Dual Therapy Approaches for Antibiotic Potentiation Against Biofilm Threats

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List of Abbreviations

EPS	Extracellular polymeric substance
DAAs	D - amino acids
LAAs	L - amino acids
QACs	Quaternary ammonium compounds
D-leu	D - leucine
D-met	D - methionine
D-trp	D - tryptophan
D-tyr	D - tyrosine
D-ala	D - alanine
D-glu	D - glucose
D-pro	D - proline
D-phe	D – phenylalanine
S. epidermidis	*Staphylococcus epidermidis*
S. aureus	*Staphylococcus aureus*
P. aeruginosa	*Pseudomonas aeruginosa*
P. fluorescens	*Pseudomonas fluorescens*
E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*
PG	Peptidoglycan
SEM	Scanning Electron Microscopy
CV	Crystal violet

MIC	Minimum inhibitory concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
DDAC	Didecyldimethylammonium chloride
CTAB	Cetyltrimethylammonium Bromide
h	Hour
AgNPs	Silver nanoparticles
CNC	Nanocellulose
PVP	Polyvinylpyrrolidone
QOSA	Quaternized Oxidized Sodium Alginate
ROS	Reactive Oxygen Species
MHB	Mueller-Hinton Broth
TSB	Tryptone Soya Broth
HCL	Hydrogen Chloride
NaOH	Sodium Hydroxide
PBS	Phosphate Buffer Saline
OD	Optimal Density
(R)	Repeat
CLSM	Confocal Laser Scanning Microscopy
SD	Standard Deviation
PI	Propidium Iodide

Abstract

Bacterial biofilms, complex communities settle in their own self-produced extracellular matrix, are a major cause of chronic and persistent infections in chronic wound and on medical devices due to their remarkable resistance to antibiotics and the immune system. This resistance is because of the biofilm matrix defense, slow bacterial growth, and the persister cells, with a minimum inhibitory concentration as much as 1000 times higher than that required for planktonic bacteria. Antibiotic resistant pathogens leading chronic infection is an indication of the necessity of new therapeutic approaches. In this study, the limitation will be met through the investigation of dual therapeutic approach that combine the use of antibiotics with the application of biofilms dispersal agents in D- amino acids (DAAs) and quaternary ammonium compounds (QACs) to enhance the sensitivity of the biofilm coated bacteria as well as the effectiveness of the treatment. The test hypothesis is that QACs and DAAs by interference with biofilm matrix and increasing bacterial susceptibility, will increase the effectiveness of antibiotics against clinically relevant bacteria such as *Staphylococcus aureus* and *Escherichia coli*. Methods used in vitro inhibition and dispersion of biofilms through the use of crystal violet staining and minimum biofilm eradication concentration (MBEC) testing, using antibiotics alone and in combination. The results indicated that the removal of biofilms by QACs alone had varying effects. However, QACs showed a strong synergistic effect by considerably reducing the quantity of antibiotic required to kill biofilm bacteria when paired with antibiotics at lower, sub-eradication concentrations. Because of their solubility and strain-specific difficulties, DAAs had little antibiofilm activity under the testing conditions. These findings suggest that dual therapy approaches hold great promise for addressing biofilm medicated resistance in chronic wound therapy. Future research direction includes in vivo confirmation, optimization of delivery system for instance in the format of alginate hydrogels, and mechanism-based investigations so further define the mechanisms of disruption of biofilms and antibiotic enhancement.

Keywords: Bacterial biofilm, Antibacterial, Antibiotics, MBEC, QACs, DAAs.

Declaration

I certify that this thesis,

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

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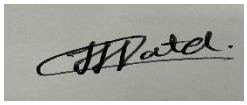
Sign: 

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Chapter 1: Literature Review

1.1 Introduction:

Complicated bacteria populations which are known as biofilms are coated in extracellular matrix which they create by their own and stick to the surfaces. The extracellular polymeric substances (EPS) make the extracellular matrix which includes proteins, lipids, polysaccharides and DNA (Donlan, 2002; Zhao et al., 2023). Antibiotics have trouble eliminating biofilms, which are organized colonies of microorganisms that attach to the surfaces and are protected in self-produced matrix. Planktonic cells change into sessile cells in response to certain signals, making the development of them a highly controlled process affected by both genetic and environmental variables (Donlan, 2002; Hall & Mah, 2017). By enabling bacteria to attach to surfaces and contribute to infectious processes, bacterial biofilms can cause both device-related and non-device-related diseases. They are also responsible for more than 80% of the infections, including multidrug-resistant scenarios (Fleming & Rumbaugh, 2017; Zhao et al., 2023).

Compared to planktonic bacteria, biofilm-based cells have the ability to withstand much higher concentrations of antibiotics, with 100-1000 times greater tolerance (Høiby et al., 2010). Because of their extracellular matrix, slow development, modified drug targets, and effective gene exchange, biofilm associated illnesses are frequently recurrent and tolerant to antibiotics (Liu et al., 2024). By increasing tolerance to antibiotics and resisting immune defense systems like phagocytosis, bacterial biofilms result in persistent infections, particularly when they infect foreign objects (Høiby et al., 2010; Sharma et al., 2019). Regrowth and recolonization of biofilms can occur quickly if they are not completely eradicated (Hall & Mah, 2017; Høiby et al., 2010). Implanted medical devices offer an attractive substrate for biofilm formation, which serves as a recurring source of infection (Høiby et al., 2010; Singh et al., 2017).

Antimicrobial drugs can only penetrate so far through the biofilm matrix. It establishes a physical barrier that retards the spread of antibiotics. Antimicrobial substances can be bound by the matrix or rendered inactive before they reach the target cells (Hall & Mah, 2017; Liu et al., 2024). Within a biofilm, cells display many physiological states. Antibiotics may be more effective against cells that are metabolically active close to the surface. Deeper layers' dormant or slowly developing

cells are frequently more resistant to therapy (Hall & Mah, 2017; Høiby et al., 2010). Cell-to-cell communication is a survival strategy used by biofilms. It synchronizes gene expression throughout the biofilm community. In response to the population density, quorum sensing controls virulence factors. It affects the processes of biofilm formation, maturation, and dissemination (Hall & Mah, 2017; Sharma et al., 2019).

Biofilms are ubiquitous challenge because they may develop on both living tissues and non-living surfaces. Although bacterial biofilms are resistant to common antibacterial medications, they play a crucial role in chronic wound infections. Because of their complex structure and defensive mechanism, which inhibit healing, innovative therapeutic approaches are required to successfully target and kill biofilm related bacteria in chronic wound management (Sanchez Jr et al., 2014). The coordinated mechanism of biofilm dispersal is investigated in the late stages of pathogen growth. The aim of the research is to find dispersive compounds that can disperse biofilm, address their resistance and improve the effectiveness of the antibiotics against chronic wound (Sanchez Jr et al., 2014). By increasing the susceptibility of bacteria to antibiotics and human immunological responses, the dispersal of biofilms can improve the effectiveness of treatment (Goswami et al., 2023; Sanchez Jr et al., 2014). The critical need to convert laboratory results into practical techniques for better clinical outcomes is highlighted by the lack of a defined clinical standard despite substantial research on anti-biofilm medicines (Goswami et al., 2023).

A potential strategy against illnesses linked to biofilms is the combination of antibiotics with biofilm dispersion agents. A promising example of such are D-amino acids (DAAs). By removing bacteria from their protective matrix, DAAs break apart biofilms and increase sensitivity to antibiotics (Sanchez Jr et al., 2014). Research on clinical isolates of *S. aureus* and *P. aeruginosa* shows that DAAs improve antibiotic activity against biofilms in vitro, avoiding the drawbacks of traditional antibiotics (Sanchez Jr et al., 2014). This strategy makes use of DAAs capacity of breaking up biofilms and work in combination with antibiotics to target biofilm-specific resistance pathways. By tackling biofilm persistence and enhancing therapeutic results, such combinations have potential to completely change the treatment of chronic wounds (Sanchez Jr et al., 2014).

Quaternary ammonium compounds (QACs) are another potential class of compounds that could be used to improve the activity of antibiotics against biofilms. QACs are a broad category of amphiphilic compounds, each characterized by a cationic ammonium head surrounded by 4 alkyl

chains, ranging in length and functional groups. They are commonly used in antiseptic and disinfectant applications, due to their effective antibacterial activity that is driven by membrane perturbation. Their mechanism of antibacterial action involves the alignment of the cationic ammonium head with the negatively charged outer surface of the plasma membrane, which is followed by an insertion of the hydrophobic tail into the lipid region of the lipid bilayer. QACs have recently been recognized for their potential to enhance the antimicrobial effect of other antimicrobial compounds, against both planktonic and biofilm-based infections (Saverina et al., 2023).

To tackle the challenge of biofilms in chronic wounds, one potential strategy is to develop combination therapies that can enhance the activity of antibiotics, and can be incorporated into a hydrogel-based application. The alginate is non-toxic, biocompatible, antibacterial, reduces swelling, retains moisture, absorbs wound fluid, and promotes skin regeneration, it is used to create innovative wound healing biomaterials (Kibungu et al., 2021). Alginate based hydrogel with dispersion agent improve wound healing by lowering inflammation and promoting the proliferative phase without interfering with the natural healing mechanism (Bounds et al., 2022).

1.2 Biofilm Formation and Challenges:

Biofilms are organized microbial communities made up of proteins, lipids, polysaccharides, and nucleic acids, including eDNA, that are encased in an EPS matrix. This hydrated combination aggregates cells and supports the structure of biofilms, forming a 3D scaffold (Yin et al., 2019). There are five essential phases in the ongoing cycle of biofilm development (Yin et al., 2019). First is attachment, which occurs when microorganisms use forces like van der Waals to attach slowly to surfaces. Second is colonization in that they use structures like sticky molecules or flagella to securely attach. The third stage is development, during which time cells divide and produce EPS matrix layers, to provide support. Fourth is Maturation stage in that, the biofilm develops in 3D structure with nutrient pathways, allowing for resource sharing and communication and lastly, fifth is active dispersal stage in that microorganism can spread and restart the cycle somewhere else when proteins of the biofilm break off as a result of internal and external cues (Yin et al., 2019). Microbes are able to colonize new areas and overcome adverse conditions because of this continues process (Yin et al., 2019).

In addition to surface-attached microbial populations, biofilms now indicate non-surface aggregates. There are two types of chronic infections: surface-associated and non-surface-associated. Although planktonic bacteria have been associated with acute infections, new research indicates that bacterial metabolic activity, rather than just aggregation, is more important for chronic infections (Sauer et al., 2022). Due to the biofilm formation, which functions as an obstacle in medicines, chronic wounds frequently resist therapy. Although biofilms are present in less than 10% of acute wounds, they can form in up to 60% of chronic wounds, which greatly delays healing and increases the risk of recurring infections even with careful treatment (Clinton & Carter, 2015). Furthermore, they have a significant impact on infections linked to medical equipment, which can result in difficult-to-treat persistent and recurring illnesses (Clinton & Carter, 2015; Rather et al., 2021).

