Quaternary Ammonium Compounds as Antibiotic Potentiators

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LIST OF ABBREVIATIONS

Abbreviation	Full name
AMR	Antimicrobial resistance
CTAB	Cetyltrimethylammonium bromide
CFU	Colony forming unit
DDAC	Didecyldimethylammonium chloride
FICI	Fractional inhibitory concentration index
MBC	Minimum bactericidal concentration
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
PI	Propidium iodine
QAC	Quaternary ammonium compounds
TSA	Tryptic soy agar
TSB	Tryptic soy broth

ABSTRACT

Antibiotics are still the most important and powerful tool in healthcare system to combat infection from pathogens. However, the rising emergence of antimicrobial resistance (AMR) is concerning and has become a serious health care system threat, resulting to the declining efficacy of antibiotics, therefore causing deaths. One possible approach to addressing the problem is to reinvigorate the activity of existing antibiotics. Quaternary ammonium compounds (QACs) are organic cationic compound with positively charge ammonium atom head that is hydrophilic in nature, binding to the negatively charged surface of bacteria via coulomb's law, attached to a hydrophobic alkyl chain. They are known to have broad-spectrum antimicrobial properties with less cytotoxic effects with low concentration. It is widely used as disinfectant to hospitals and other establishments and have also been used as antiseptic solution. However, despite the antimicrobial property of QAC, previous studies have not been through profound investigation of the potential of QAC to improve the activity of antibiotics.

This study aims to improve the antimicrobial activity of the existing conventional antibiotics by permeabilizing the bacterial cell membrane through QACs, hence, promoting the influx of the antibiotics. To test this, we measure the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the QACs; cetyltrimethylammonium bromide (CTAB) and didecyldimethylammonium chloride (DDAC) and use the subinhibitory concentration to then combine with antibiotics against two clinically relevant strain of Gram-positive bacteria which is *Staphylococcus aureus (S. aureus)* and a Gram-negative bacteria which is *Escherichia coli (E. coli)* then measure the MIC and MBC value to see if there is any reduction as compared to the value by itself. The antibiotics that were used for this study are the following: Cefazolin; Colistin; Daptomycin; Gentamicin; Kanamycin; Levofloxacin; Tetracycline; Vancomycin. The choice of the antibiotics was based on their different mode of action against Gram-positive and Gram-negative pathogens.

We tested a wide range of combinations and observed that in some cases there was no change in antibiotic activity, while in other cases the activity was increased significantly, that indicates improved antibiotic activity. We investigated further to measure whether these interactions were synergistic in nature and reported 2 synergistic interactions such as kanamycin combined with CTAB against *E. coli* and tetracycline combined with CTAB against *S. aureus*. We further characterized the activity of those two synergistic interactions by looking at time-kill kinetics and resulted with a dramatically decrease in bacterial growth after 2 hrs in both combination and completely eradicated after 24 hours. Lastly, we aimed to elucidate the

mechanism of enhanced antibiotic activity by evaluating the permeability triggered by QACs, and we did not report a clear trend between permeability and antibiotic activity, suggesting other factors are contributing to the effect. These results validated the combination approach, and the investigation justifies further research into how this can be applied in a biomedical application.

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;

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed by: Janine Domingo Bolo

Date: 2nd June 2025

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The use of antimicrobial agent against infection started during the ancient civilizations, wherein they used the concoction of different plants and moulds that has antibacterial properties (Muteeb et al., 2023). It was year 1928, when sir Alexander Fleming, a bacteriologist unintentionally discovered one of the most significant interventions in the field of medicine; the penicillin discovery (Gaynes, 2017; Hutchings et al., 2019). It happened when he noticed a zone around an invading fungus in a plate of agar without any bacterial growth. The efficacy was first tested year 1939 in a group of mice; they injected a strain of *Streptococci* to 8 mice and injected 4 of them with penicillin and the rest was untreated and served as positive control. The next day, the 4 untreated positive control were found dead while the rest with penicillin were still alive. After multiple purification process of penicillin, they decided to test the effectiveness in clinical year 1941. It was a police officer with severe infection and complication who first received penicillin as treatment. The condition of the policeman started to show improvement the day after they administered penicillin. However, the subject died after few weeks because of the limited supply of the treatment (Gaynes, 2017).

The discovery of antibiotics by Sir Alexander Fleming as treatment is considered the most significant breakthrough in the medical field of 20th century because different medical procedure became possible and arguably extended lifespan by over 20 years (Hutchings et al., 2019). However, with the abuse and misuse of antibiotics like ceasing of treatment too early resulting to incomplete clearance of infection that causes exposure of bacteria to the known antibiotics allowing them to develop resistance, the efficacy of antibiotics has been compromised (Muteeb et al., 2023). Antimicrobial resistance (AMR) has become a serious global health threat causing high mortality annually of approximately 0.7 million and it is expected to increase up to 15 times by the year 2050 (Papkou et al., 2020). On the other hand, given the slow rate of discovery and translation of antibiotic alternatives, conventional antibiotic treatment remains the most impactful current intervention against infection. However, bacterial infections again have become a serious problem after decades of using it as treatment because of the quick emergence of bacterial resistance (Ventola, 2015).

1.2 Conventional antibiotics

At present, antibiotics are still the most powerful intervention against infections from pathogens (Yao et al., 2023). It works by either stopping the bacteria from reproducing and growing or killing the bacteria directly (Preeti Patel, 2023). In relation to that, antibiotics mechanisms are divided into two parts; bacteriostatic, which refers to the ability of the antibiotics to stop or inhibit the growth of the bacteria; bactericidal which refers to the directly killing of the bacteria (Pankey & Sabath, 2004).

Bacteriostatic antibiotics works by disrupting the essential intracellular functions of the pathogens (Pankey & Sabath, 2004). For example, fluoroquinolones like levofloxacin interfere with DNA replication of the pathogen (Preeti Patel, 2023), vancomycin and beta-lactam antibiotics works by stopping the bacterial cell peptidoglycan synthesis which leads to cell lysis. Daptomycin disrupts the outer membrane of the bacteria. Moreover, bacteriostatic antibiotics like tetracycline blocks the protein synthesis by binding to the bacterial ribosomes (Khanal, 2025).

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Adapted from Khanal, 2025 BioRender

Figure 1: Schematic summary of antibiotics different mode of action

(Khanal, 2025) The image represents a bacterial cell with labels indicating the sites of action for different antibiotics, which includes those affecting cell wall synthesis such as beta lactams, vancomycin, cell membrane integrity; Polymyxins, folate synthesis like sulfonamides and trimethoprim, nucleic acid synthesis or quinolones and rifampicin, and protein synthesis such

as tetracyclines, aminoglycosides targeting the 30S subunit; macrolides, clindamycin, linezolid targeting the 50S subunit.

