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Keep It Natural: Ditching Conventional Antibiotics and Treating Infections in Chronic Wounds with Fatty Acids

Abstract

As bacterial biofilms become increasingly resistant to antibiotics, an emerging alternative to using traditional antibiotics is inhibiting biofilms with fatty acids and antiseptics. An urgent application of this approach is to eliminate the bacterial biofilms associated with chronic wounds. Undecanoic Acid (UDA) is a fatty acid that exhibits anti-virulence and biofilm-degrading properties, making it a strong candidate as alternative treatments for antibiotics. Additionally, cetyltrimethylammonium bromide (CTAB), a component of common topical antiseptics, acts as a "detergent" in the presence of biofilms and enhances biofilm degradation. Because of this property, CTAB presents the opportunity to be paired with UDA to inhibit biofilms in Gram-negative bacteria. In our project, the combination of UDA and CTAB was explored and studied using *Vibrio harveyi* biofilms, so as to replicate the environment of an infected chronic wound. Tested independently and in mixture, the degradation and inhibition abilities of UDA and CTAB on a *V. harveyi* biofilm were observed. Our data suggested that UDA had no inhibitory effect on biofilm growth and that CTAB has very potent antibacterial properties in the presence of two strains of *V. harveyi*. Additionally, after completing two experiments to determine the minimum inhibitory concentration of CTAB, we were able to narrow down the margin in which this concentration may be found.

Introduction

Over 90% of chronic wounds become infected by bacterial biofilms (Attinger & Wolcott, 2012). Not only do chronic wounds give rise to infections, but they also pose a tremendous financial burden on the healthcare system. It is estimated that the healthcare system dedicates roughly $28 billion to $97 billion dollars annually to treating chronic wounds (Sen, 2019). Even so, chronic wounds are difficult to treat, especially since bacterial biofilms are prone to colonizing within the open wound itself.

A bacterial biofilm is a collection of dense bacteria encased by a protective, adhesive coating (Donlan, 2002). Since bacteria have the ability to quickly spread through their host, biofilms growing in chronic wounds pose a threat to the patient's entire body, making chronic wounds increasingly difficult to treat and an even more serious diagnosis (Omar, 2017). As medical treatments for chronic wounds and infections have evolved, so have bacteria; many bacteria are genetically resistant to conventional antibiotics. Bacteria also produce a sticky substance that serves as their protective barrier, making it especially challenging to penetrate their structure (Saxena, 2019). Researchers are exploring alternative inhibitory treatments for bacterial biofilms that might accelerate both chronic wound closure and reduce healthcare expenses. Some of these alternatives were explored in our study.

Biofilm formation. Biofilm formation begins with the stage of initial attachment, when the bacteria adhere to a surface and colonize to form a film (**Figure I**). When bacteria colonize on a human surface, such as a chronic wound, the extracellular membrane of the bacteria must bind to the fibrous proteins on this surface (Ruhal & Kataria, 2021). Then, the biofilm can form the matrix, the “armor” of the biofilm. The matrix encases the biofilm with proteins and polysaccharides (Ruhal & Kataria, 2010). Extracellular polymeric substances (EPS) compose a

large portion of the bacterial matrix. The EPS improves the biofilm community by immobilizing biofilm cells so that they can relay information with one another (Flemming & Wingender, 2010). Serving as the biofilm's primary source of nutrients and immune defense as a protective membrane, the EPS preserves biofilm viability while also blocking penetration by any antibiofilm agent, such as antibiotics (Ruhal & Kataria, 2021). Virulent bacteria that don't adhere to the biofilm are dispersed and attach to a nearby surface. Dispersion of a biofilm refers to the degradation, or erosion, of bacterial colonies in a biofilm and their reattachment to the biomass (Paluch et al., 2020). Within the biofilm, the bacteria are able to freely grow and divide under the extracellular matrix to further spread and increase the biomass of the biofilm. This cycle within the human body allows for biofilms to quickly populate, further spreading an infection. To prevent infection, we rely on antibiotics through their antibacterial properties, the ability to prevent the growth of or even kill bacteria and other microorganisms (Bentley & Bennett, 2003).