Antibiotic tolerance is one of the main problems that biofilms in clinical settings provide. Antibiotic tolerance in bacteria found in biofilms can reach 1000 times higher levels than in bacteria found in planktonic environments (Clinton & Carter, 2015). There are multiple reasons for this increased tolerance: (1.) Antibiotic penetration is physically restricted by the extracellular matrix. (2.) Bacteria in biofilms are less vulnerable to antibiotics that target active cell processes because of their slow growth rates. (3.) Persister cells, which are extremely tolerant to antibiotics, can be found in biofilms (Clinton & Carter, 2015).

In addition to antibiotic resistance, biofilms provide other difficulties. By inhibiting the identification of pathogen-associated molecular patterns and restricting the penetration of immune cells and antibodies, the biofilm matrix shields bacteria from host immunological responses. This defense mechanism adds to the enduring and recurrent nature of infections linked to biofilms (Clinton & Carter, 2015; Rather et al., 2021). Cells in various physiological states live within the same biofilm, exhibiting phenotypic variety in biofilms as well. This diversity promotes resistance to treatment and enables survival under a variety of environmental stressors. For instance, cells that are metabolically active and close to the surface may be more vulnerable to antibiotics, whereas slower-growing or dormant cells are typically more resilient to them (Clinton & Carter, 2015; Rather et al., 2021).

Persister cells restore biofilms when antibiotics stop working, maintaining persistence and the initial antibiotic sensitivity which made them more difficult to treat. Bacterial EPS causes entire removal more difficult by strongly attaching biofilms to wound beds. After biofilms are removed, residual cells reproduce the biofilm, causing recurrent infections that are frequent in chronic wounds (Clinton & Carter, 2015). By managing gene expression in relation to population size, quorum sensing allows bacteria to coordinate to the biofilm development and the production of virulence factors(Clinton & Carter, 2015). Quorum sensing, cell-to-cell signaling, and external factors all influence the production of biofilms, which results in organized microbial colonies. These biofilms create serious risks to the food, healthcare, and veterinary sectors by causing persistent infections, antibiotic resistance, immunological escape, and food spoiling (Rather et al., 2021).

Figure 1 :Cycle of biofilm formation with different stages in the microorganism (Yin et al., 2019). Biofilm formation has five different stages: I. Attachment: microbial cells slowly attach to the surface. II. Colonization: microbial cells securely attach to the surface through sticky molecules like flagella, pili, etc. III. Development: cells divide and produce an extracellular polymeric substances (EPS) matrix to provide support. IV. Mature: biofilm forms 3D structure to stabilize. V. Active dispersal: protein of biofilm breaks, and microorganisms can spread and restart the cycle.

1.3 Roles of DAAs in Dispersal and Inhibition of the Biofilm:

A number of reports have shown that certain combinations of DAAs can inhibit biofilm growth of pathogens. By interacting with structural components, D-leucine, D-methionine, D-tryptophan and D-tyrosine (D-leu, D-met, D-trp, D-tyr) from *Bacillus subtilis (B. subtilis)* biofilms prevent the development of new biofilms while dispersing existing biofilm (Warraich et al., 2020).. They improve efficacy against antibiotic resistance when combined with antibiotics, providing strategy to overcome treatment problems. Amyloid fibers that structurally connect biofilm cells are released in response to DAAs (Warraich et al., 2020). Bacteria mostly proliferate in wounds as biofilms, which are communities of one or more species covered in an extracellular matrix that the bacteria produce on their own to protect and increase their survival (Sanchez Jr et al., 2014).

Bacterial peptidoglycan (PG) is made up of necessary DAAs, specifically D-alanine and D-glutamic acid (D-ala, D-glu), which combine to create cross-linking stem peptides that give the molecule structural stability and resistance to osmotic stress (Aliashkevich et al., 2018). While the production of exopolysaccharides is unaffected, the disruption of the protein component of the EPS caused by DAAs (D-tyr, D-pro, D-phe) inhibits the formation of *Staphylococcus aureus (S. aureus)* biofilm without preventing initial attachment of bacteria. Compared to individual amino acids (500μM each), a combination of these DAAs at concentrations under 100μM significantly inhibits microcolony growth into mature biofilms (Aliashkevich et al., 2018). Furthermore, the mixture breaks up existing biofilms. There are contradictory findings, indicating variations in the experimental setup or strain-specific reactions. This indicates the complex structure and potential of DAAs as drug that target biofilms (Aliashkevich et al., 2018).

The effects of D-tyr on *B. subtilis* (a Gram-positive bacteria) and *Pseudomonas aeruginosa* (*P. aeruginosa)* (a Gram-negative bacteria) biofilms were species-specific and concentration-dependent. D-tyr prevented the production of biofilms in both species at low concentrations (5nM) and high concentrations (200μM), but it had no impact at intermediate doses (1-10μM) (Aliashkevich et al., 2018). EPS were affected differently by D-tyr, in *P. aeruginosa* exopolysaccharides climbed at low concentrations and reduced at higher levels, whereas extracellular proteins declined (Aliashkevich et al., 2018). On the other hand, *B. subtilis* revealed no change in exopolysaccharides and an increase in extracellular proteins. The complex nature of DAAs interaction with bacterial biofilms and the requirement for modified applications are highlighted by these contradictory reactions, which point to different, concentration-dependent mechanisms of action among species (Aliashkevich et al., 2018).

As an equimolar mixture, D-met, D-phe, D-trp concentration of ≥5mM dispersed *P. aeruginosa* and *S. aureus* biofilms with increased efficiency. DAAs improved the effectiveness of antibiotics against clinical isolates by reducing *S. aureus* minimum biofilm inhibitory concentration (MBIC) from 32 to 8 μg/ml and achieving >2-log bacterial reduction when combined with antibiotics (Sanchez Jr et al., 2014). By dispersing biofilms and releasing bacteria for better treatment, DAAs increase the effectiveness of antibiotics. This is an effective approach to chronic infections (Sanchez Jr et al., 2014). By lowering biofilm bacterial counts and inhibitory concentrations, DAAs (D-met, D-phe, and D-trp) dispersed *S. aureus* and *P. aeruginosa* biofilms in vitro and

improved the effectiveness of antibiotics (Sanchez Jr et al., 2014). By exchanging D-ala in peptidoglycan, DAAs (such as D-leu and D-met) prevent the production of bacterial cell walls while also changing the physicochemical characteristics and structure of cell walls (Su et al., 2022).

Antibiotic efficiency is increased and resistance, which can include matrix-mediated process, which is challenged by targeting the components of the biofilm matrix. As anti-biofilm agents, amino acids have potential for use in wound healing dressing and coating medical equipment like implants as well as catheters, which is providing dual-action techniques against chronic infections and preventing the growth of antibiotic resistance (Warraich et al., 2020).

1.4 The antibacterial and antibiofilm activity of QACs:

Broad-spectrum bactericidal substances with a simple amphiphilic structure, QACs have a hydrophilic quaternary ammonium group and up to four hydrophobic alkyl chains (Verderosa et al., 2019). They work against bacteria by disruption of bacterial plasma membrane, which results in cell lysis and cytoplasm leakage. Commercially important for infection control, QACs are commonly used as preservatives, disinfectants, and antiseptic (Verderosa et al., 2019).

The dialkyl-quaternary ammonium compound didecyldimethylammonium chloride (DDAC) has bactericidal, virucidal, and fungicidal qualities and is utilized in several products (Anderson et al., 2016). A study that modelled *Bacillus cereus (B. cereus)* and *Escherichia coli (E. coli)* exposed to sub-MIC DDAC over a 48-hour period. It demonstrated the concentration-dependent bacteriostatic and bactericidal action of DDAC (Pedreira et al., 2022). Using CTAB in combination therapy to treat biofilm the enzymes like lipase, protease, β-glucanase and α-amylase demonstrated modest biofilm removal and CFU decrease against *Pseudomonas fluorescens (P. fluorescens)* (Araújo et al., 2017). While cetyltrimethylammonium bromide (CTAB)-protease combinations counteracted removal, CTAB with β-glucanase or α-amylase combinations improved biofilm removal. Protease reduced the biofilm mass over the time, whereas biofilms treated with β-glucanase developed again. The effectiveness of CTAB was sometimes diminished by enzymes, and complete eradication was not obtained (Araújo et al., 2017).

The antibacterial activity of DDAC against *E. coli* was strong, with a MIC of 1.3 mg/L. Fluorescent probe-labeled liposomes indicated a phase transition in membrane fluidity, which was correlated with protein and β-galactosidase leakage at 3-4 mg/L. According to these findings, DDAC destroys cells by rupturing the membrane integrity, which allows macromolecule leakage and at larger concentrations, blebs develop post-disruption (Yoshimatsu & Hiyama, 2007).

CTAB, a surfactant, is frequently used in topical antiseptics because it reduces the attachment qualities of bacterial cells (Araújo et al., 2017). Araújo et al. showed that by breaking down EPS, CTAB improves the enzymatic breakdown of *Pseudomonas* and *P. fluorescens* biofilms, lowering biofilm mass by 30% at 35 mg/L in comparison to enzymes alone. Although total biofilm eradication was not achieved, CTAB-enzyme combinations reduced metabolic activity, indicating cell death (Araújo et al., 2017; Carney & Perkins, 2023). Using a rotating device, the biofilm's physical stability was also investigated. The variation of the mass remaining on the surface allowed for the evaluation of the surfactant's impact on the stability of the biofilm (Simões et al., 2005). The kinetics at sub-MIC concentrations, when adaptation, growth, stationary, and death phase can be observed, are ignored by these static values (Pedreira et al., 2022). These concentrations did not yield the MIC of CTAB, but we were able to identify a range for the MIC which is between 83 µM and 96 µM for CTAB (Carney & Perkins, 2023). They work in combination with other substances such as enzymes and amino acids to improve corrosion prevention and biofilm dispersal (Carney & Perkins, 2023; Zhou et al., 2023).

In time-kill assay, the combination of DDAC and Si-QAC increased antimicrobial activity, indicating that lower concentration successfully treat multidrug-resistant hospital-associated illnesses (Chun et al., 2024). α,α-Disubstituted β-amino amides mached the effectiveness of chlorhexidine (CHX) in reducing *S. aureus* biofilm viability by 45-50 µM in 1 hour. CTAB demonstrated similar susceptibility to planktonic cells as CHX but less effect on biofilms (Ausbacher et al., 2023). Through membrane rupture, CTAB deactivates planktonic cell, although it has trouble with bacterial biofilms. Despite not achieving complete inactivation, laminar-flow biofilms are more susceptible than turbulent ones. Biofilms demonstrated respiratory recovery after treatment, and biofilm detachment was not induced by CTAB alone (Simões et al., 2005).