1.3 Antimicrobial resistance: emergence and mechanisms

1.3.1 Emergence of AMR

Resistance to antibiotics is a natural phenomenon that refers when the mutation of the bacteria happened after the exposure to the antibiotics. This results to the cease of bacteria to respond or be affected to the antibiotics that they were once vulnerable, which made the treatment ineffective and impossible to cure (Chinemerem Nwobodo et al., 2022) (Mancuso et al., 2021). The emergence of bacterial resistance is due to different factors. One of those is the use of different antimicrobial agents whether as therapeutic or to promote growth in poultry, hog and in other farm raised animals. Arguably, this can cause the microorganisms to become resistant to the drug, and later transmitted to human pathogens as food, spreading the resistance (Knobler SL, 2003). Moreover, the effect of antibiotics that spreads active drug throughout the body even to the untargeted areas is called systemic side effect, the wasted from human with more active drugs then will contaminate the environment and could also contribute to the emergence of AMR (Wegener et al., 2017). Furthermore, despite the fact that AMR is a natural phenomenon, researchers said that the primary culprit of the spread is the abuse of antibiotics (Mancuso et al., 2021) and it has been predicted by Sir Alexander Fleming, 17 years after he discovered penicillin, he warned the public about overuse of antibiotic as they continue to use it (Ventola, 2015).

In other advanced countries, the top contributing factor of microbial resistance is the doctor's inappropriate prescribing of antibiotics. In contrary, to most third world countries, the top contributor is the improper usage or self-medication. This is because of the lack of implementation of the protocol in dispensing antibiotics or for some cases because of socioeconomic status that they are incapable of paying the doctor's fee, resulting to self-medication (Mancuso et al., 2021). This practice is most common at some parts of the globe such as Africa, some parts of Asia, America and Europe, it comes to the point that it seemed like basic commodities and is readily available in the free market and can be purchased by anyone even without prescription or without proper diagnosis despite being illegal, and worst this is dispensed by a unauthorized person (Llor & Bjerrum, 2014). Along with this is the patient's discipline, for instance; they tend to not follow the instruction given by the physician, the reason may be they forget to take it on time or cease the treatment at all the moment they feel better resulting to incomplete clearance of infection (Knobler SL, 2003). Emergence of

AMR happens when antibiotics only kill a portion of pathogen population, it will act as an evolutionary selective force to those individual cells that have favourable mutation allowing them to survive at high concentration.

1.3.2 Mechanisms of AMR

The emergence of the AMR to current antibiotics resulted in the discussion of alternative ways to counter AMR, by in dept understanding the mechanisms of resistance to the known antibiotics (Dhanda et al., 2023). There are different types of resistance mechanism; (a) modification of target or changing of the structure of the molecule within the bacteria, wherein the antibiotics failed to bind to the target; (b) the activation of enzyme present in the bacterial cell that target the antibiotics, such as ß-lactamases to inactivate the ß-lactam ring present in penicillin and other antibiotics of the same class (Douafer et al., 2019).; (c) efflux pumps overexpression that eject the antibiotic outside of the cell; (d) impermeability of the cell membrane stopping the entry of the antibiotic (Douafer et al., 2019).

Antibiotic resistance mechanism can be categorized into two groups; Intrinsic resistance and acquired resistance. Intrinsic resistance also known as inherent resistance refers to the natural, inherent ability of the bacteria to resist the activity of a certain class of antibiotics due to their structural or functional characteristics (Habboush Y, 2023). An example intrinsic resistance is vancomycin simply doesn't work against Gram-negative bacteria because it cannot permeate the outer cell membrane of the bacteria under normal circumstance as its mechanism of action is to disrupt the peptidoglycan (Patel S, 2023).

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Figure 2: Schematics for the intrinsic resistance mechanism

(BioRender, 2025) Antibiotic A enters into periplasm via a porin protein and binds to penicillin-binding proteins (PBPs) which are constituents of the peptidoglycan synthesis. Antibiotic B enters only via porin but is thereafter pumped out from the periplasm by an antibiotic efflux pump and therefore fails to reach its site of action. Antibiotic C is unable to permeate through the outer membrane porin either based on size or through any other factor, hence, not finding access to the periplasm or intracellular target. These are intrinsic mechanisms that exist normally within bacteria, helping them evade the action of certain antibiotics.

Acquired resistance on the other hand develops when bacteria, through genetic mutation, acquire the ability to resist an antibiotic they were previously vulnerable. This can occur because of spontaneous mutations or through uptake of resistance genes from other bacteria (Habboush Y, 2023). The example of this is a strain of *S. aureus,* this bacteria has developed resistance to ß-lactam antibiotics including methicillin, penicillin and other related drugs. The bacteria produce an enzyme called ß-lactamase that breaks down the ß-lactam and inactivating the antibiotics. However, researchers have developed a way to counter this type of resistance using antibiotic potentiation (Ali Alghamdi et al., 2023).

The activity execution of the antibiotics with intracellular targets is mainly depending on whether they can go inside the cell to the target by permeating the cell membrane. However, different class of antibiotics has different response in case of permeabilization of cell

membrane. With Gram-positive bacteria the cell envelope has peptidoglycan and the cell membrane itself only. As for the gram-negative bacteria, the cell envelope is composed of outer cell membrane, peptidoglycan and inner cell membrane, respectively (Dhanda et al., 2023), whereas some antibiotics like vancomycin cannot permeate through the outer cell membrane to reach the periplasm and inner cell membrane under normal circumstances, making them inherently resistant to the antibiotics as its mode of action is to inhibit the synthesis of peptidoglycan (Douafer et al., 2019). After understanding the mechanism of microbial resistance, researchers then come up with the ideas on how to improve the efficacy of the existing antibiotics (Dhanda et al., 2023) using antibiotic potentiation as oppose to working on drug discovery that takes decades.

1.4 Antibiotic potentiation

Antibiotic potentiators are the nonantibiotic substances that enhances the efficacy of antibiotics. An increased in permeabilization of cell membrane is one way we are looking to potentiate the antibiotics by promoting the influx of the antibiotics (**Figure 3**) (Paul et al., 2023). Gram-negative bacteria are highly resistant to some antibiotics because of their extra layer of outer membrane that is reinforced by lipopolysaccharides (LPS) and proteins that block the entry of antibiotics (Chan et al., 2021). LPS forms a tough barrier that repels hydrophilic antibiotics, while outer membrane proteins exclude hydrophobic agents. To overcome this, outer membrane permeabilizers can be used in combination with antibiotics. These compounds weaken the outer membrane by disrupting the LPS layer, increasing membrane permeability and allowing antibiotics to penetrate more effectively, potentially enhancing their antibacterial activity (Farrag et al., 2019). Farrag and team found that combining natural phytochemicals, such as thymol and gallic acid as permeabilizers with antibiotics significantly increased the susceptibility of resistant bacterial isolates. Permeabilizers disrupted the bacterial outer membrane by releasing LPS, enhancing antibiotic entry (Vaara, 1992). This synergistic approach offers potential for using existing antibiotics to treat resistant bacterial infection.

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Figure 3: Schematic representation of antibiotic potentiation by permeabilization.

(i) Gram-negative bacteria outer cell membrane that prevents the entry of antibiotics. (ii) Antibiotic potentiators disrupt the integrity of the outer membrane allowing the entry of drug (Chan et al., 2021)

However, there are other ways to potentiate the activity of antibiotics. One classic example of antibiotic potentiation that is already available in the market is clavulanic acid which is a β lactamase inhibitor, combined with amoxicillin under the brand name *Augmentin* (Huttner et al., 2020). β-lactam antibiotics works by inhibiting the peptidoglycan synthesis, then the bacteria develop resistance to them by the enzyme called beta lactamase which breaks down the β lactam ring, inactivating the antibiotics. It works by preventing the activity of beta-lactamase from happening, hence resensitizing the bacteria to beta lactam. A similar principle is used with an alternative β-lactamase inhibitor, Zosyn (Queenan & Bush, 2007).