Antibiotic Mechanism and Resistance. Antibiotics are the traditional treatment for bacterial infections, including those in, or originating in, chronic wounds. This treatment targets virulent bacteria by either inhibiting further growth or inducing cell death (Kohanski et al., 2010). To do this, antibiotics cause DNA breakage within the cells, therefore preventing DNA synthesis and ultimately leading to cell death. However, this produces a protective stress response in the bacteria, decreasing its susceptibility to the antibiotic (Kapoor et al., 2017). Antibiotics aim to penetrate the cell walls of bacteria through small channels called porins, the vehicle for diffusion of the treatment through the lipid bilayer of the cell. In response, the bacteria decrease the number of porin channels, preventing this diffusion from occurring (Kapoor et al., 2017). If the bacteria experience selective pressure from an antibiotic, genes within its DNA will mutate and become antibiotic-resistance genes (Kumar et al., 2020). Within a biofilm,

the tightly packed bacteria can easily transmit their antibiotic-resistance genes, thus rendering any given antibiotic ineffective. These mechanisms demonstrate how antibiotic resistance increases in a bacterial biofilm where penetration by an antibiotic is limited.

Undecanoic Acids as Antibiofilm Agent. Salini and colleagues explored the efficacy of combining fatty acids and auxins (growth-regulating plant hormones) to degrade the biofilms formed by bacterial model system *Vibrio harvey*i under *in vitro* and *in vivo* conditions (Salini et al., 2019). Specifically, Undecanoic Acid (UDA), a fatty acid with highly potent antibacterial properties, was tested in conjunction with two plant hormones known as auxins: Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA). When administered alone, UDA didn't effectively reduce biofilm biomass; however, it demonstrated promise when mixed with auxins IAA and IBA. Indole (IAA and IBA) is speculated to control virulence factors in Gram-negative bacteria by inhibiting quorum sensing, which is a sequence of cell-cell communication steps that regulate the formation of biofilms (Salini et al., 2019). Similarly, UDA successfully controlled biofilm growth via quorum sensing inhibition in *Serratia marcescens* (Salini et al., 2015). After seeing the potential of IAA, IBA, and UDA separately, Salini and colleagues wanted to investigate the results when all the agents were combined.

CTAB as Antibiofilm Agent. The surfactant cetyltrimethylammonium bromide (CTAB) reduces bacteria cells' adhesive properties, which is why it is a common ingredient in topical antiseptics (Araujo et al., 2017). In this way, CTAB controls and reduces biofilm development. Araújo and colleagues measured the efficacy of surfactant CTAB as an enhancer of enzymes that degrade biofilms produced by *Pseudomonas* and *P. fluorescens* (Araujo et al., 2017). The bacteria culture of *P. fluorescens* was grown at about 30℃ in a phosphate buffer with glucose, peptone, and yeast extract (Araujo et al., 2017). The results demonstrated that the addition of

CTAB enhanced the enzymatic degradation of EPS and biofilm formation. As reported in the study, 35 mg/L of CTAB decreased biofilm mass by almost 30% as compared to solely enzymatic degradation. Also, metabolic activity in the biofilm was not detectable when CTAB was incorporated in the enzyme treatment, thus indicating cell death in the organism.

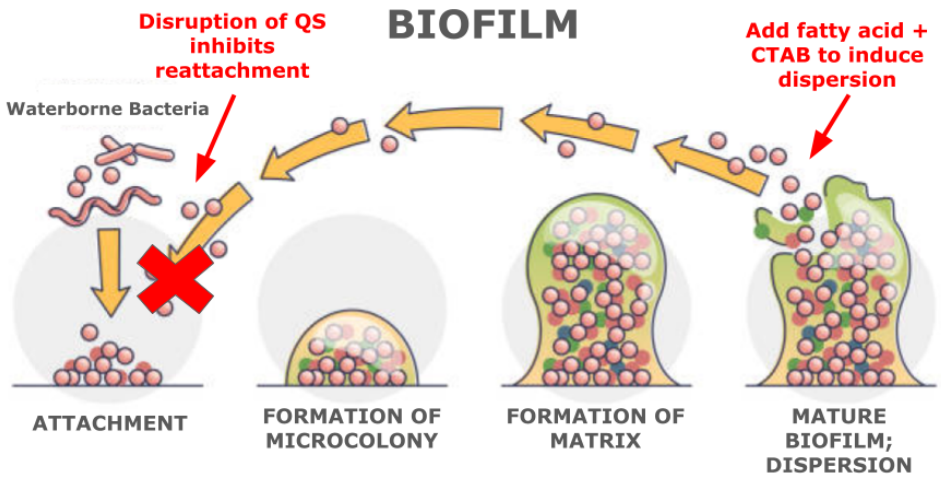


Figure I. A visual representation of how the fatty acid and CTAB interfere with the stages of biofilm formation (Adapted from Sauer et al., 2022).