1.5 Alginate-Based Hydrogel Formation for Wound Healing:

According to histological research, modified alginate dressings have a great potential for treating chronic infected wounds in both medical and veterinary scenarios because they encourage full re-epithelialization, thicker neoepithelium, dense collagen, and little inflammation (Atepileva et al., 2024). Although gas exchange and painless application are provided by alginate-based dressing, their structural strength and antibacterial qualities are insufficient, which demands the addition of other agents for efficient wound care (Atepileva et al., 2024). Time-dependent drug delivery is made possible by natural hydrogels like alginate, chitosan, and dextran, which also have advantages over synthetic hydrogels for controlled therapeutic release, including biocompatibility, biodegradability, bacteriostatic effects, wound-healing qualities, and tissue-like dynamics (Babavalian et al., 2015). Alginate hydrogel cross-linking is decreased by low calcium levels, which causes softer, porous forms. These hydrogels reduce tissue damage during dressing changes by effectively absorbing wound exudate and facilitating medication distribution. Despite having less mechanical strength, their high-water content keeps the wound area wet, which aids in healing (Shi et al., 2023).

Alginate hydrogels absorb exudate, retain moisture, and help in the healing process (El-Sayed et al., 2024). Even if alginate's endotoxin level is different, it shows biocompatibility defends its medical application. With a concentration on hydrogel composition and the impact of alginate on important functional aspects in these biomedical domains, this study explores its function in antimicrobial applications, controlled drug release, cancer therapy, and skin restoration (Tomić et al., 2023). Through variable release, gelling, and degradation, the addition of bioactive medication and nanocarriers to alginate-based biomaterials improves wound healing. By increasing surface area and improving mechanical strength, drug bioavailability, and tissue contact, the design of these hydrogels as microneedles enhances controlled therapeutic delivery and healing results (Kibungu et al., 2021). In order to improve coagulation and antibacterial qualities in wound healing, alginate is mixed with biopolymers like fibrinogen, nisin, and EDTA (Tomić et al., 2023).

When it comes to drug delivery, alginate is a flexible biopolymer that works well for releasing medications with low or high molecular weights as well as those with poor solubility. Customized therapeutic delivery systems are made possible by their safety, biocompatibility, biodegradability,

unique structure and simple production (Tomić et al., 2023). Hydrogels with increased mechanical strength and high-water absorption were produced by combining alginate with silver nanoparticles (AgNPs) and nanocellulose (CNC). These substances showed low cytotoxicity and concentration-dependent antibacterial action against *S. aureus, E. coli, P. aeruginosa*. The polyvinylpyrrolidone (PVP) stabilized 3D-printed scaffolds have potential for use in medical applications which require infection control and biocompatibility (Tomić et al., 2023). A self-healing dialdehyde alginate or polyethyleneimine hydrogel containing strontium ions was produced by Lu et al. It sticks to wounds, heals itself, and releases strontium ions which stimulates collagen deposition and angiogenesis, speeding the healing of skin defects and sports injuries (El-Sayed et al., 2024).

1.6 Wound Healing Application:

In chronic wounds, biofilms are organized microbial communities that are surrounded by an EPS matrix of proteins, lipids, DNA, and exopolysaccharides. In order to facilitate food exchange and resistance to host immune system and treatments, they must stick to the surface, proliferate, and differentiate into protective structures (Diban et al., 2023). Chronic wound inflammation inhibits cell division and healing by disrupting the normal balance of growth factor which is lytic enzymes. Exudate that is high in nutrients, maintains bacterial biofilms, which increases infection and chronic inflammation (Goswami et al., 2023).

Chronic wounds stop the inflammatory phase because of ongoing innate immune activation, which causes increased collagenase activity. This will affect the growth factor, macrophage polarization, and enhance the tissue injury which will restrict the healing process by sustaining inflammation (Versey et al., 2021). Although it has been proven, long-term exposure to biofilm in wounds might delay the formation of protective innate immunological memory by maintaining immune cell activity (Versey et al., 2021). Research showed that 60% of chronic wounds had biofilms, compared to 6% of acute wounds while performing the SEM and light techniques. Small samples and their uneven distribution could make it difficult to determine the existence of biofilms (Percival et al., 2015).

In chronic wounds, bacterial biofilms that are less metabolically active and wrapped in EPS, resist treatment and increase inflammation, which delays tissue healing (Clinton & Carter, 2015). By causing the release of amyloid fibers, which structurally connect cells, DAAs (D-leu, D-met, D-

trp, D-tyr) prevent the formation of biofilms and disperse those that already exist. These DAAs, which were isolated from *B. subtilis* biofilms, disperse microbial aggregation and provide another way of preventing biofilm persistence (Warraich et al., 2020).

For the treatment of diabetic wounds, a quaternized oxidized sodium alginate (QOSA) hydrogel material was created that has effective hemostatic, antibacterial, self-healing, and injectable qualities (Ma et al., 2024). Alginate-based wound dressings can reduce bacterial infections at the wound site, absorb excess wound fluid, and keep the surrounding environment physiologically moist (Aderibigbe & Buyana, 2018). This shows the potential uses of multifunctional hydrogel materials containing QOSA in promoting antibacterial activity and speeding up skin wound healing (Ma et al., 2024).

1.7 Experimental Methods for Biofilm and Antibiotic Testing:

Experimental techniques and antibiotic testing for biofilm is important for determination of the DAAs and QACs effectiveness. Bacterial biofilm assay divided in two parts biofilm inhibitory assay and biofilm dispersal assay. Biofilm inhibition assay can determine the inhibitory effect of the compounds on bacterial biofilm. For biofilm inhibition assay, bacterial biofilm was grown with various concentrations of DAAs and QACs for 48 h in 96-well plate (Warraich et al., 2020). For biofilm dispersal assay, bacterial biofilm were grown in 96-well plate for 24 h and then treated with various concentrations of DAAs and QACs for 24 h which can determine the dispersal effect of the compounds on the bacterial biofilm. These was followed by crystal violet staining and then quantified viable cells by taking absorbance (Warraich et al., 2020). Study showed, biofilm quantification can identify that the DAAs inhibits the biofilm formation in *S. aureus and P. aeruginosa,* when detached cell were removed from the surfaces (Kolodkin-Gal et al., 2010).

The particular type of DAAs determine the minimum inhibitory concentration (MIC) at which biofilm formation may be inhibited (Ampornaramveth et al., 2018). According to guidelines of Clinical and Laboratory Standards Institute, antibiotic susceptibilities were measured by determination of MICs (Sanchez Jr et al., 2014). MBEC assay stands for Minimum Biofilm Eradication Concentration. In order to measure the minimum biofilm eradication concentration (MBEC), biofilms were treated with increasing antibiotic concentrations by using MBEC assay plates. The lowest antibiotic concentration that prevented visible development after six hours of

recovery, known as MBEC which demonstrates that biofilm bacteria were effectively eradicated after antimicrobial treatment (Sanchez Jr et al., 2014). The study of MIC and MBEC shows that DAAs disperse biofilm and have the ability to enhance the antibiotic efficiency by releasing bacteria (Sanchez Jr et al., 2014).

1.8 Antibiotic Potentiation by DAAs and QACs:

To determine increased efficiency in infections associated with biofilms, combination DAAs (D-met, D-phe, D-trp) with antibiotics were tested against planktonic bacteria and biofilm of *S. aureus* and *P. aeruginosa* strains (Sanchez Jr et al., 2014). DAAs increased anti-biofilm activity against *S. aureus* when combined with rifampin, clindamycin, or vancomycin. Rifampin showed >2-log bacterial decrease, as shown by its MBIC dropping to 8µg/ml from 32-64µg/ml alone. The combination of clindamycin and vancomycin decreased viable counts by 1.5-2 logs, indicating that they worked in coordination with other clinical isolates (Sanchez Jr et al., 2014). When DAAs are combined with antibiotics, they improve treatment of biofilm-associated infections by dispersing communities of bacteria, overcoming resistance, and increasing efficiency of antibiotics against biofilms (Sanchez Jr et al., 2014).

Pyridine-based QACs demonstrated stable, strong antibacterial action against bacteria, with MICs and MBCs 166-32 times lower than the conventional antiseptics (Frolov et al., 2022). In comparison to independent treatments, Cu^{2+} works in combination with QACs to reduce P. aeruginosa biofilm minimum bactericidal concentration by 128 times while preserving broad-spectrum efficacy (Harrison et al., 2008). Synergistic combinations of antibiotics that exhibit strong, broad-spectrum action against Gram-positive infections with minimal development of resistance which follows repeated exposure (Van de Vliet et al., 2024). QACs can promote plasmid transfer and the evolution and spreading of antibiotic resistance genes by increasing bacterial membrane permeability and reactive oxygen species (ROS) generation (Han et al., 2019). Compared to QACs, newly synthesized soft QACs with labile amide bonds exhibit reduced toxicity, membrane preservation, and bacteriostatic activity. They provide safer, more environmentally friendly alternatives for antimicrobial treatments with less resistance and environmental effect by blocking bacterial growth and protein synthesis (Crnčević et al., 2025).

1.9 Research Gap and Research Questions:

Significant gaps remain in the conversion of anti-biofilm methods into clinically effective treatments, despite advances in our understanding of biofilm-mediated antibiotic resistance. Although QACs and DAAs have potential as biofilm dispersal agents, their effectiveness is frequently constrained by concentration and species dependent variability, issues with solubility in complex media, and insufficient interaction with antibiotic delivery systems (Aliashkevich et al., 2018; Frolov et al., 2022). DAAs like D-tyr, for example, suffer from solubility in nutrient-rich conditions and show paradoxical effects at intermediate doses, which lowers their bioavailability and repeatability (Aliashkevich et al., 2018; Sanchez Jr et al., 2014). Similarly, despite their broad-spectrum activity, QACs have limited clinical relevance because to issue with cytotoxicity and lack of tailored administration (Hympanova et al., 2020; Saverina et al., 2023).

The majority of current research ignores the combined potential of dual therapies and their translation to in vivo wound conditions in favor of single-agent approaches or in vitro models (Bjarnsholt, 2013; Goswami et al., 2023). Moreover, alginate-based hydrogels are being investigated for wound healing, nevertheless, their potential for co-delivering antibiotics and dispersion agent is still unclear, especially when it comes to biofilm recurrence and host-microbe interactions (Bounds et al., 2022; Kibungu et al., 2021). By examining synergistic DAAs and QACs within optimized hydrogel delivery systems, this study fills these gaps and attempts to overcome translation, toxicity, and solubility hurdles in the treatment of chronic wounds.

1. To what extend can DAAs and QACs be used to improve the effectiveness of antibiotics against bacterial biofilms of the pathogens that is associated with the chronic wounds?
2. Which combinations of DAAs and QACs and antibiotics can be used to combat biofilms of wound associated pathogens?
3. Does the combined treatment work at concentration that can be applied into an alginate-based hydrogel?

1.10 Hypothesis:

Dual therapy approaches for combining conventional antibiotics with other non-antibiotic compounds, with anti-biofilm or antibacterial activity, will enable more effective clearance of biofilms composed of bacteria frequently associated with chronic wounds, in comparison to antibiotics alone.

1.11 Specific Aims:

1. To assess the ability of DAAs and QACs to inhibit biofilm formation, dispersed the pre-established biofilms, and potentiation of the antibiotics against clinically relevant pathogens.
2. To develop an alginate-based hydrogel with antibiofilm properties to enhance the effectiveness of treatment with antibiotics against infections associated with wounds.