1.5 Quaternary ammonium compounds (QACs)

The use of QACs as antimicrobial agent started during the beginning of the 20th century (Jacobs, 1916). QACs are surfactant with net positive ammonium atom head that is hydrophilic attached to the hydrophobic alkyl chain (Camagay AV, 2023; Kawabata & Nishiguchi, 1988), with this chemical structure, it affords it amphipathic nature where the main mode of action is to disrupt cell membrane (Jennings et al., 2015). QACs are normally light in colour and crystalline powder and are highly soluble in water (Song et al., 2018). In brief, through Coulomb's force the hydrophilic head electrostatically attracts to the negatively charged phosphate head of the plasma membrane (Kawabata & Nishiguchi, 1988), while the

hydrophobic alkyl chain slides itself to the lipid layer of the plasma membrane which results to the disruption of its integrity and increasing the permeability (Buffet-Bataillon et al., 2012), these cause effects such as leakage of cell contents such as DNA and RNA (Kawabata & Nishiguchi, 1988), depolarization of the cell membrane, generation of reactive oxygen species (ROS) and ultimately cell death. One primary mechanism is that the ability of QACs to disrupt bacterial membrane is a property that may lead to antibiotic potentiation (Kawabata & Nishiguchi, 1988; P. Gilbert, 1985).

1.5.1 Cetyltrimethylammonium Bromide (CTAB)

CTAB is a cationic surfactant having a structure that consist a positively charged ammonium head group $[N(CH_3)_3]Br$, attached to a long hydrocarbon tail ($C_{16}H_{33}$) (Janosevic-Lezaic et al., 2014), Its widely used as disinfectant and topical solution, one example of the product that is currently being used by medical professional is Cetrimide (Bonnet et al., 2020). Previous studies conclude that CTAB treatment against *E. coli* cells led the bacteria to a generation of state of superoxide; a type of oxidative reactive species (ROS) and hydrogen peroxide (H_2O_2). It was also proposed that superoxide generation was based by inhibiting SoxS; a protein that activates genes as a result of oxidative stress, functions and reduced Manganese superoxide dismutase (Mn-SOD); an enzyme mainly antioxidant that prevents cells from oxidative damage, activity. (Nakata et al., 2011).

1.5.2 Didecyldimethylammonium Chloride (DDAC)

DDAC is a cationic QAC with a molecular structure of two decyl chains, two methyl groups and one chloride counterion ($C_{22}H_{48}CIN$) (Tezel et al., 2008). It has a broad spectrum against both Gram-positive and Gram-negative bacteria. DDAC acts as a membrane-active disinfectant and acts as bactericidal effects via electrostatic binding and membrane permeabilization (Gerba, 2015).

1.5.3 QAC Structural characteristics

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Figure 4: Chemical structure of the QACs (A) CTAB and (B) DDAC (Janosevic-Lezaic et al., 2014; Tezel et al., 2008)

Related literature review says that the size of the alkyl chain determined the antibacterial property of a polymer (Baudrion et al., 2000; Nadagouda et al., 2022), it has been found that the longer the alkyl chain, the stronger the antimicrobial activity (Daoud et al., 1983). In comparison to this, antimicrobial peptides (AMPs) which are naturally occurring peptides made up of 10-100 amino acids, also with a positive or negative charge depending on their type, often act by forming pores in microbial membranes or by binding to intracellular targets to disrupt essential processes, such as protein synthesis (Zhang et al., 2021). Both QACs and AMPs target microbial membranes, primarily using electrostatic interactions to disrupt membrane integrity (Dan et al., 2022). Their effect is similar, as with QAC being synthetic, broad-spectrum disinfectants and AMPs being natural, multipurpose peptides. Both QAC and AMP are widely accepted in relation to antimicrobial activity as their primary mode of action involves disrupting the cell membrane (Jiao et al., 2017).

1.5.4 Antimicrobial mechanisms

Quaternary ammonium compounds (QACs) belong to the organic cationic compounds that are known in the industry as antibacterial agents (Morrison et al., 2019). QAC are compounds commonly used as disinfectant in hospital, clinical setup and was used by the doctors for pre-op disinfection of hands over century. They also have been used in different products such as; eye drops or lubricant, treatment for the acne, face cleanser, nasal

decongestant solution, hair products, make up and make up remover products and many more (Buffet-Bataillon et al., 2012). It is also currently use in a dental industry to prevent and treatment of oral disease like periodontitis. Moreover, QACs appear to improve the antibacterial activity of implants such as dimethylaminododecyl methacrylate (DMADDM) hydantoin, a preservative and antimicrobial agent and hydroxyapatite filled with polydopamine on the titanium surface found to be infection resistant (Li et al., 2023). QACs have long been used as effective antibacterial agents having 95 drugs currently marketed. They come with many different R groups, and specifically CTAB and DDAC differ by chain size and number of chains, which may govern their influence on the membrane (Dan et al., 2022; P. Gilbert, 1985).

Furthermore, some bacteria are also resistant to QAC, and a major contributor to QAC resistance is overexpression of efflux pumps, which expel QACs and other antimicrobial agents from bacterial cells. This resistance can also be acquired through mobile genetic elements, such as plasmids and transporins, which facilitate the transfer of QAC-specific efflux pumps genes (Tezel & Pavlostathis, 2015). Co-resistance and cross-resistance can emerge as a result. Though efflux inhibitors like verapamil and reserpine have shown potential in vitro to block these pumps, more research is needed to improve their efficacy and safety for clinical use, highlighting the need for further exploration of strategies to mitigate QAC toxicity and resistance (Tischer et al., 2012).

Prolonged exposure to sub-inhibitory concentrations of QACs can select bacteria with lower susceptibility, ultimately leading to the development of QAC-resistant strains (Jiao et al., 2017). These bacteria undergo several adaptive mechanisms, including modifications to the cell membrane structure, increase biofilm formation, and enhanced efflux pump activity, which contribute to reduced QAC-induced activity (McBain et al., 2002).

1.5.5 QACs as antibiotic potentiators

In June 2023, the researchers developed a hydrogel formula of modified silica nanoparticles (MPSi) functionalized with CTAB (MPSi-CTAB) to inhibit the growth of *staphylococcus* and *candida* strains, they performed the experiment in vitro, MPSi-CTAB has shown an antimicrobial effect on methicillin-resistant *S. aureus* ATCC 700698 having an MIC and MBC of 0.625 mg mL−1 and 1.25 mg mL−1 respectively, it also demonstrate a reduced MIC and MBC for *Staphylococcus epidermis* (*S. epidermis*) ATCC 35984 giving them a positive outcome. Moreover, it exhibits decreased MIC values by 32 and 16 folds respectively when synthesized with ampicillin and tetracycline having a low toxicity in eukaryotic fibroblast

(Martins da Silva Filho et al., 2023). Although QACs can be toxic, they are still being used but at a reasonable lower concentration (Camagay AV, 2023).

1.6 Applications of QAC /Antibiotic combination treatment

Lambert and team conducted a study modelling a synergistic effect of combining Ethylenediaminetetraacetic acid (EDTA) with QAC and some specific antibiotics (Lambert et al., 2004). It was found that the minimum inhibitory concentrations of both QAC and antibiotic oxacillin and cefamandole were reduced by 3-10 times. More significantly, the MIC of ampicillin was reduced by factor from 1524 to 21 mg l^{-1}, when combined with 500 mg/L of EDTA (Lambert et al., 2004), this indicates a strong enhancement of antimicrobial efficacy through the use of EDTA in combination with QAC and certain antibiotics. The possible application is similar to what Martins de Silva and team have formulated, a hydrogel, topical cream, wound healing dressing or a topical solution for pre operative surgeries (Martins da Silva Filho et al., 2023).