Research Objectives

To test the efficacy of UDA and CTAB as biofilm inhibitors when combined, varying combinations of these agents were added to *Vibrio harveyi*. Our experiment aimed to determine the concentrations of UDA and CTAB that would most efficiently reduce biofilm mass and cell viability when given in combination. We predicted that if both UDA and CTAB are biofilm inhibitors, then combining them will decrease the biomass of *Vibrio harveyi* biofilm at lower concentrations than when used separately. Research objectives included our primary question: what will the experimental outcomes be from the combinations of UDA (27 uM, 54 uM) and CTAB (96 uM)? We predicted that the addition of these agents would induce dispersion and inhibit bacterial reattachment, as shown in **Figure I**. Additionally, a mutant strain of *Vibrio*

harveyi (designated BB170) allowed us to further explore if this treatment targeted the "AI-2" pathway as depicted in **Figure II.** *Vibrio harveyi* contains two possible quorum sensing "pathways" (AI-1 and AI-2) which can be targeted for biofilm inhibition. Also, considering that CTAB has very potent antibacterial properties in the presence of *V. harveyi*, our objective was to determine the minimum inhibitory concentration of CTAB, which is the lowest concentration of CTAB where biofilm inhibition can be observed. This value has not been reported in the literature for *V. harveyi.*

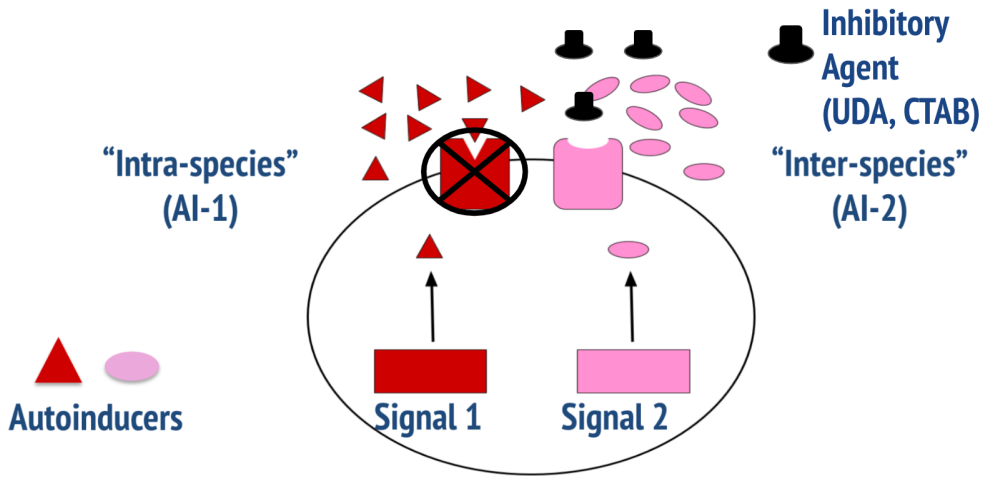


Figure II. The basic pathways of quorum sensing as observed in *Vibrio harveyi* bacteria. Autoinducers bind to the active site of the "sensor," and stimulate gene expression. This gene expression produces virulence factors such as biofilms, among other group behaviors.The BB170 strain of *Vibrio harveyi*, with a disabled AI-1 receptor, can be treated with UDA to see if these fatty acids target the AI-2 pathway.

Importance and Impact

Through the evolution of bacteria to become resistant to many conventional antibiotics, the treatment of chronic wounds has become more difficult and places a financial burden on both the patients and the healthcare system. Chronic wounds are a common diagnosis, in which many suffer from infections and require treatment from medical professionals. Our work breaks new ground since previous studies of UDA and CTAB in combination have not been explored as a

treatment method for an actively forming biofilm. The efficacy of an additional fatty acid, UDA, as an antibiofilm agent has not been previously tested on biofilms at different concentrations. It is unknown what mechanism UDA uses to degrade biofilms in the *Vibrio harveyi* model system. The literature has not previously determined which autoinducer pathway these fatty acids target, or how they perform on a variant with a disabled pathway. CTAB has not been tested in combination with fatty acids, only with enzymes that enhanced its biofilm inhibition efficacy. The mechanism of how CTAB interacts with fatty acids in a *Vibrio harveyi* biofilm is unknown. Promising concentrations of these antibiofilm agents have been identified; however, there are no data documenting how they perform together (Salini et al., 2019; Santhakumari et al., 2017; Araújo et al., 2017). Our work is designed to address these questions and provide insight to the interactions between UDA and CTAB.

