Furthermore, biofilm formation is influenced by many factors, such as bacterial motility and intercellular communication [9]. Each of the three bacterial strains displayed unique morphological characteristics that were retained even when cultured together. This could be attributed to the unique properties of each bacterial strain, such as bacterial motility, cellular communication, and EPS composition, which influence and direct the emergence of biofilm. This could also lead to varying responses to the treatments.

NAC was shown to be an effective treatment for dismantling the biofilms from *Ec, Pa,* and *Sx*, individually or in combination. Specifically, the effect of NAC alone was statistically significant for all three bacteria. Moreover, when considering the individual bacteria, the effect of NAC alone was better than in combination with the antibiotics. We have previously shown that application of NAC to chronic wounds in the diabetic db/db mice, a model that we have developed, leads to the breakdown of biofilm, resulting in the healing of the chronic wounds [11].

The combination of NAC with antibiotics demonstrated variable effects on treatment efficacy in dismantling the biofilms of individual bacteria. Gentamicin (G), for instance, abrogated the efficacy of NAC for *Ec* and *Sx*,

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whereas Ciprofloxacin (C) inhibited the efficacy of NAC for *Pa* biofilms. A study that used EBV-transformed lymphoblastoid cells showed that G was able to catalyze the production of ROS in the presence of iron and/or arachidonic acid [14]. Furthermore, another study that used human fibroblasts showed that C induced lipid peroxidation and decreased intracellular glutathione concentration in a dose-dependent manner. Both measurements indicate an increase in ROS [15]. As NAC is an antioxidant, the variation in its efficacy could be explained by the differing levels of ROS induced by the antibiotics. As stated previously, these inhibitory effects are not consistent across all three individual strains of bacteria, as G inhibits NAC's effectiveness in *Ec* and *Sx*, but not *Pa*. Conversely, the efficacy of NAC was decreased by the presence of C in *Pa* biofilms.

A significant reduction in the efficacy of the treatments was observed in the presence of oxidative stress, *Ec*+*Sx* being an exception. However, the treatments, especially NAC+G+C, remained generally effective at dismantling the established biofilm. A study testing the antioxidant capabilities of NAC found that, although NAC reacts with H_2O_2, it does so at a slow rate [16]. Therefore, the suppression of the efficacy of NAC to dismantle biofilm in the presence of H_2O_2 may be attributed more to temporal limitations than to its capability as an antioxidant. In addition, it is noteworthy that in conditions with added oxidative stress, in biofilms where *Pa* may make up a larger proportion of the biofilm, such as in the cases of *Ec*+*Pa* and *Pa*+*Sx*, the effects of NAC were diminished greatly. In contrast, this effect was not observed when all three bacterial strains were in combination, potentially due to an increase in the proportion of *Ec* and *Sx*.

Gram-negative bacteria possess an outer membrane that acts as an additional permeability barrier, which gram-positive bacteria do not have [17]. Because *Pa* is a gram-negative bacterium, its resistance to NAC, when compared with *Sx*, may be explained by the presence of this additional outer membrane, which decreases permeability to NAC. In addition, unlike Enterobacteria, Pseudomonads lack general diffusion porins in their outer membranes, resulting in lower outer membrane permeability [18]. Although both *Ec* and *Pa* are gram-negative bacteria, lower permeability to NAC may be exhibited by *Pa*, which could explain why *Pa* has greater resilience to NAC's inhibitory effects. Regarding the treatment and its decreased efficacy in the presence of *Pa*, *in vivo*, the biofilms that form on chronic wounds will likely involve a plethora of different strains. Therefore, it is likely that the treatment of NAC will be effective in dismantling established biofilms due to the lower likelihood of *Pa* dominating the biofilms and the competition the bacteria face with each other.

Diabetes has been associated with delayed wound healing, often correlating with the onset of wound chronicity [19]. Because a characteristic condition of diabetes is hyperglycemia, d(+)-glucose was added to the established biofilms. The addition of glucose, much like the addition of H_2O_2, attenuated the ability of our treatments to dismantle established biofilm. Although both NAC and NAC+G+C have their effects abrogated by the addition of glucose, the inhibitory effects of NAC are bolstered by the addition of the antibiotics, as NAC+G+C is a significantly better treatment than NAC alone. Under hyperglycemic conditions, the treatments remained generally effective at dismantling biofilm, with the exceptions of *Ec*+*Pa* and *Pa*+*Sx*, where the effects of both NAC and NAC+G+C were statistically insignificant. Even with *Pa* alone, although both our treatments remain significantly effective, the impact of NAC alone is minor. As observed with the addition of H_2O_2, in biofilms where *Pa* might make up a larger proportion of the bacteria, the efficacy of our treatments is decreased drastically, possibly due to the reduced permeability of *Pa* in comparison to the other bacteria. However, as stated previously, biofilms *in vivo* are composed of multiple strains of bacteria, which makes it more likely for our treatments to remain effective at dismantling established biofilms. In addition, the inhibitory effects of the added glucose might be two-pronged. Glucose is an energy source for the bacteria, which could aid in their growth. In addition, a study that researched the glycosylation of low-molecular-weight thiols found a possible mechanism for such a process in Lincomycin A Biosynthesis, which occurs in the bacterium *Streptomyces lincolnensis* [20]. The glycosylation of thiol groups is possible through both enzymatic and chemical means. Direct reactions between glucose and the thiol group of NAC may result in inactivation of NAC, thereby reducing its efficacy. However, our treatments are still broadly effective, especially when all three bacteria are combined

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Therefore, NAC could prove to be an effective treatment to dismantle established biofilms. As biofilms contribute to wound chronicity, a treatment that abrogates their impact could serve as a treatment for chronic wounds, which would decrease the impact chronic wounds have on the global healthcare system.

CONCLUSIONS

N-acetyl cysteine, particularly with the aid of Gentamicin and Ciprofloxacin, was shown to significantly dismantle *in vitro* established biofilm, with or without oxidative stress and elevated glucose levels. Although the addition of the latter two diminished its effects, the effects of NAC remained statistically significant. Because NAC was consistently effective in dismantling the biofilms formed by *Ec+Pa+Sx*, it could prove to be an effective treatment *in vivo* against biofilm. This is significant because biofilms contribute to wound chronicity, a widespread global health concern. Therefore, NAC has the potential to effectively treat chronic wounds, although it is important to note that, as this study was conducted *in vitro*, it may not fully mimic the situation *in vivo*. In future experiments, we will perform molecular studies after treatment with NAC, to determine the impact of NAC on a more molecular level. We will also perform experiments to determine the impact of NAC on bacteria grown in media that more closely resemble the chronic wound environment to further investigate the therapeutic potential of NAC.

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