1.7 Safety and toxicity

QACs are known to interact with microbial cell membranes at concentrations above MIC leading to membrane disruption and leakage of cellular contents (Tischer et al., 2012). QAC is the most useful amongst most surfactant for their microbicidal action, however, it is also one of the most toxic for mammalian cells. In order to maximize the safe potential of QAC for its properties, understanding the toxicity mechanism is important. Study shows that QAC's toxicity at sublethal concentration intervene through mitochondrial distress of the cell resulting to less production of energy that leads to apoptosis, hence, a much higher concentration leads to necrosis (Inacio et al., 2013). A group pf researchers tested genotoxicity of two kinds of QAC which are benzalkonium chloride (BAC) and dimethyldioctadecylammonium bromide (DDAB) (Ferk et al., 2007).

Generally, QACs as disinfectant are low risk to eukaryotes as those products are of low concentration. This supports that our proposed first hypothesis "Sub-MIC concentrations of QACs can be used to increase the activity of antibiotics against bacterial pathogens" is safe in eukaryotic cells as we are aiming to use a sub-MIC, meaning a concentration lower than the MIC for application, ensuring the safety use of the compound.

1.8 Bacterial Gram-stain

Staphylococcus aureus (S. aureus) and *Escherichia coli (E. coli)* are both one of the most clinically relevant bacterial species, causing a wide range of human infections (Prestinaci et al., 2015). *S. aureus* is a Gram-positive spherical-shaped bacteria that is commonly causing a skin and nasal passages infection like pneumonia (Tong et al., 2015) and a major contributor with diarrheal infection among children and adults (Song et al., 2018). Moreover, *E. coli* is a Gram-negative rod-shaped bacteria that is most commonly seen as harmless in the gastrointestinal tract of animals and human (Russo & Johnson, 2000), except some strain that could cause foodborne diseases, urinary tract infection and neonatal meningitis (Kaper et al., 2004). The versatility and pathogenic capabilities of both bacteria is the reason the target of public health interventions and research (Cassini et al., 2019).

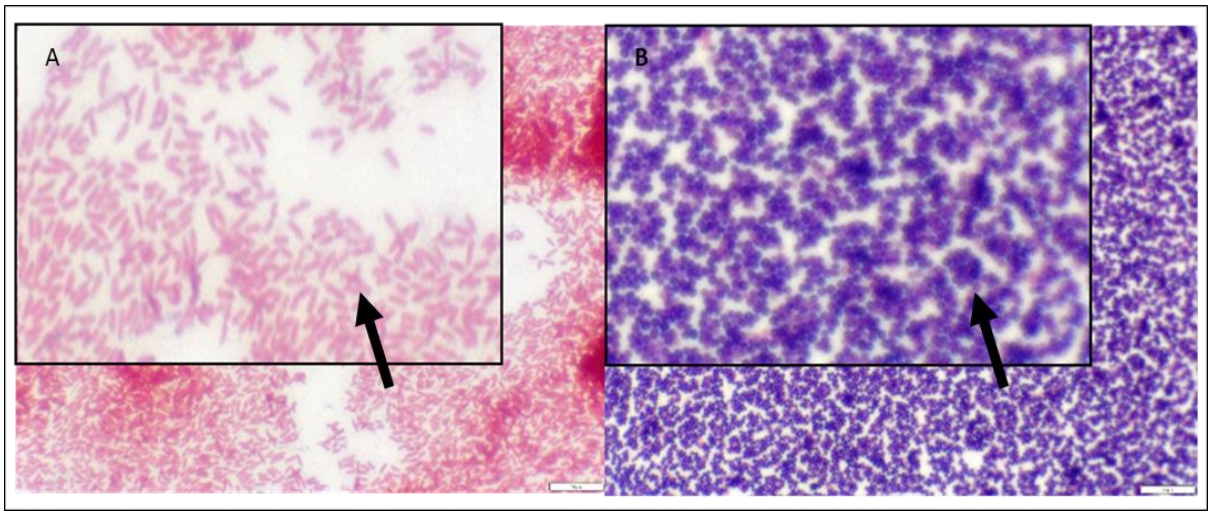

Figure 5: Microscopic image of Gram-negative and Gram-positive bacterial stain (A) Gram-stained *E. coli* (B) Gram-stained *S. aureus*

1.9 Research question

Can QAC be used to enhance antibiotic activity?

Interestingly, QAC exhibit a wide range of antimicrobial activity against microorganisms including fungi, viruses and bacteria, showing more efficacy against gram-positive bacteria (Dan et al., 2022). Disrupting the outer cell membrane of gram-negative bacteria is an excellent way to enhance the efficacy of antibiotics or other antimicrobials that are inherently resistance to these pathogens (Jennings et al., 2015). This approach allows previously ineffective antibiotics, particularly those designed for gram-positive bacteria to

penetrate the cell membrane and execute their antibiotic activity (MacNair & Brown, 2020; Pottier et al., 2023)

Researchers studied on how to increase the permeability of the cell by disturbing its integrity by means of adjuvants (Dhanda et al., 2023). By disrupting the outer membrane, it shows that the rules governing antibiotic entry into gram negative bacteria change, allowing even hydrophobic antibiotics to penetrate more easily. This disruption not only bypasses existing resistance mechanisms but also helps overcome spontaneous resistance that may develop during treatment. Additionally, outer membrane perturbation reduces the bacteria's ability to form biofilm, which are structured communities of bacteria that are often highly resistant to antibiotics. (MacNair & Brown, 2020).

1.10 Research gap

Previous studies have shown the broad-spectrum of antibacterial activity of QACs and its potential use in industry, that includes the disinfectant products, topical solution and many more. However, QAC's potential to improve the antibiotic activity by increasing the permeability of the bacterial membrane to possibly promote the influx of antibiotics, enhancing its activity has been left undiscovered. Therefore, the importance to further investigate and maximize the potential of QACs is important to address the declining efficacy of antibiotics and to prevent the spread of microbial resistance.

1.11 Hypothesis and Aims

1.11.1 Hypothesis

a. Sub-inhibitory concentrations of QACs can be used to increase the activity of antibiotics against bacterial pathogens.
b. Sub-inhibitory concentrations of QACs can sensitize Gram-negative bacteria to vancomycin which they are inherently resistant to.

1.11.2 Aims

a. Investigate the potential for QACs to potentiate antibiotic activity
b. Elucidate the mechanism of antibiotic potentiation
c. Investigate the potential for QACs to broaden the spectrum of antibiotics

1.12 Relevance to Medical Biotechnology

Antibiotics are the most powerful solution to fight bacterial infections (Ventola, 2015), without it, no medical surgeries that save millions of people could be possible (Hutchings et

al., 2019). However, with the rapid emergence of antimicrobial resistance, its efficacy has been compromised (Muteeb et al., 2023). If the direction of the declined efficacy of the antibiotic continue the way it is at present, without actions, it might lead to the increased morbidity and mortality and therefore might affect the well-being of most living organisms including human and animals (Hutchings et al., 2019). With the help of the previous research studies, we are aiming to address the research gap and potentiate the existing antibiotics. This research study could bring back the efficacy of the antibiotics and therefore improve bell-being of the patients (Ventola, 2015).