Through efforts like our project, we can potentially provide an alternative to conventional antibiotics, hence reducing the rates of antibiotic resistance and ensuring more effective treatment for patients. To discover the minimum inhibitory concentration (MIC) of CTAB allows treatments to be created using this agent. The minimum inhibitory concentration is important to know for any antibacterial agent so that treatments are effective and safe. Ultimately, we have narrowed down the concentration range in which the MIC can be found, which provides information for future studies looking to use CTAB in any given antibiofilm treatment.

Materials

Various microbiology media, cultures and supplies were used to conduct our project's experiments. The *Vibrio harveyi* mutant strains BB120 (wild type) and BB170 were available from previous student projects. Reagents were purchased from SigmaAldrich (ethanol, methanol, DMSO, PBS 10x, Undecanoic Acid, Cetyltrimethylammonium Bromide, crystal violet,

resazurin) and filter-sterilized when necessary. Black-walled sterile 96-well microplates (Costar 3603) were used for resazurin assays; clear, round-bottom 96-well microplates were used for crystal violet staining assays. A pH meter was used in the preparation of media.

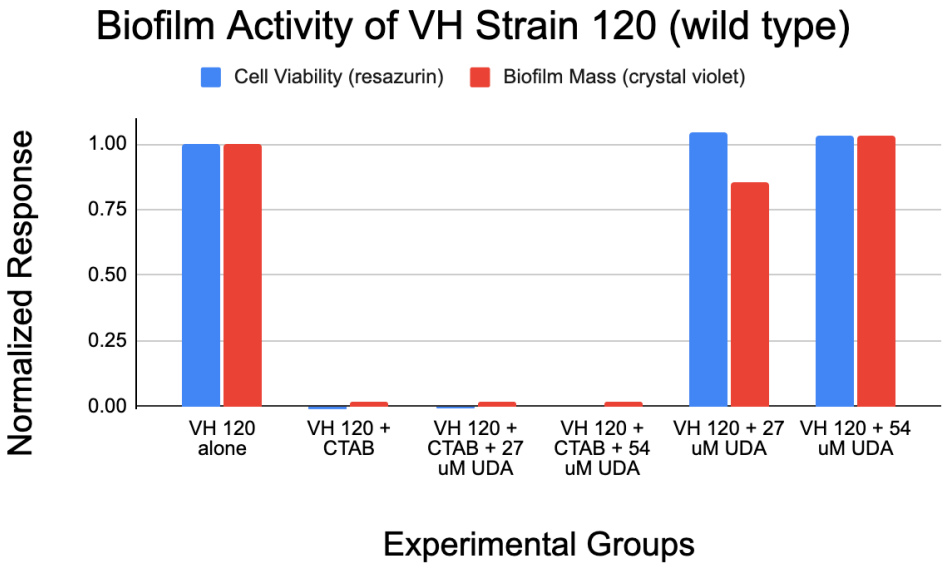
Methods. All studies were conducted using proper aseptic techniques, including disposal of sterilized biological materials. All *Vibrio harveyi* strains (BB120 & BB170) were grown in a sterilized Difco Broth (MB) medium and were incubated at 30℃ while constantly shaking (Vikram et al., 2010). Stock solutions of Undecanoic Acid and CTAB were prepared in ethanol in order to address solubility issues; diluted stocks were prepared using Difco MB and mixed with the *Vibrio* strains at the appropriate time. The wells in the microtiter plate (96 well plate) contained less than 1% of ethanol (as proposed by Vikram et al., 2010) to minimize any interference of the ethanol on bacterial growth. Undecanoic Acid was tested at different concentrations (27-54 uM final concentrations) and CTAB was tested at different concentrations (0.86-96 uM).