Chapter 2: Methodology

2.1 Materials and Bacterial Strains:

Mueller-Hinton Broth (MHB) from OXOID by Thermo Fisher Diagnostics, Plate Count Agar, Vegitone by Sigma-Aldrich from Millipore, Phosphate Buffer Saline used pre-made tablets of Ammonium phosphate monobasic from Sigma-Aldrich, Tryptone Soya Broth (TSB) from OXOID by Thermo Fisher Diagnostics, Hydrogen Chloride (HCl), Sodium Hydroxide (NaOH), DAAs and L-amino acids (LAAs) by Sigma-Aldrich (D-Tryptophan (≥ 98%), D-Leucine (99%), D-Tyrosine (99%), D-Methionine (≥ 98%), L-Tryptophan (≥ 98%), L-Leucine (≥ 98%), L-Tyrosine (≥ 98%), and L-Methionine (≥ 98%)), QACs (DDAC, and CTAB) by Sigma-Aldrich, Gram's Crystal Violet Solution, and Gram's Decolorizer Solution from Sigma-Aldrich, Gram-positive bacterial strains - *Staphylococcus aureus* (*S. aureus* ATCC 25923), and *Staphylococcus epidermidis* (*S. epidermidis* ATCC 12228), Gram-negative bacterial strains – *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 15692), and *Escherichia Coli* (*E. coli* ATCC 25922), Antibiotics - Vancomycin, Colistin, Kanamycin, and Tetracycline obtained from Sigma-Aldrich.

2.2 Antibiotics and Compounds:

Following bacterial strain were used with the different concentration of antibiotics. For *S. epidermidis* vancomycin (0.15 to 1,024 µg/ml), *P. aeruginosa* colistin (0.15 to 1,024 µg/ml), *S. aureus* tetracycline (0.125 to 1,024 µg/ml), *E. coli* kanamycin (0.125 to 1,024 µg/ml) were used. Antibiotics were ordered from Sigma-Aldrich. Stock solutions were made as recommended and diluted to the relevant concentrations in PBS. DAAs were also purchased from Sigma-Aldrich and then prepared a stock solution in water or 1 M HCl which was followed by filter sterilization. From that stock solution, DAAs were diluted into the MHB, and the diluted concentrations were 10 nM, 100 nM, 1 µM, 500 µM, which was neutralized with 1 M NaOH (pH 7 to 7.4) when require. QACs were purchased from Sigma-Aldrich and then prepared the stock solution in PBS and then followed by filter sterilization. From the stock solution, QACs were diluted in range of 2 to 2,048 µg/ml.

2.3 Crystal Violet Assay for Biofilm Quantification:

The biofilm assay was performed by using DAAs and QACs with the controls and 5 replicates to check whether the compounds are inhibiting or dispersing the bacterial biofilm. The overnight culture was prepared and adjusted with MHB with concentration at $1x10^7$ CFU/ml and that is approximately 0.001 optimal density 600 nm (OD_{600}) and followed by measuring with Thermo Scientific Nanodrop. By using diluted bacterial culture in specific media and in desired concentrated compound (DAAs, and QACs) for biofilm formation was performed in non-pyrogenic, Polystyrene, 96-well plates purchased from Corning Incorporated Costar® for 48 h but after 24 h incubation period, culture medium was removed and replaced with particular fresh media without disturbing the biofilm formation in each well for dispersal and inhibition. Following 48 h incubation, the culture media was removed from all the wells and were washed with the PBS (And then replaced with the 200 µl, 0.1% crystal violet diluted in milliQ water. Follow 10 minutes incubation and gently rinse the plate 2 times and place 200 µl decolorizer (50% acetone, 50% ethanol) and again incubate for 5 minutes, aliquot 100 µl dissolved stain in a new 96-well plate and read the plate into the plate reader by measuring optimal density at 590 nm (OD_{590}).) and biomass of the biofilm was determined by measuring optimal density at 590 nm (OD_{590}) of crystal violet solubilized in decolorizer.

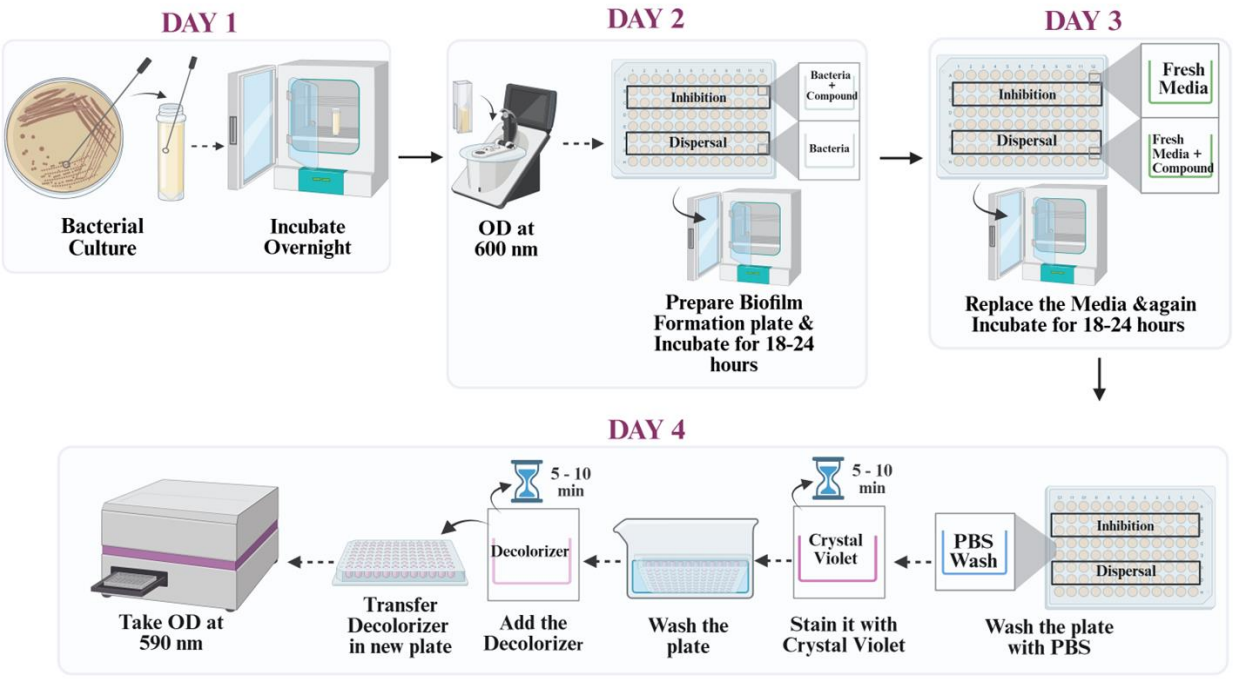

Figure 2: Crystal Violet Biofilm assay: the schematic diagram of the experimental method for crystal violet assay for dispersion and inhibition of the biofilm in 96-well plate.

2.4 Antibiotic Susceptibility of Bacteria:

2.4.1 Minimum Inhibitory Concentration (MIC) Assay:

Antibiotic susceptibility of bacterial strains was assessed from the determination of the MIC which is recommended by the Clinical and Laboratory Standards Institute (Humphries et al., 2021). The overnight bacterial culture was prepared and diluted in the MHB with the bacterial concentration at 1 x 10^7 CFU/ml (0.001 OD_{600}) which was measured by Thermo Scientific Nanodrop. The MIC was performed in triplicates. Diluted culture media was aliquot in the 96-well plate from Corning Incorporated Costar® with the media control and positive control at 2-fold serial dilution, where the starting concentration was 320 µg/ml with the final bacterial cell concentration of 5 x 10^5 CFU/ml in each well. The plates were incubated for 24 h at 37°C and followed by observation and measurement of the minimum antibacterial concentration which can inhibit bacterial growth.

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Figure 3: MIC assay: Minimum inhibitory concentration will be used to determine the standard measurement of the antibiotic activity for checking the susceptibility (Barnes et al., 2023).

2.4.2 Minimum Biofilm Eradication Concentration (MBEC) Assay:

To assess the bacterial biofilm susceptibility to antibiotics, and antibacterial agent by determine the MBEC. The overnight culture was prepared and adjusted with MHB with concentration at $1x10^7$ CFU/ml and that is approximately 0.001 optimal density 600 nm (OD_{600}) and followed by measuring with Thermo Scientific Nanodrop. By using diluted bacterial culture in specific media and in desired concentrated QACs for biofilm formation was performed in MBEC Assay® Biofilm Inoculator (96-well plate with pegs) purchased from innovotech inc. with biofilm control and sterile control for 18-20 h incubation at 37°C on orbital shaker. New 96-well plate was set as wash and challenge plate with sterile PBS and range of antibiotics (2-1024 µg/ml) respectively. After 18-20 h incubation, transfer the MBEC Assay® Biofilm Inoculator lid on wash plate and give the rest for 10-15 seconds and again transfer the MBEC Assay® Biofilm Inoculator lid on the challenge plate and incubate that plate for 18-20 h. Again, set a new 96-well plate as recovery plate inoculated with MHB. Transfer the lid from the challenge plate to the recovery plate. To check the bacteria will grow or not, retrieve the cells from the biofilm into the fresh culture media by putting it into the ultrasonic bath for sonication for about 1 minute and followed by removing MBEC Assay® Biofilm Inoculator lid, replacing that with sterile 96-well plate lid and incubate that plate for 18-20 h on orbital shaker at 37°C. After incubation place the plate into the plate reader and shake it for 5 seconds and measure the optimal density at 600nm (OD_{600}).