1.13 Summary

The combination of quaternary ammonium compounds (QACs) with conventional antibiotics offers a promising strategy to combat antibiotic resistance by enhancing antibiotic penetration, offering a promising strategy to enhance the efficacy of existing conventional antibiotics as the pace of drug discovery has dramatically slowed down. QACs may promote increased penetration of antibiotic, by disrupting the integrity of cell membranes through its electrostatic interactions, making resistant bacteria more susceptible to antibiotic treatment. QACs ability to disrupt cell membrane can be synergistically combined with antibiotics to overcome resistance mechanisms that has become a global threat, particularly Gram-negative bacteria where the outer membrane acts as a barrier. This dual action approach, utilizing QACs to potentiate antibiotics, provides an innovative pathway to combat antibiotic resistance. Further research into optimizing QAC-antibiotic combinations could lead to new, more effective treatments for resistant infections.

CHAPTER 2: MATERIALS AND METHODS

This experiment was conducted at Biomedical Nanoengineering Laboratory (BNL) at Flinders Medical Centre (FMC)

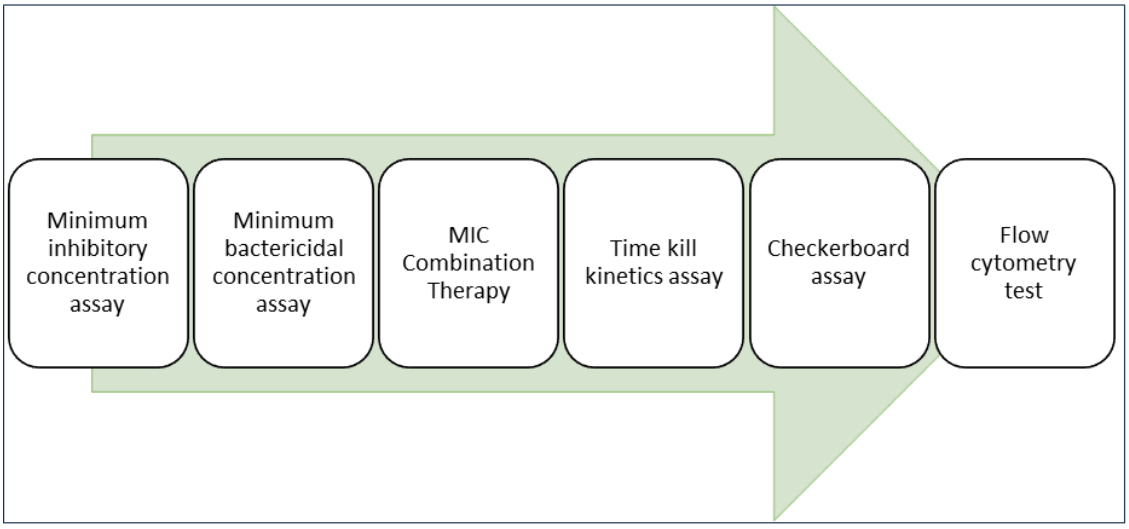

Figure 6: Summary of the methodology from identifying the MIC of all the QACs and antibiotics to identifying the MBC; obtaining the potentiating capability of QAC to antibiotics against bacterial strain; studying the time of the combination could kill a bacterial strain; cross checking the MIC and MBC validity through checkerboard assay; to finally analysing how each antimicrobial agents affect the bacterial cell.

2.1 Materials

All antibiotics and reagents used for this research are from Sigma Aldrich, unless otherwise stated. Cetyltrimethylammonium Bromide (CTAB); Didecyldimethylammonium chloride (DDAC), Cefazolin; Colistin; Daptomycin; Gentamicin; Kanamycin; Levofloxacin; Tetracycline; Vancomycin; *Escherichia coli; Staphylococcus aureus*; Mueller Hinton Broth (MHB); Tryptic Soy Broth (TSB); Phosphate-buffered Saline (PBS); Agar ; 96-well microtiter plates; Petri-dish; 5mL centrifuge tube; 50 mL tube; 15 mL tube; Micropipette; (Eppendorf, Thermo Fisher); Multichannel pipette (Eppendorf, Thermo Fisher); serological pipette (Eppendorf, Thermo Fisher); Biosafety cabinet; Incubator; Spectrophotometer (Thermo Fisher); Plate reader; Compound microscope; Flow Cytometer; Propidium Iodine

2.2 Minimum Inhibitory Concentration (MIC) Assay

Brief overview of MIC assay

MIC assay is currently used in research laboratories especially in the microbiological research to study antibiotic resistance mechanisms, identification of emerging drugs targets and assessments of the promise of the novel drug candidates (Brennan-Krohn et al., 2017). MIC assay is an important way to determine bacterial strain susceptibility to an antimicrobial molecule of interest. In summary in order to do this assay, a pure culture of the known bacterial strain is standardized to 10^7 colony forming units (CFU)/mL (OD_{600}) and treated to a various antibiotic concentration for 16-24 hours at 37 °C (Kaderabkova et al., 2024). After the incubation period the bacterial growth for each antibiotic concentration is being evaluated using a plate reader, where the MIC value is the lowest concentration required to stop the growth of the bacterial strain which strictly observed in vitro to get an accurate results (Kowalska-Krochmal & Dudek-Wicher, 2021).

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Figure 7: Schematics of the MIC set up (A.) transferring of media to the 96-well plates (B.) Introduction of the antibiotics, serial dilution, and exposure to the bacteria (C.) incubation for 16-24 hours at 37°C

Experimental details

A colony of bacterial strain is inoculated in Mueller Hinton Broth (MHB), under strictly observed controlled conditions of 37 °C within a range of 16-24 hours in a shaking incubator. In a 96-well plate 95µL of MHB is supplied in all columns in triplicate except the first column which should be 170µL of MHB. An antibiotic having a high concentration ranging from 160µg/mL and up is introduced in the first column all in triplicate where there is 170µL of MHB having a total volume of 195µL (Appiah et al., 2017). A 10-fold serial dilution was then performed leaving a final well volume of 100µL. A negative control in triplicate is supplied with just 95µL of media without bacteria and a positive control with 95µL and bacteria to ensure the validity of the result. A standardized bacteria of $1x10^7$ cfu/mL is then introduced to all wells including the positive control and incubated within 16-24 hours at 37°C (CMI, 2003).

2.3Minimum Bactericidal Concentration (MBC) assay

Brief overview of MBC assay

MBC is the concentration required to kill 99.9% of the bacteria, it is being performed after getting the MIC concentration. It is important to determine whether the antibacterial agent is bacteriostatic or bactericidal (Andrews, 2001). Bacteriostatic refers to the antibacterial agent that inhibit or supress the growth of the bacterial strain while the bactericidal are the antibacterial agents that kills the bacteria or shows a reduction of 99.9% of the bacterial strain (Bernatova et al., 2013)

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Figure 8: **Schematics of the overview on how to get the minimum bactericidal concentration of the antibacterial agent**.(A) getting the aliquot from the wells with no visible

growth (B) Plating the aliquot to the Tryptic soy agar (TSA) (C) Incubation of the plate for 16-24 hours at 37°C.

Experimental details

To summarize, 10µL aliquot has been taken from the wells of the 96-well MIC plate starting from the wells without visible growth of up to at least 4th well from the MIC value and plate it into petri dish with tryptic soy agar (TSA) and incubate up to 16-24 hours at 37 °C. After the incubation period, the lowest concentration with no growth is considered the minimum bactericidal concentration of the antibacterial agent (Andrews, 2001; Mah, 2014).