We acknowledge important work done by previous students in adapting experimental protocols for our studies, as adapted from Packiavathy and colleagues (2013). For the biofilm assays, separate cultures of *V. harveyi* strains were grown overnight at 30°C in Difco media. The bacteria was diluted 1:100 in fresh media. One hundred microliters of media + *V. harveyi* strain was added to 50 µl of concentrated CTAB, UDA, or CTAB + UDA solution, filling 150 µl of each well. Control groups included wells without any of these solutions added. The solutions were then incubated for 16-24 hours at 30°C. Resazurin and crystal violet assays were used to quantify cell viability and biofilm formation, respectively. These assays followed published protocols taken from papers used in our Literature Review. Resazurin assays were performed using a Molecular Devices Gemini fluorescence microplate reader, and the crystal violet assays

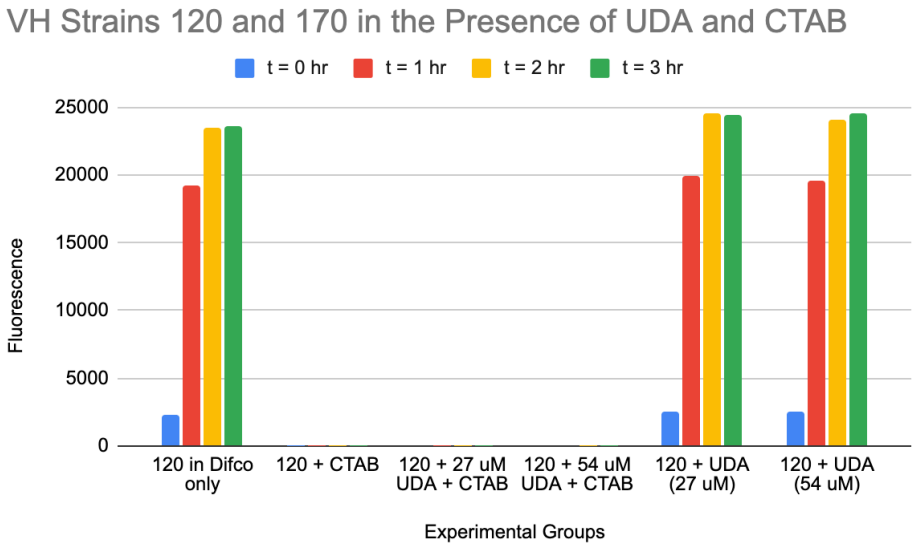
were performed using a Molecular Devices ThermoMAX microplate reader. Data was copied into a spreadsheet program (Google Sheets) and processed manually. “Media only” blank values were averaged and subtracted from the signal to give a corrected value for each well. Bar charts were prepared and the experimental samples were normalized to the control samples.

Results

We completed four experiments throughout this Project. For the first experiment, we tested UDA at 54 uM and 27 uM when administered to the two *V. harveyi* strains. This experiment showed that UDA did not have an inhibitory effect on biofilm growth. For our second experiment, we repeated the conditions from the first experiment, as well as including both of these concentrations in combination with 96 uM CTAB. We also tested CTAB alone to observe the antibiofilm properties of this agent on its own. The results for this experiment can be seen in **Graphs I** and **II**. Consistent with our results from experiment #1, UDA alone had no inhibitory effect on the biofilms of either strain. CTAB had an effect on the biofilm, but ultimately killed the bacteria.

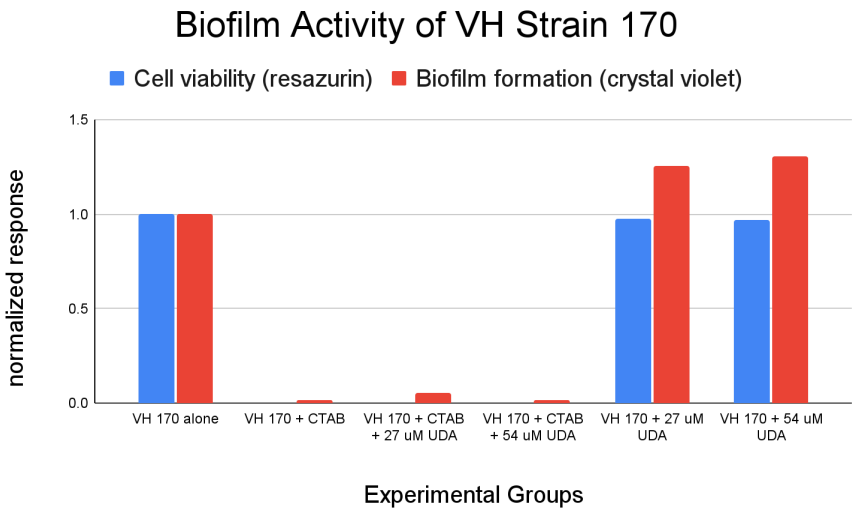


Graph 1: Biofilm activity of *Vibrio harveyi* strain 120 in the presence of UDA (0, 27 uM, 54 uM) and CTAB, in varying combinations. (Data are from Study #2).