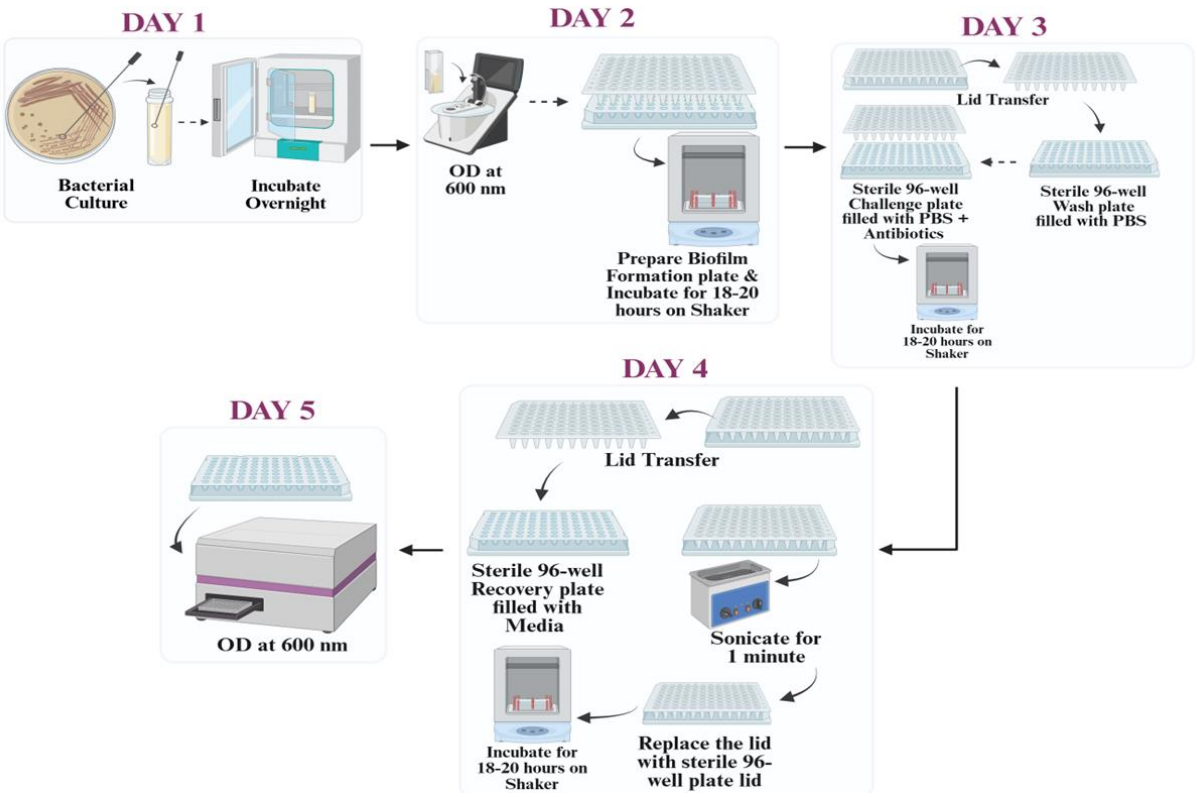


Figure 4: MBEC assay: detailed procedure of minimum biofilm eradication concentration assay.

2.4.3 Live-Dead cell viability Assay:

To examine the antibacterial activity of the QACs and the combination of the QACs with the antibiotics against *S. aureus* and *E. coli*, we used LIVE/DEAD™ Bacterial Viability Kit (Invitrogen, MA, USA). The overnight bacterial culture of *S. aureus* and *E. coli* were prepared and diluted in MHB to the 0.001 (10^6 CFU/ml) OD. Followed by 18-20 h incubation of the diluted bacterial culture with the specific concentrated QACs in sterile µ-Slide 24-well polystyrene plate from Corning Incorporated Costar® and after the incubation, the culture media was removed from the wells and then it was washed with sterile PBS. After that, it was stained with the SYTO®9 and propidium iodide (PI) in the dark condition and then incubate that for 10-15 minutes. A Confocal Laser Scanning Microscope from Germany (CLSM-ZEISS LSM 880) was used for the sample determination. For the cell viability, red and green cells were counted by using the software, and by the formula below, the cell viability (%) was calculated and compared with the control and processed and analyzed with the GraphPad Prism version 10.4.2.

$$\text{Cell Viability (\%)} = \frac{\text{Live cells}}{\text{Total cells}} \times 100$$

2.5 Statistical Analysis:

All the experimental data were presented as mean ± Standard Deviation (SD) of all the 3 or 5 replicates using one-way ANOVA followed by Dunnett's *post hoc* multiple comparison test which is processed by using GraphPad Prism Version 10.4.2 for windows, GraphPad Software. The experimental differences were examined to be statistically significant at *P* values of <0.05. There are different symbols for statistical differences, which are * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$ and ns = $P > 0.05$.

Chapter 3: Results

3.1 Determination of Biofilm Inhibition and Dispersal:

To analyze the potentiation and application of DAAs, we tested whether DAAs effectively inhibit or disperse the biofilm of *S. epidermidis* and *P. aeruginosa* by performing crystal violet assay. Equimolar concentrations have been used to combine the DAAs and performing the biofilm inhibition assay.

This result demonstrated that LAAs were significant at concentration 10 nM for *S. epidermidis* and showed higher crystal violet (CV) absorbance, indicates greater amount of biofilm which means biofilm inhibition was not occurred and there was no significance showed in DAAs **(Figure 5 A)**. For *P. aeruginosa,* at concentration 100 nM and 500 µM showed highly significance and 500 µM(R)(R=repeat) showed significance at $P<0.05$ for both treatments, DAAs and LAAs **(Figure 5 B)**. This result demonstrates that, in some concentrations of DAAs and LAAs, we observed significant difference in CV absorbance, however there was no concentration-dependent trend, which suggesting that the differences reported were due to technical error or natural variation.

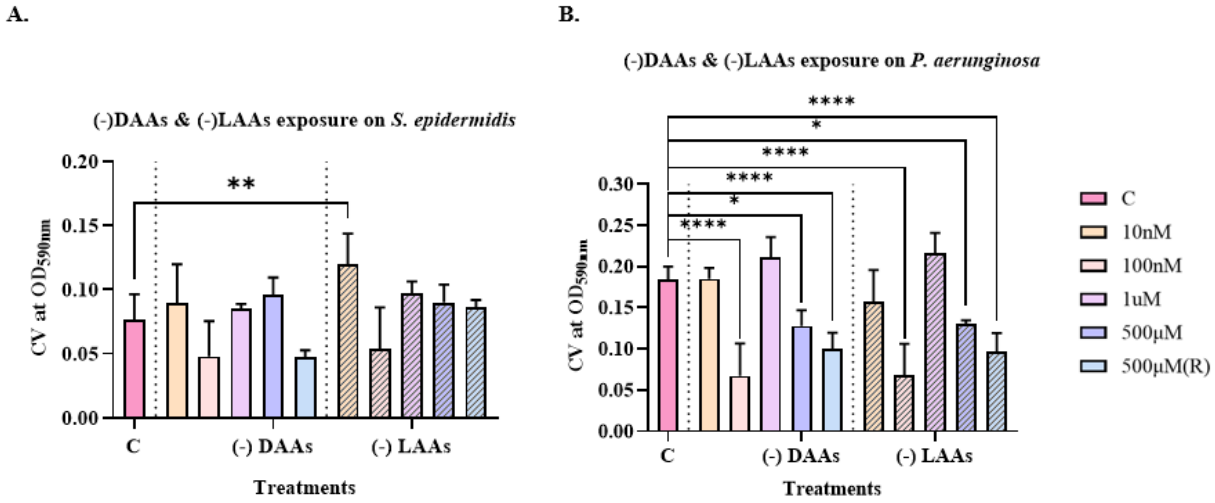


Figure 5: Exposure of DAAs and LAAs on S. epidermidis and P. aeruginosa biofilm inhibition.

(A) Biofilm inhibition was assessed by measuring the absorbance of solubilized crystal violet from stained biofilm of *S. epidermidis* at exposure of DAAs and LAAs at different concentrations (10 nM, 100 nM, 1 µM, 500 µM, and 500 µM(R)). (B) Similar treatment concentration has been

used to assess the biofilm inhibition by measuring the absorbance of solubilized crystal violet from stained biofilm of *P. aeruginosa*. Data demonstrate here as a mean ± SD, where n=5. Statistical significance was determined by one-way ANOVA with multiple comparisons (compare to the control), where *=p<0.05, **= p<0.01, ****= p<0.0001, (R)=repeat, (-) inhibition.

Equimolar concentrations have been used to combine the DAAs and performing the biofilm dispersal assay. This result illustrated that DAAs were significant at concentration 1µM for *S. epidermidis* and LAAs were highly significant at concentration 500µM. This result also showed higher CV absorbance, indicates the greater amount of biofilm which means biofilm dispersion was not occurred **(Figure 6 A)**. For *P. aeruginosa,* at concentration 100 nM and 500 µM(R) showed significance at P<0.05 and 500 µM showed highly significance at P<0.0001 for DAAs treatment. In contrast, LAAs treatment showed significance at concentration 100 nM and 500 µM at P<0.01 and P<0.0001 respectively **(Figure 6 B)**. This result demonstrates that, in some concentrations of DAAs and LAAs, we observed significant difference in CV absorbance, however there was no concentration-dependent trend, which suggesting that the differences reported were due to technical error or natural variation.

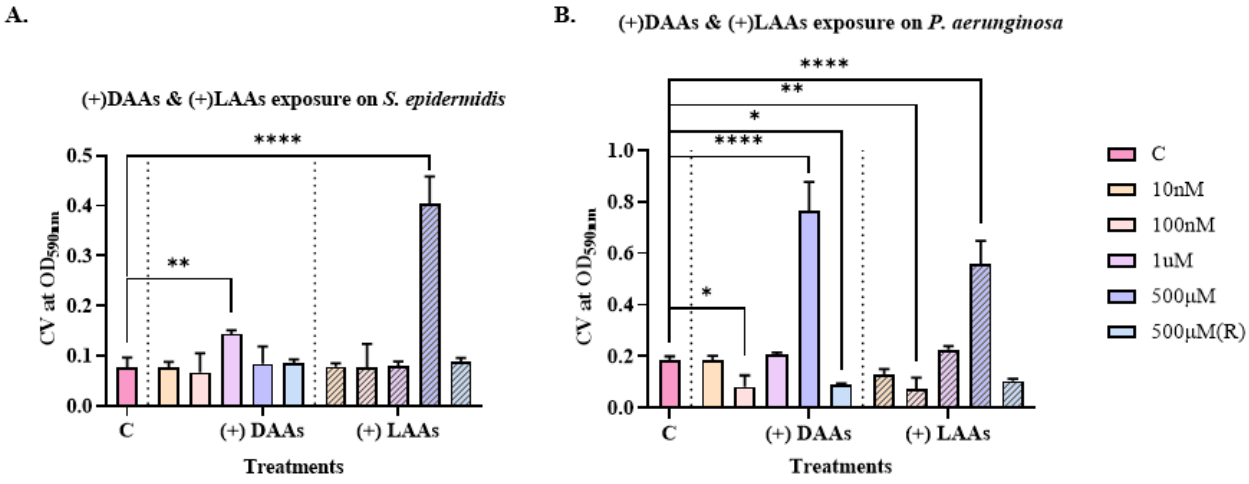


Figure 6: Exposure of DAAs and LAAs on S. epidermidis and P. aeruginosa biofilm dispersion.

(A) Biofilm dispersion was assessed by measuring the absorbance of solubilized crystal violet from stained biofilm of *S. epidermidis* at exposure of DAAs and LAAs at different concentrations (10 nM, 100 nM, 1 µM, 500 µM, and 500 µM(R)). (B) Similar treatment concentration has been

used to assess the biofilm dispersion by measuring the absorbance of solubilized crystal violet from stained biofilm of *P. aeruginosa*. Data demonstrate here as a mean ± SD, where n=5. Statistical significance was determined by one-way ANOVA with multiple comparisons (compare to the control), where *=p<0.05, **= p<0.01, ****= p<0.0001, (R)=repeat, (+) dispersal.