2.4 Time-kill kinetics assay

Brief overview of the assay

Time kill kinetics assay principle is basically to evaluate when does the antibacterial agents kills the bacterial strain by measuring the bacterial viability over time (Sunberg, 2021). It is an important technique to characterize the bactericidal activity of certain antimicrobial drug, especially when studying the differences between the effectiveness of a certain antimicrobial agent alone and when combined with another antibacterial agent (Montero et al., 2021).

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Figure 9: Schematic of the time kill assay procedure (A) 6 isolated tubes to be tested; (1) antibiotics in MHB with bacteria, (2) antibiotic + QAC in MHB with bacteria, (3) QAC in MHB with bacteria, (4) MHB with bacteria, (5) QAC by itself (6) MHB without bacteria. (B) Incubation time in a shaker, time interval having, 15 minutes; 30 minutes; 60 minutes; 120 minutes; 24 hours (C) After each incubation period, each tube is transferred to 96-well plates for serial dilution (D) Plating 10µL aliquot from 0-5 and incubate for 16-24 hours at 37°C.

Experimental detail

This assay used 6 isolates: (1) antibiotics in MHB with bacteria, (2) antibiotic + QAC in MHB with bacteria, (3) QAC with bacteria, (4) MHB with bacteria, (5) QAC by itself (6) MHB without bacteria, it is important note that tubes 5 and 6 were utilized to ensure that the media used in not contaminated and tubes 3 and 4 represent the positive control where we expected the growth to continue. The concentration used is the MBC value of the antibiotics and half of the MIC value of the QAC as one of the aims of this project is to use the

subinhibitory concentration of QAC to potentiate the antibiotics. For the first tube, the MBC value of the antibiotic in pure MHB is combined with the standardized bacteria of 10^7 cfu/mL (OD_{600}). Second tube is MBC value of antibiotics with subinhibitory concentration of QAC in MHB with the standardized bacteria of 10^7 cfu/mL (OD_{600}). Third tube is subinhibitory concentration of QAC in MHB with the standardized bacteria of 10^7 cfu/mL. Fourth tube is pure MHB with the standardized bacteria of 10^7 cfu/mL. Fifth tube is MHB with QAC at subinhibitory concentration with no bacteria. Sixth tube is MHB alone with no bacteria. Fifth and Sixth tube is there to ensure that the media is not contaminated. After the preparation of all the tube, it is then quarantined in a shaker incubator for the span of 15, 30, 60 and 24 hours. While it is inside the shaker, preparation of the 96-well plate and 6 petri dish is being held. For the 96-well plate, the set up was vertical instead of the normal horizontal set up, the first 3 columns are for the first tube, 4-6 are for the second tube, 7-9 are for the third tube, 10-12 are for the fourth tube and the fifth and sixth tube are in the columns A-B (1-3) and A-B (4-6) in duplicate **(Figure 9).** Columns 2-6 is filled with 180µL of MHB and the rest of the succeeding rows too then wait until the first-time interval is done.

After 15 minutes, the first 3 columns are filled with 200µL of aliquot from the first tube and do the same for the rest of the tubes **(Figure 9C)**. Transfer 20µL from first column and serially dilute until 6th column, then discard the last 20µL leaving a total volume of 180µL for each well. Plate a 10µL aliquot from each well with different concentration from the tubes and leave in the incubator for 16-24 hours at 37°C (Adusei et al., 2019). Repeat the same process after 30, 60, 120 minutes and 24 hours. After 24 hours, after doing the same process, streak the 6 tubes into TSA and leave for 16-24 hours at 37°C to see if there are any growth in any of the tubes.

After 16-24 hours incubation period, the colony forming unit is calculated using this formula:

$$\text{CFU/mL} = \frac{\text{Number of colonies}}{\text{Volume plated (mL)}} \text{ x dilution factor}$$

2.5 Synergy checkerboard Assay

Brief overview of the assay

Synergy checkerboard assay is used to determine and compare the effectiveness of an individual antimicrobial agent and in combination with other antibacterial agents. The result is then represent the Fractional inhibitory concentration index value (FICI) (Pharma, 2023). The

FICI index value is calculated using the formula below, where *Compound A* refers to the MIC of compound A, and Compound $A_{(Combined)}$ represents the MIC of compound A when combined with another compound. The same allocation is applied to *Compound B* and Compound $B_{(Combined)}$.

$$FIC_A = \frac{Compound\ A_{(Combined)}}{Compound\ A}$$

$$FIC_B = \frac{Compound\ B_{(Combined)}}{Compound\ B}$$

$$FICI = FIC_A + FIC_B$$

The table below is the summary of the FICI interpretation, whereas synergistic effect (≤ 0.5) indicates significant enhancement of antimicrobial activity, Additive ($> 0.5 – 1.0$) shows mild enhancement but not true synergy. While indifferent ($> 1.0 – 4.0$) means no meaningful interaction between the two compounds, and finally the antagonistic (> 4.0) interprets as the combination reduces efficacy.

Table 1: Fractional inhibitory concentration index (FICI) equivalent value interpretation

FICI value	Equivalent	Interpretation
≤ 0.5	Synergistic	Significant enhancement of antimicrobial activity
$> 0.5 – 1.0$	Additive	Mild enhancement, but not true synergy
$> 1.0 – 4.0$	Indifferent	No meaningful interaction
> 4.0	Antagonistic	The combination reduces efficacy

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Figure 10: Schematic representation of checkerboard assay where the combination of the 2 compounds (QAC + antibiotics) is determined. (A) Antibiotics (compound A) 10-fold serial dilution (B) QAC (compound B) 10-fold serial dilution. (C) Combined antibiotic and QAC synergy result, blue colour is the antibiotic MIC while the red is the QAC MIC.

Experimental details

Antibiotics starting concentration used for this assay is 4x its MBC value while the QAC is 4x of its half MIC value since we wanted the plots to be in the middle and to achieve the desired concentration after the dilution. The desired concentration is calculated using the formula $C_1V_1=C_2V_2$ (Biosciences, 2023). This assay used 2 plates; first being just the antibiotics alone, columns 2-10 is filled with pure MHB while the column 1 is filled with MHB and antibiotics. A 10-fold dilution was performed leaving a final volume of 100µL for each well. Second plate is QAC alone, same process for the plate 1 except for this, it was serially diluted vertically having the column H and row 12 excluded (CD, 2025). When the 2 plates are ready, transfer the plate 1 to plate 2, mix well and discard 100µL from each well leaving a final volume of 100µL then add the 1 x 10^7cfu/mL of bacteria for each well, incubate within 16-24 hours at 37°C.

2.6 Flow cytometry assay

Brief overview of flow cytometry assay

Flow cytometer is an important instrument used for measuring different characteristics of individual cell like their size and internal complexity while move through the measuring device. It works by detecting how cells scatter light with the help of propidium iodine (Adan et al., 2017). It is useful for this experiment as we are studying synergistic effect of combined antimicrobial agents and we wanted to know the difference of how antibiotics by itself and in combination with QAC affects the cells like by quantifying the reactive species oxygen (ROS) production (Mondal & Singh, 2022).