Graph II: Biofilms of *Vibrio harveyi* (strains 120 and 170) were allowed to form in the presence of UDA and CTAB, and the cell viability (amount of live cells) of the resulting biofilms was observed using fluorescence 595 after three hours in incubation. This graph presents results for the control group and the experimental groups with UDA, CTAB + UDA, and CTAB alone. There was no bacterial growth in the wells that contained CTAB, but the wells treated with UDA alone grew in a similar way as the control (bacteria alone).

For our third experiment, we further explored CTAB by attempting to find the minimum inhibitory concentration, or MIC. In this experiment, we used CTAB at concentrations 13.7 uM, 6.86 uM, 3.43 uM, 1.7 uM, and 0.86 uM. The results from this experiment demonstrate that the MIC of CTAB does not lie within this range, as all of the bacteria treated with CTAB grew consistently with the control. For our fourth experiment, we repeated the MIC exploration with new and higher concentrations. The anti-biofilm effects of CTAB were explored at concentrations 83 uM, 66 uM, 50 uM, 33 uM, and 17 uM. Consistent with the results from experiment #3, the MIC was not determined from these concentrations but we were able to determine a range for the MIC (between 83 uM and 96 uM).



Graph III: Biofilm activity of *Vibrio harveyi* strain 170 in the presence of UDA (0, 27 uM, 54 uM) and CTAB, in varying combinations. (Data are from Study #2). The presence of CTAB prevented any biofilm activity.

Discussion & Conclusion

The results from **Graph I** and **Graph III** with UDA and CTAB both refute and support our hypothesis. The findings of this particular experiment helped us confirm that CTAB has antibiofilm properties and prevents bacterial growth. On the contrary, we observed that UDA had no effect on biofilm formation as we initially hoped based on our analysis of the published literature on UDA. These results for VH strain 120 and 170 remained consistent with each other (compare **Graph I** and **Graph II**).

According to all our graphs, UDA is not a viable quorum sensing inhibitor. At concentrations of 27 uM and 54 uM, UDA had no effect on biofilm growth. Thus, we can conclude that UDA did not inhibit gene expression as we had predicted; active gene expression leads to the production of virulence factors and biofilms. The effects of UDA on VH strain 120 and strain 170 did not differentiate from each other; therefore, we are unable to determine which pathway it targets, if any.

Graph I, II, and **III** indicates that CTAB has very potent antibacterial properties in the

presence of both strains of *V. harveyi* and triggers cell death. This confirms our proposal that the incorporation of CTAB halted all metabolic activity, thus indicating cell death. The results support our hypothesis that CTAB controls and prevents biofilm development. After completing two full experiments with both UDA and CTAB, the data suggested that UDA had no effect on biofilm formation, and so we focused the remainder of our study entirely on CTAB.

Considering our data from **Study 3** and **Study 4** (data now shown here), we can conclude that the minimum inhibitory concentration of CTAB suggestively lies between 83 uM and 96 uM. At a low concentration range (0.86 uM-13.7 uM), CTAB had no observable effect on biofilm growth. All the bacteria grew at the same rate as the control group, thus indicating that these concentrations were far too low to interfere with biofilm growth. With that being said, we can conclude that the MIC of CTAB is much higher. At a higher concentration range (17 uM-83 uM) the results did not vary. All the bacteria continued to grow with the control, even in the presence of CTAB at 83 uM. Since CTAB killed all the bacteria at a concentration of 96uM but had no observable effect on bacterial growth at 83uM, we can conclude that the MIC lies somewhere in between that range.

Fatty acids could be substantial antibiofilm and antivirulence agents against microbial biofilms. Researchers speculate that since fatty acids possess both hydrophilic (water-loving) and hydrophobic (water-repelling) properties, they can easily penetrate cell membranes and disrupt the biofilm's extracellular matrix (Burdge & Calder, 2015). Our work evaluated how one fatty acid might interfere with the biofilm formation steps, and future studies based on our work can explore how fatty acids might be used to treat pre-formed biofilms associated with chronic wounds.

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