To move further with the research and assess the biofilm inhibition and dispersal we decided to perform crystal violet assay by using different bacterial strains to check whether the maximum concentration of DAAs can inhibit or disperse the biofilm of *S. aureus* and *E. coli*. The result showed increased CV absorbance of the *S. aureus* biofilm for both treatment DAAs and LAAs which indicates no biofilm inhibition was occurred. It also showed significance at p<0.05 and p<0.001. The result did not showed any significance for biofilm dispersal for both treatments because the CV absorbance was higher **(Figure 7 A)**. In contrast, *E. coli* doesn't have any effect on biofilm formation with both the treatment and no significance have been noted for that **(Figure 7 B)**.

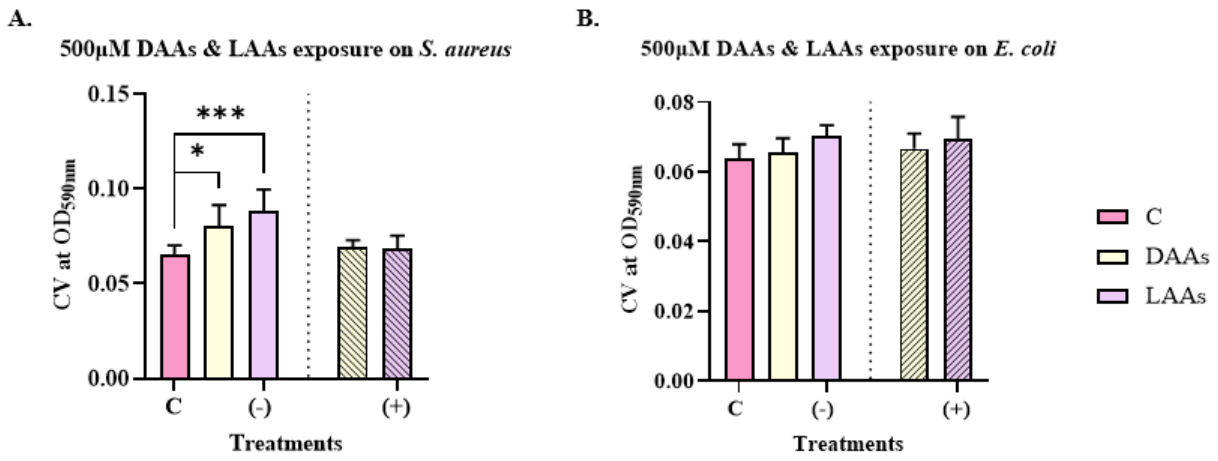


Figure 7: Exposure of 500µM DAAs and LAAs on S. aureus and E. coli biofilm inhibition and dispersion.

(A) Biofilm inhibition and dispersion was assessed by measuring the absorbance of solubilized crystal violet from stained biofilm of *S. aureus* at exposure of DAAs and LAAs at 500µM concentration. (B) Similar treatment concentration has been used to assess the biofilm inhibition and dispersion by measuring the absorbance of solubilized crystal violet from stained biofilm of *E. coli*. Data demonstrate here as a mean ± SD, where n=5. Statistical significance was determined

by one-way ANOVA with multiple comparisons (compare to the control), where *=p<0.05, ***= p<0.001, (-) inhibition, (+) dispersal.

Overall, this result shows that the DAAs don't have any effect on biofilm inhibition and dispersal for both bacterial strains at this concentration. Due to solubility issue of the DAAs we won't be able to perform the crystal violet assay for biofilm quantification with the higher concentration. As we were not able to replicate DAAs-mediated biofilm inhibition or dispersion that was reported in related literature, we decided to pivot the project to use different compounds for the same original purpose of antibiotic potentiation.

Half x MIC of individual QACs has used to analyze the potentiation and application of QACs, we tested whether QACs effectively inhibit or disperse the biofilm of *S. aureus* and *E. coli* by performing crystal violet assay. As mentioned, half x MIC concentration was used which was 0.25 µg/ml concentration for both DDAC and CTAB on *S. aureus* and for *E. coli* respectively 2 µg/ml and 8 µg/ml concentration has been used for DDAC and CTAB. The result data showed no significant effect of the QACs on biofilm biomass with the inhibition and dispersal approaches, for *S. aureus* **(Figure 8 A)**. For *E. coli,* CTAB showed significant effect on biofilm inhibition and dispersal both at p<0.05 **(Figure 8 B).**

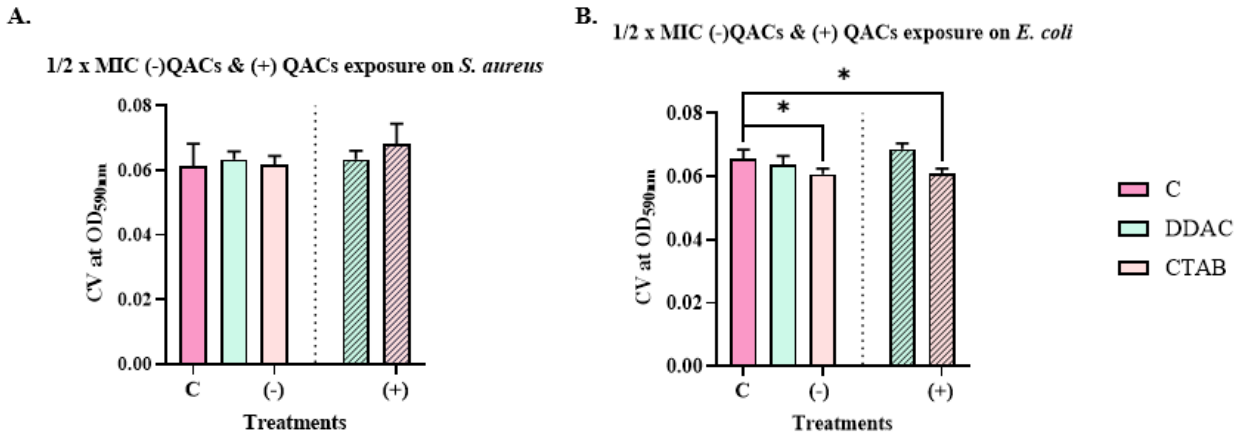


Figure 8: Exposure of half x MIC of QACs on S. aureus and E. coli for biofilm inhibition and dispersion.

(A) Biofilm inhibition and dispersion was assessed by measuring the absorbance of solubilized crystal violet from stained biofilm of *S. aureus* at exposure of QACs at half x MIC (0.25 µg/ml) concentration. (B) Respectively 2 µg/ml and 8 µg/ml concentration for DDAC and CTAB has been

used to assess the biofilm inhibition and dispersion by measuring the absorbance of solubilized crystal violet from stained biofilm of *E. coli*. Data demonstrate here as a mean ± SD, where n=5. Statistical significance was determined by one-way ANOVA with multiple comparisons (compare to the control), where *=p<0.05, (-) inhibition, (+) dispersal.

Overall, this result shows that the biofilm inhibition and dispersal did not affect by the treatment of the QACs on *S. aureus*. For *E. coli* CTAB showed biofilm inhibition and dispersal both but DDAC didn't showed any significance on the biofilm formation. So, we performed another crystal violet assay by doubled the MIC of the QACs for both bacterial strains.

2 x MIC of individual QACs has used to analyze the potentiation and application of QACs, we tested whether QACs effectively disperse the biofilm of *S. aureus* and *E. coli* by performing crystal violet assay. As mentioned, 2 x MIC concentration was used which was 1.0 µg/ml concentration for both DDAC and CTAB on *S. aureus* and for *E. coli* respectively 8 µg/ml and 32 µg/ml concentration has been used for DDAC and CTAB. The result data showed no significant effect of the QACs on biofilm dispersal for *S. aureus* **(Figure 9 A)** as well as *E. coli* **(Figure 9 B)**.

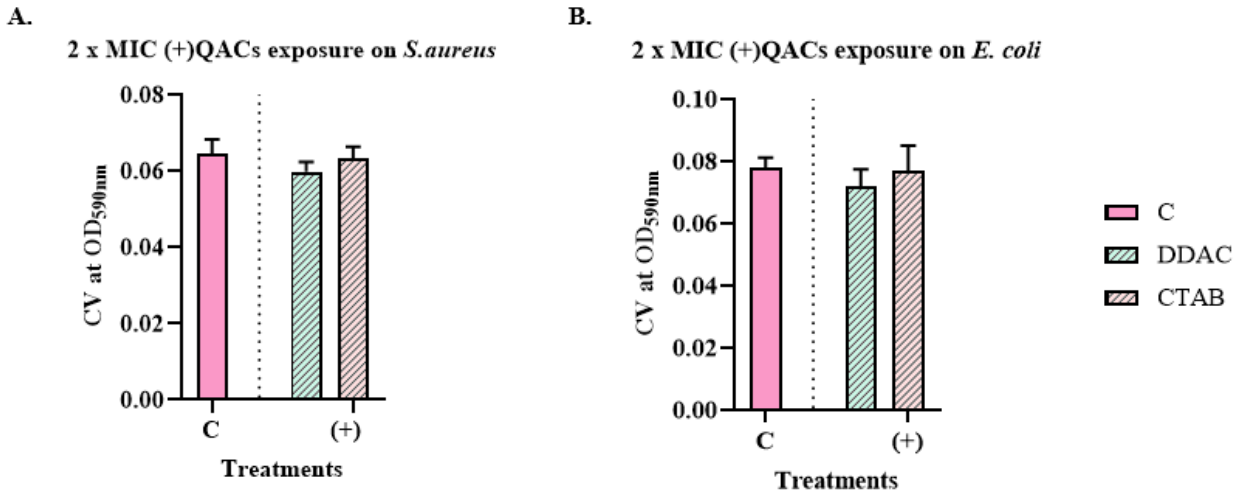


Figure 9: Exposure of 2 x MIC of QACs on S. aureus and E. coli for biofilm dispersion.

(A) Biofilm dispersion was assessed by measuring the absorbance of solubilized crystal violet from stained biofilm of *S. aureus* at exposure of QACs at 2 x MIC (1.0µg/ml) concentration. (B) Respectively 8µg/ml and 32µg/ml concentration for DDAC and CTAB has been used to assess the biofilm dispersion by measuring the absorbance of solubilized crystal violet from stained biofilm of *E. coli*. Data demonstrate here as a mean ± SD, where n=5. Statistical significance was

determined by one-way ANOVA with multiple comparisons (compare to the control) where, (+) dispersal.

Overall, this result shows that the treatment of QACs on both bacterial biofilms didn't affect and no significance has been showed. But the QACs have the potential to act as an antimicrobial agent so to check the effectiveness and antibacterial activities we performed the MIC and MBEC assay.

3.2 Antibiotic Susceptibility of Bacteria Determination:

3.2.1 Determination of Minimum Inhibitory Concentration (MIC):

To determine if QACs could enhance the effect of conventional antibiotics against bacteria within biofilms, antibiotic susceptibility, minimum inhibitory concentration assay was performed with the QACs on both bacterial strains. The MIC of antibiotic for *S. aureus* was determined for tetracycline which was 0.5 µg/ml and the MIC of DDAC and CTAB was also 0.5 µg/ml **(Table 1)**. Similarly, the MIC of antibiotic for *E. coli* was determined for kanamycin which was 8 µg/ml and the MIC of DDAC and CTAB was respectively 4 µg/ml and 16 µg/ml **(Table 1)**. Overall, the MIC indicates the lowest concertation of the antibiotics and QACs for bacterial strain *S. aureus* and *E. coli*.