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Figure 11: **Schematic of the flow cytometry analysis to measure cell permeability using propidium iodine**. (1) Preparation of the bacteria, exposure to QACs (2) Treating cells with 1 µg/mL PI to assess the membrane integrity of the bacterial cell (3) Samples were analysed using flow cytometer (4) Data analysis using GraphPad Prism 10

Experimental details

Analysis of cell permeability was performed using flow cytometry, in a CytoFLEX S flow cytometer (Beckman-Coulter, Brea, CA), equipped with violet laser side-scatter (VSSC) for small particle scatter detection. Briefly, overnight bacterial culture was diluted to a final concentration of 10^7 cfu/mL and then treated with DDAC or CTAB at subinhibitory concentrations for 1h and incubated at 37 °C on an orbital shaker (120 RPM). In parallel, cells from treated and untreated groups were exposed to propidium iodide (PI) at a concentration of 1µg/mL. Samples were immediately measured with the flow cytometer at a flow rate of 100 µL/min over 2 minutes. Phosphate buffered saline, PI only, and unstained bacterial samples

were used as controls to determine background noise. All "events" detected by the instrument higher than the background noise were recorded. Cell events positive for PI were classified as "permeable", while PI-negative events were classified as impermeable. Overall cell permeability of the cell population was calculated by the following:

$$\text{Permeability (\%)} = \frac{\text{Permeable cells}}{\text{Total cells}} \text{ x } 100$$

2.7 Statistical analysis

In this project, a one-way analysis of variance (ANOVA) was conducted to establish the statistical significance between the means of different sample groups. It was chosen to see the different treatment effects on bacterial growth; this enables us to identify significant variations between the treatment conditions. All statistical analysis were conducted using Graphpad Prism and Microsoft excel, with a significance level set at $P < 0.05$.

CHAPTER 3: RESULTS

3.1 MIC and MBC assay of QACs and antibiotics by itself and combined results

The experiment began with determining the MIC value of the two QACs which are the CTAB and DDAC alone against the two bacterial strains, for Gram-positive, we used *Staphylococcus Aureus (S. aureus*) and for Gram-negative, we used *Escherichia coli (E. coli).* After determining the MIC and MBC values of the two QACs by itself, the next step was measuring the MIC value of the different antibiotics sample by itself as a foundation in order to see if there is any difference with the values after combining with QACs.

To be able to determine whether a QAC could potentially increase the antibacterial activity of antibiotics, this study chose different antibiotics with different mode of action against Gram-positive and Gram-negative bacteria. The following antibiotics used are Cefazolin; Colistin; Daptomycin; Gentamicin; Kanamycin; Levofloxacin; Tetracycline; Vancomycin. After all the MIC value was determined, we then used the 0.5x and 0.25x of MIC value of each of the QACs and then combine it with different variety of antibiotics with different mode of actions and measure the MIC value to compare before and after the combination treatment to test the first hypothesis. Cefazolin and vancomycin inhibit the peptidoglycan of the Gram-positive bacteria (Mahdi, 2018; Watanakunakorn, 1984), while the daptomycin disrupts the bacterial cell membrane (Huang, 2020), colistin targets the outer membrane of the Gram-negative bacteria (Abou-Zeid et al., 1978; Yahav et al., 2012). Gentamycin and kanamycin on the other hand interferes with protein synthesis by binding the ribosomal subunit that leads to bacterial cell lysis. Tetracycline also targets the 30S subunit to block the transfer ribonucleic acid (tRNA) attachment (Athauda et al., 2023; Pearson et al., 2025; Ramachanderan & Schaefer, 2021). Lastly, levofloxacin inhibits the DNA replication by targeting the deoxyribonucleic acid (DNA) gyrase and topoisomerase IV (Vardanyan & Hruby, 2016).

Table 2: Summary of the antibiotics utilized to combined with QACs

Antibiotics	Mode of Action	*S. aureus*	*E. coli*
Cefazolin	Peptidoglycan synthesis	Not tested	Tested
Colistin	Membrane interference	Not tested	Tested
Daptomycin	Membrane interference	Tested	Not tested
Gentamicin	Protein synthesis	Not tested	Tested
Kanamycin	Protein synthesis	Tested	Tested
Levofloxacin	DNA synthesis	Tested	Tested
Tetracycline	Protein synthesis	Tested	Not tested
Vancomycin	Peptidoglycan synthesis	Tested	Tested

The MIC values were determined by measuring the optical density (OD) at 600 nm after the incubation period with different concentrations of CTAB and DDAC. Complete inhibition of *S. aureus* with CTAB was seen at a concentration of 1µg/mL and above **Figure 12A**. While with DDAC, the complete inhibition of *S. aureus* was observed at a concentration of 0.5 µg/mL as seen in **Figure 12B**. Moreover, with the strain of *E. coli*, MIC value of CTAB as shown in **Figure 12C** was observed at a concentration of 16µg/mL, whilst with DDAC MIC value was observed at a concentration of 4µg/mL as shown in **Figure 12D**. Based on these results, the half (0.5) and the quarter (0.25) subinhibitory concentration of CTAB and DDAC against *S. aureus* and *E. coli* is the standard concentration that we used to test potentiating ability of QACs when combined with different antibiotics.