Table 1: The MIC of QACs and antibacterial agent alone against S. aureus and E. coli.

MIC of *S. aureus*		MIC of *E. coli*	
DDAC	0.5 µg/ml	DDAC	4 µg/ml
CTAB	0.5 µg/ml	CTAB	16 µg/ml
Tetracycline	0.5 µg/ml	Kanamycin	8 µg/ml

3.2.2 Determination of Minimum Biofilm Eradication Concentration (MBEC):

To determine if QACs could enhance the effect of conventional antibiotics against bacteria within biofilm, antibiotic susceptibility, minimum biofilm eradication concentration assay was performed with QACs on both bacterial strains *S. aureus* and *E. coli*. It also, determine the concentration which could eradicate the pre-established bacterial biofilm completely. The MBEC for antibiotics and QACs were determined initially and then to check the susceptibility of QACs, the MBEC was

performed in the combination of the QACs and antibiotic. For combination of the compounds and antibiotics half MBEC was used as an initial concentration and followed by quarter half for CTAB MBEC.

The result showed that the individual MBEC of DDAC, CTAB and tetracycline is 32 µg/ml, 1024 µg/ml and >2048 µg/ml respectively for *S. aureus*. MBEC of tetracycline in presence of ½ MBEC DDAC is 1024 µg/ml. MBEC of tetracycline in presence of ½ MBEC CTAB is 64 µg/ml. This represents the reduction of MBEC when we combine the QACs with the antibiotics **(Table 2)**.

Table 2: The MBEC of QACs and Tetracycline against S. aureus, shows the concentration which could eradicate the pre-established bacterial biofilm.

MBEC of compounds against *S. aureus*	
Treatments	MBEC
DDAC	32 µg/ml
CTAB	1024 µg/ml
Tetracycline	>2048 µg/ml
Tetracycline + ½ MBEC DDAC	1024 µg/ml
Tetracycline + ½ MBEC CTAB	64 µg/ml

The result showed that the individual MBEC of DDAC, CTAB and kanamycin is 64 µg/ml, 16 µg/ml and >1024 µg/ml respectively for *E. coli*. MBEC of kanamycin in presence of ½ MBEC DDAC is 512 µg/ml. MBEC of tetracycline in presence of ½ MBEC CTAB is 1024 µg/ml. This represents the reduction of MBEC when we combine the QACs with the antibiotics **(Table 3)**.

Table 3: The MBEC of QACs and Kanamycin against E. coli, shows the concentration which could eradicate the pre-established bacterial biofilm.

MBEC of compound against *E. coli*	
Treatments	MBEC
DDAC	64 µg/ml
CTAB	16 µg/ml

Kanamycin	>1024 µg/ml
Kanamycin + ½ MBEC DDAC	512 µg/ml
Kanamycin + ½ MBEC CTAB	1024 µg/ml

3.2.3 Determination of Live-Dead cell viability:

Bacterial viability has been determined for the effect of the QACs and their combined treatment with antibiotics against *S. aureus* and *E. coli*. By using the LIVE/DEAD™ Bacterial Viability Kit, we analyzed the bacterial viability of untreated, QACs treated, antibiotic treated, and treated with the combination of QACs and antibiotics of *S. aureus* and *E. coli*.

In this viability test green fluorescence color showed the healthy live cells absorption of SYTO®9 dye **(Figure 10 A, B, C, E, and F)**, and unhealthy dead cells appeared as red fluorescence color due to absorption of PI dye **(Figure 10 D, G)** under a confocal laser scanning microscope (CLSM). The result shows reduction of the live cells in combination treatment of the DDAC or CTAB and antibiotic by ± 91.1% and 97.7% respectively, and with the extreme significant effect (****=p<0.0001) **(Figure 10 H)**.

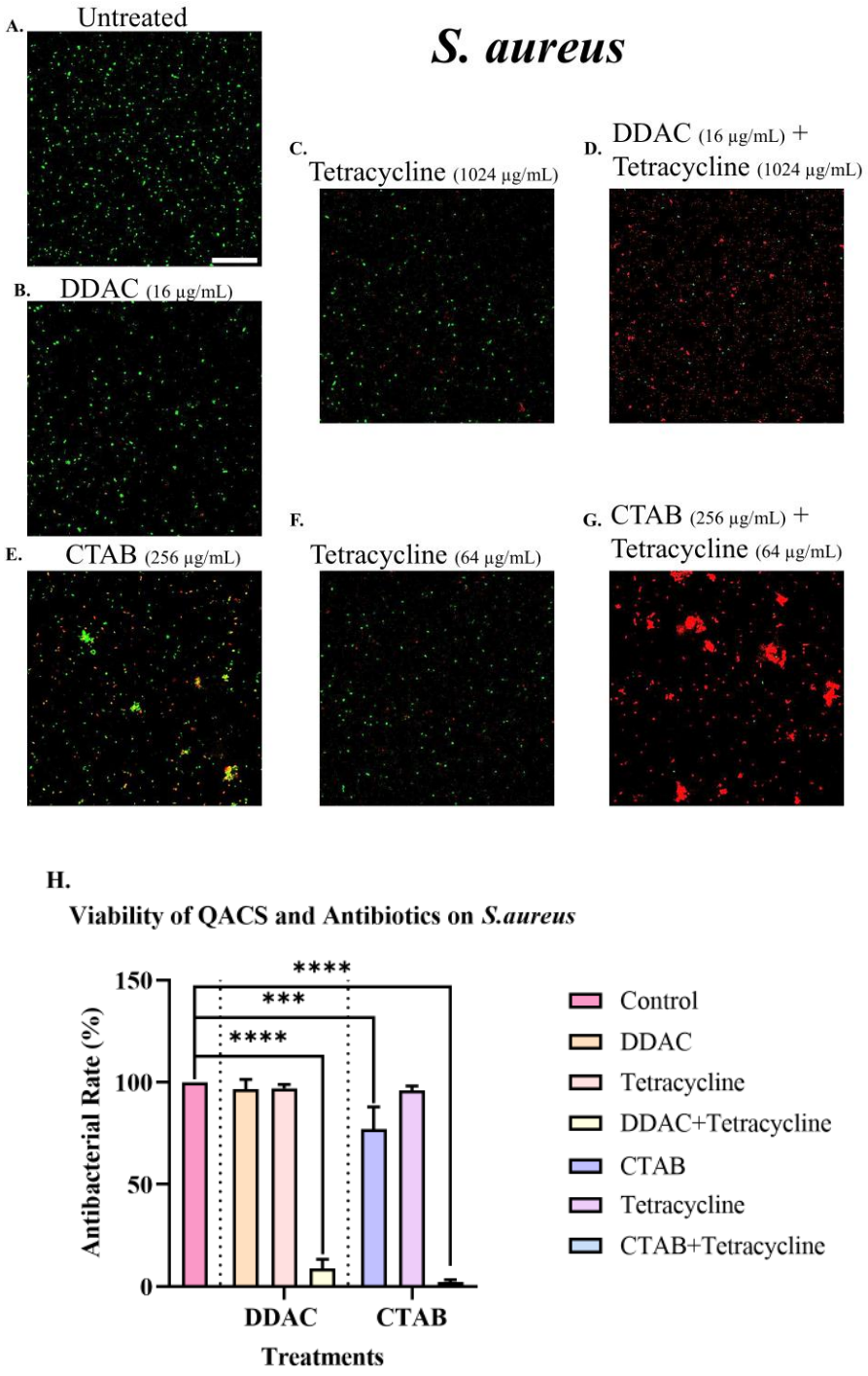
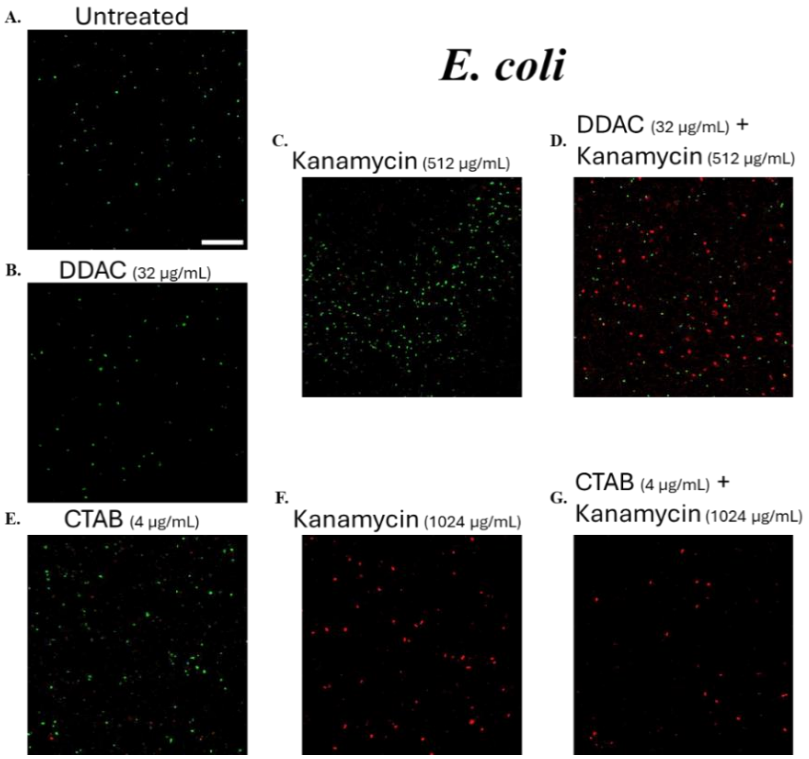


Figure 10: CLSM image of S. aureus grown on pre-sterile coverslip in microtiter plate for 18- 20 h at 37°C.

The figure indicates the staining of the cell with untreated. (A), treated QACs (B, E), treated with antibiotics (C, F), and treated in a combination of the QACs and antibiotics (D, G) at different concentrations where the green color shows live bacteria and red color shows dead bacteria. (H)

The antibacterial rate of the cell was measured with all the treatments and untreated sample as a control. Data demonstrate here as a mean ± SD, where n=3. Statistical significance was determined by one-way ANOVA with multiple comparisons (compare to the control), where ***=p<0.001, ****=p<0.0001.

In this viability test green fluorescence color showed the healthy live cells absorption of SYTO®9 dye **(Figure 11 A, B, C, E, and F)**, and unhealthy dead cells appeared as red fluorescence color due to absorption of PI dye (Figure 9 D, G) under a CLSM. The result shows reduction of the live cells in combination treatment of the DDAC or CTAB and antibiotic by ± 63.5% and 100% respectively, and with the extreme significant effect (****=*p*<0.0001) **(Figure 11 H)**. These results showed that QACs have the ability to enhance the effectiveness of the antibiotics.



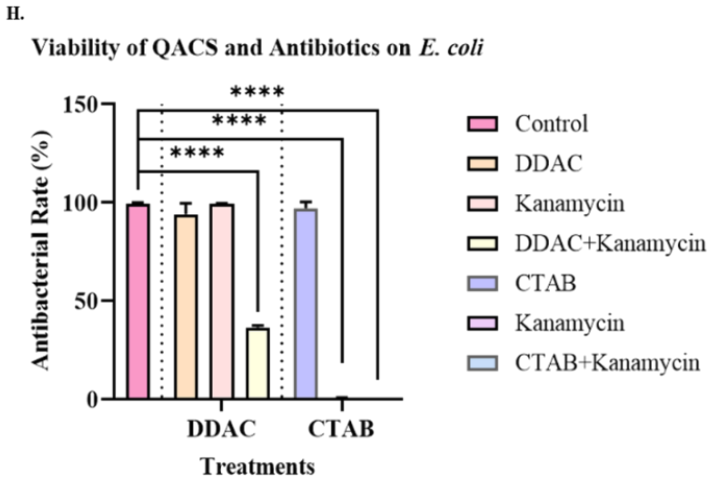


Figure 11: CLSM image of E. coli grown on pre-sterile coverslip in microtiter plate for 18- 20 h at 37°C.

The figure indicates the staining of the cell with untreated (A), treated QACs (B, E), treated with antibiotics (C, F), and treated in a combination of the QACs and antibiotics (D, G) at different concentrations where the green color shows live bacteria and red color shows dead bacteria. (H) The antibacterial rate of the cell was measured with all the treatments and untreated sample as a control. Data demonstrate here as a mean ± SD, where n=3. Statistical significance was determined by one-way ANOVA with multiple comparisons (compare to the control), where ***=p<0.001, ****=p<0.0001.

Chapter 4: Discussion

Various mechanism signaling employ cell-density-dependent signaling to control adhesion, matrix creation, the transition from planktonic to biofilm, pathogenicity, and nutrient acquiring, hence regulating the bacterial biofilm formation (Kalia et al., 2023). Cells can be shielded from antimicrobial agents and the host's immune by the structural characteristics of biofilm (Abdel-Aziz & Aeron, 2014). In this present study, various concentration of DAAs and LAAs exposure on *S. epidermidis* and *P. aeruginosa* results showed the high absorbance of crystal violet staining, indicates that the formation of biofilm is increased for both inhibition and dispersal assay which means DAAs did not provide a significant reduction in biofilm.

This result aligns with the previous research conducted by Leiman et al. (2013), which found that the precipitation in nutrient-rich conditions can significantly decrease the bioavailability and efficacy of DAAs and that their solubility is crucial component of their antibiofilm activity. In their trials, they found that DAAs worked best when completely dissolve and the aggregation resulted in loss of function which is observed in this results as well (Leiman et al., 2013). Another study also discovered that DAAs could only disperse *S. aureus* biofilms when they were included in formulations and at quantities that guaranteed total solubility. They found the DAAs had an ability to form a group at higher concentrations, which could simultaneously stabilized biofilm formations by reducing the dispersal effect and possibly forming local microenvironment. Which can be describe that the why DAAs result showed steady effect on some concentration. In the results of that dispersal also can show the opposite effect (Hochbaum et al., 2011). Interestingly, on the other hand, another research showed strong biofilm dispersal using DAAs. However, both investigations used newly made, fully dissolved DAAs solution, and in order to maximize solubility and stability, they frequently used media. The research demonstrated that the availability and physical condition of the chemicals in experiment as well as concentration affect, how effective DAAs are (Kolodkin-Gal et al., 2010; Sanchez Jr et al., 2014).

This result suggests that the compound's physicochemical limits in MHB, rather than an inherent failing, are the cause of the DAAs' lack of efficiency in the trials. Since the formulation and delivery route may impact the success of failure of DAAs based therapeutics, this is an important concern for laboratory studies and clinical translation (Davey & O'toole, 2000; Leiman et al., 2013). In previous study presented by Ramon-Perez et al. showed that numerous pathogenic and

non-pathogenic strain of S. epidermidis have been shown to demonstrate various levels of sensitivity to DAAs during biofilm formation (Ramón-Peréz et al., 2014). All of the DAAs that were examined decreased the formation of biofilms for certain strains, but just a few of them or none at all had an inhibitory effect on other strains (Aliashkevich et al., 2018). According to a Kolodkin-Gal et al., biofilm dissociation by DAAs is highly dependent on the experimental setup. This research paper addressed inconsistencies in the activity of DAAs as biofilm disassembly agents and variations in the active concentrations (Aliashkevich et al., 2018; Bucher et al., 2015; Kolodkin-Gal et al., 2010). The main factors that explain the variations in the concentration of DAA needed to inhibit biofilm development are the pre-culture medium, the growth phase, the inoculation ratio, and the removal of spent medium prior to the inoculation (Aliashkevich et al., 2018; Bucher et al., 2015).

Since the initial finding that biofilm dissolving in *B. subtilis* was caused by a mutation in the strain utilized in that investigation, the impact of DAAs on biofilm formation has lately come under investigation (Kao et al., 2017; Leiman et al., 2013). The impact of DAAs on *P. aeruginosa* biofilm development has been investigated in a number of previous investigations. These investigations, however, failed to provide compelling evidence of an effect (Kolodkin-Gal et al., 2010). For instance, Brandenburg et al. demonstrated that *P. aeruginosa* biofilm development was suppressed by both D-try and L-try. The impact of D-met, D-phe, and D-try on biofilm dispersal in six P. aeruginosa strain was examined by Sanchez et al. They discovered that biofilm dispersal was strain-dependent. Additionally, unlike the Kolodkin-Gal et al. research, D-tyr was shown to be ineffective in *P. aeruginosa* biofilm dispersal by Sanchez et al. Lastly, Sanchez et al. also found harmful effects of DAAs on the survival of *P. aeruginosa*, which complicated the interpretation of their findings (Brandenburg et al., 2013; Kao et al., 2017; Kolodkin-Gal et al., 2010; Sanchez Jr et al., 2014; Sanchez Jr et al., 2013; Sanchez et al., 2013).

In this present study we still wanted to pursue the approach of combination therapy, so we transitioned to using QACs for their known antibacterial activity (Saverina et al., 2023). For this we have performed the crystal violet assay for biofilm quantification at half MIC and 2 x MIC and we observed no reduction occurred in biofilm biomass and the CV absorbance of the QACs have increased in compared with control biofilm which indicates that there were no biofilm inhibition or dispersal occurred. The previous study result of scanning electron microscopy (SEM) showed

following benzalkonium chloride (BC) treatment, the matrix of those modified biofilms thickened and became similar. When bacteria are exposed to antibacterials on a regular basis, biofilms including more virulent and resistant bacteria may form. Either phenotypic adaptation, genetically acquired resistance, or both may contribute to this adaptive resistance. Using a disinfectant can promote biofilm growth and tolerance rather than eliminating biofilms and killing microbes. Given that it can occur in clinical settings where the use of antibiotics is inevitable, this must be serious concern (Machado et al., 2012).

While we did not observe the reduction in biofilm biomass, we wanted to determine whether QACs could promote the activity of the conventional antibiotics against biofilm cells and we established the MBEC assay of individual QACs and once we got the MBEC quantities, we reduced the concentrations of QACs to sub-MBEC concentrations and then measured the tetracycline MBEC in presence and absence of the QACs. For these results, we observed considerable decrease in tetracycline MBEC when combined with the QACs at sub-MBEC concentrations which supported our hypothesis that combination therapies could be used to improve the activity of antibiotics against biofilms. The previous studies also showed synergetic effect of QACs when combined with antibiotics but there were some limitations for this needs to be addressed first (Boyce, 2023; Wang et al., 2025).

Limitation:

The solubility and stability of DAAs, especially at higher concentrations in nutrient-rich media like MHB, is another significant drawback. According to previous studies, the low solubility of some hydrophobic DAAs like D-leu and D-try led to precipitation and decreased bioavailability in this study, which in turn limited their anti-biofilm effectiveness. Other research has acknowledged this problem, showing that DAAs worked best when completely dissolved and dispersed equally. In addition to decreasing DAAs' direct interaction with biofilm matrix, precipitation can result in irregular dosage and erratic outcomes. In order to guarantee consistent and repeatable anti-biofilm effects, this limitation implies that future research should concentrate on improving DAAs formulations, perhaps by using solubilizing agents or different delivery methods.

Although QACs showed strong antibacterial and anti-biofilm efficacy in this investigation, there may be disadvantages to using them. Because of their recognized cytotoxic qualities, QACs can harm mammalian cells, slow the healing of wounds, or interfere with the beneficial host microbiota

when used frequently or in high concentrations. In chronic wound conditions where tissue regeneration and immunological function are crucial, QAC doses that are effective against biofilms in vitro might not be safe or tolerated in vivo. Though biocompatibility and safety evaluations are necessary prior to clinical application, even though recent developments in QAC chemistry have attempted to lessen toxicity while preserving antibacterial activity.

Both DDAC and CTAB observed effectiveness varied with species and strain, which limits their potential for widespread clinical application. As mentioned in the results and previous studies showed that the variations in matrix composition, genetic regulation and environment adaption might cause distinct bacterial strains and species to react differently to dispersal agents. Furthermore, the efficacy of single-agent or even dual-therapy techniques may be further complicated by the existence of polymicrobial biofilms, which are prevalent in chronic wounds. This variation emphasizes the necessity of tailored or focused treatment depending on the unique microbial composition of every infection. The long-term implications of recurrent usage of dispersion agents, including DAAs and QACs, are still unknown, despite the fact that they are intended to increase antibiotic potency and decrease resistance. Particularly if the drugs are used widely or incorrectly, there is a chance that bacteria will evolve adaptive defenses against them.

Future Directions:

We needed to determine the biocompatibility and safety of using QACs at the concentrations that provide antibiotic potentiation activity. If these concentrations are safe to use, we need to determine how they can be applied in a wound healing application, such as an alginate-based hydrogel. If the concentrations are not safe for clinical use, then we can still aim to learn more about the mechanisms responsible for antibiotic potentiation, because these principles may be applied to different combination therapies using compounds that are compatible.

Conclusions

In medicine, biofilms are a severe problem because they can cause chronic, repeated and frequently multi drug resistant infection that complicate wound healing and make maintenance of the medical devices more complicated. In addition to protecting bacteria from medication and immune attack, the complex extracellular matrix formed by microbial biofilms allows for quick regrowth and recolonization after partial removal of the biofilms. Novel strategies must be employed to overcome these obstacles because traditional therapeutic methods generally fail to tackle the specific resistance mechanisms of biofilms. Dual therapy is one of the promising methods of improving the success rate of treatment, in which antibiotics are used in combination with biofilm dispersing agent such as DAAs and QACs can also be used in complex drug delivery systems like alginate hydrogels, which facilitate wound healing by stimulating the regeneration of tissue and suppressing inflammation. In this study, results showed that when QACs were combined with the antibiotic, the antibiotics action on the pathogens could be strengthened effectively. Even with such breakthroughs, there are several problems that continue to exist, such as the requirement of ideal dosage preparations, development of resistance, and differing efficiency of antibacterial medications against distinct bacterial groups.

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