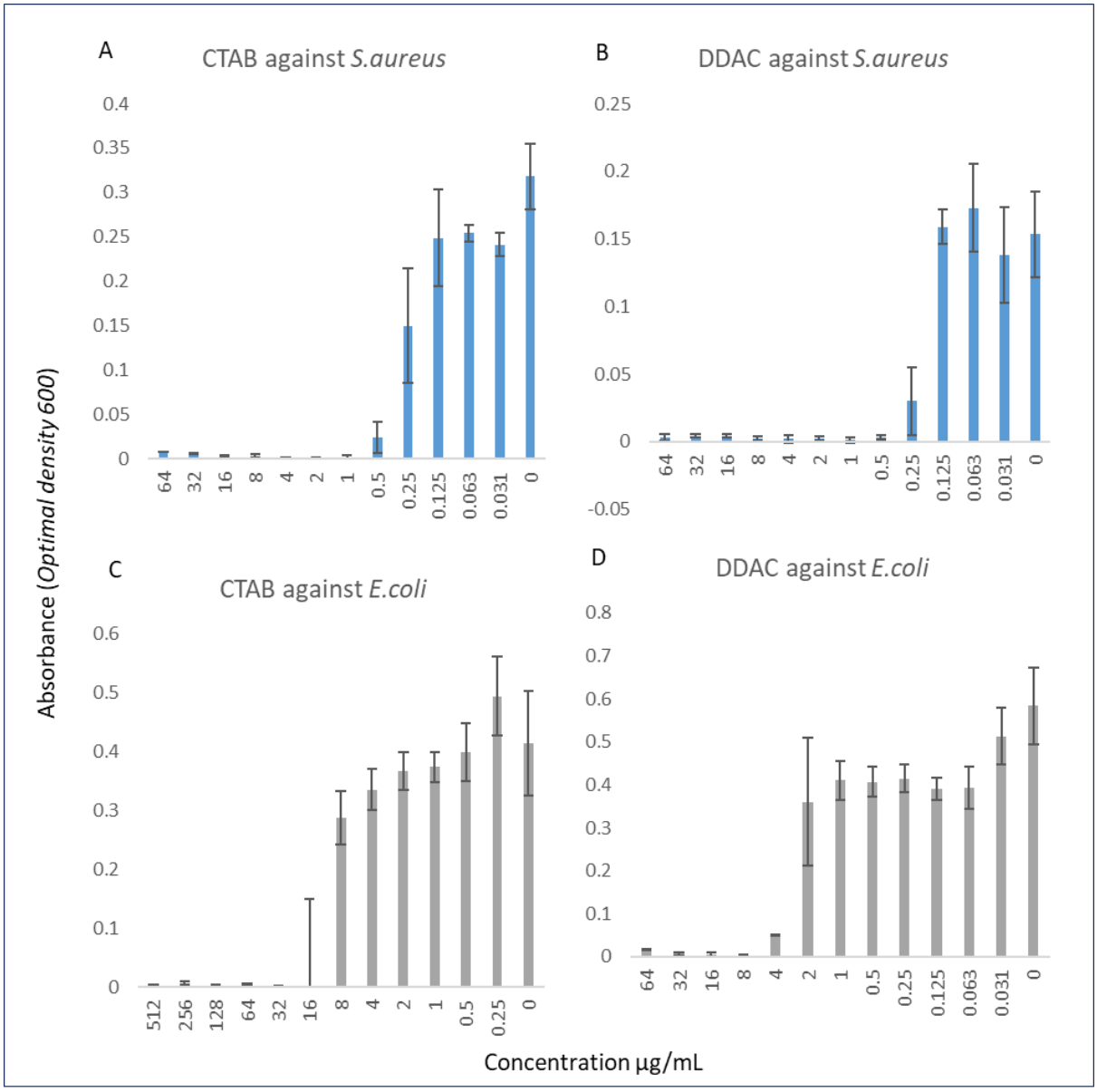


Figure 12: **MIC results of CTAB and DDAC against *E. coli* and *S. aureus* with 3 replicates and a positive control without QAC** (A) CTAB MIC value against *S. aureus* (B) DDAC MIC value against *S. aureus* measured at 600 nm (C) CTAB MIC value against *E. coli* (D) DDAC MIC value against *E. coli* measured at 600 nm

3.1.1 MIC of antibiotics and in combination with QACs subinhibitory concentration

The MIC has been done by measuring the optimal density (OD) at 600nm after the incubation period with a range of kanamycin concentration and in combination with 0.5x and 0.25x MIC value. As demonstrated in **Figure 13A**, the growth of the bacteria was completely inhibited at a higher concentration of the antibiotic, but the minimum has been observed at concentration 8µg/mL being the MIC value. The combination of kanamycin and 0.5x and 0.25x CTAB MIC value **(Figure 13B-C)** was observed having a significant difference of 2-fold

reduction of 4µg/mL. Moreover, the combination of kanamycin and 0.5x and 0.25x DDAC MIC value **(Figure 13D-E)** was observed having no significant difference of 8µg/mL.

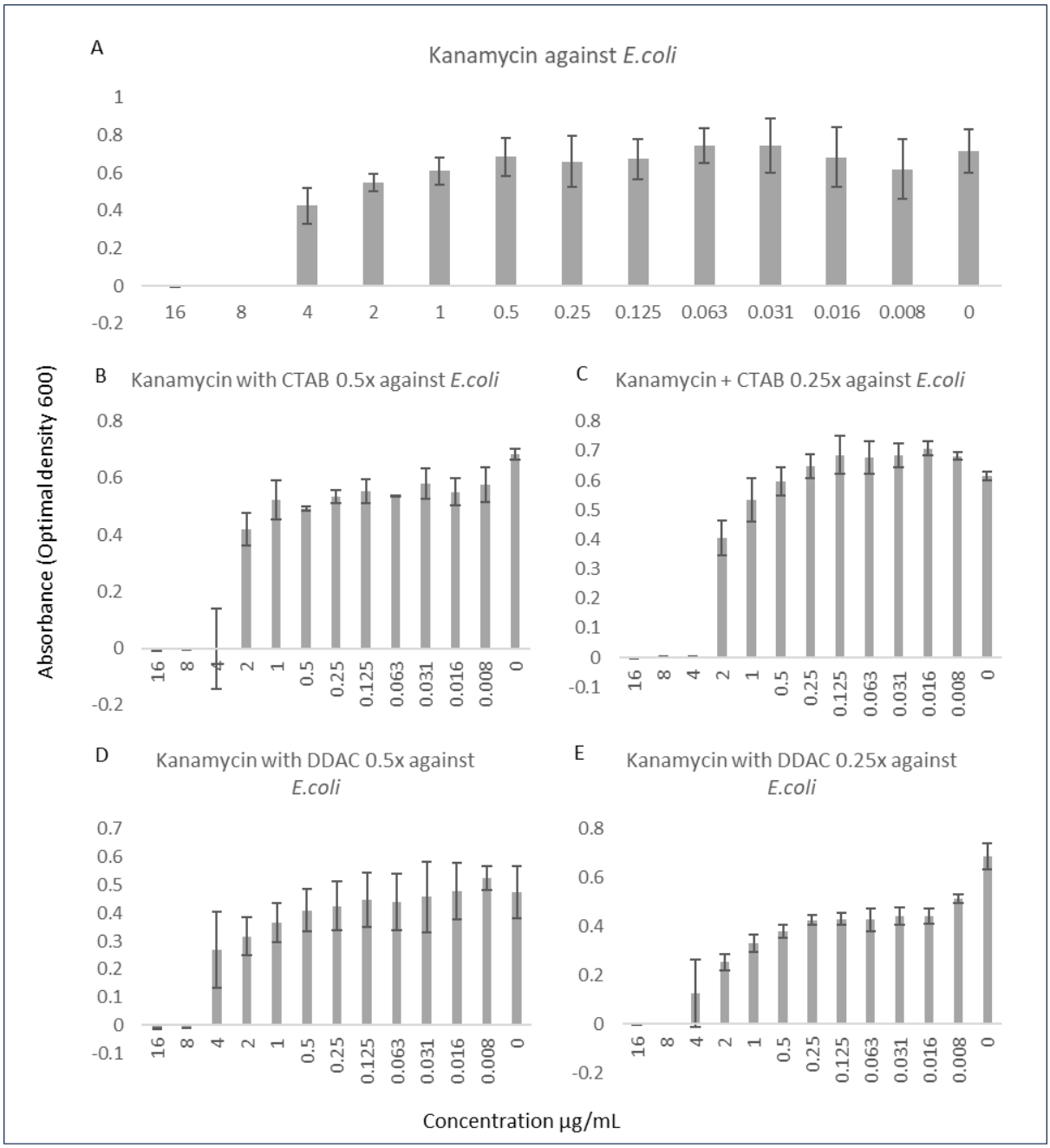


Figure 13: MIC of kanamycin against *E. coli* with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm (A) kanamycin by itself against *E. coli* (B) Combination of kanamycin and 0.5x CTAB MIC value (C) Combination of kanamycin and 0.25x CTAB MIC value (D) Combination of kanamycin and 0.5x DDAC MIC value (E) Combination of kanamycin and 0.25x DDAC MIC value

The MIC value of kanamycin by itself against *S. aureus* has been observed at concentration of 2µg/mL being the MIC, but the growth of the bacteria was visibly inhibited at a higher concentration of the antibiotic as demonstrated in **Figure 14A.** Moreover, the combination with 0.5x and 0.25x CTAB MIC value **(Figure 14B-C)** was observed having no significant difference of concentration of 2µg/mL. While the combination of 0.5x and 0.25x DDAC MIC value **(Figure 14D-E)** was observed having significant 2-fold reduction of 1µg/mL.

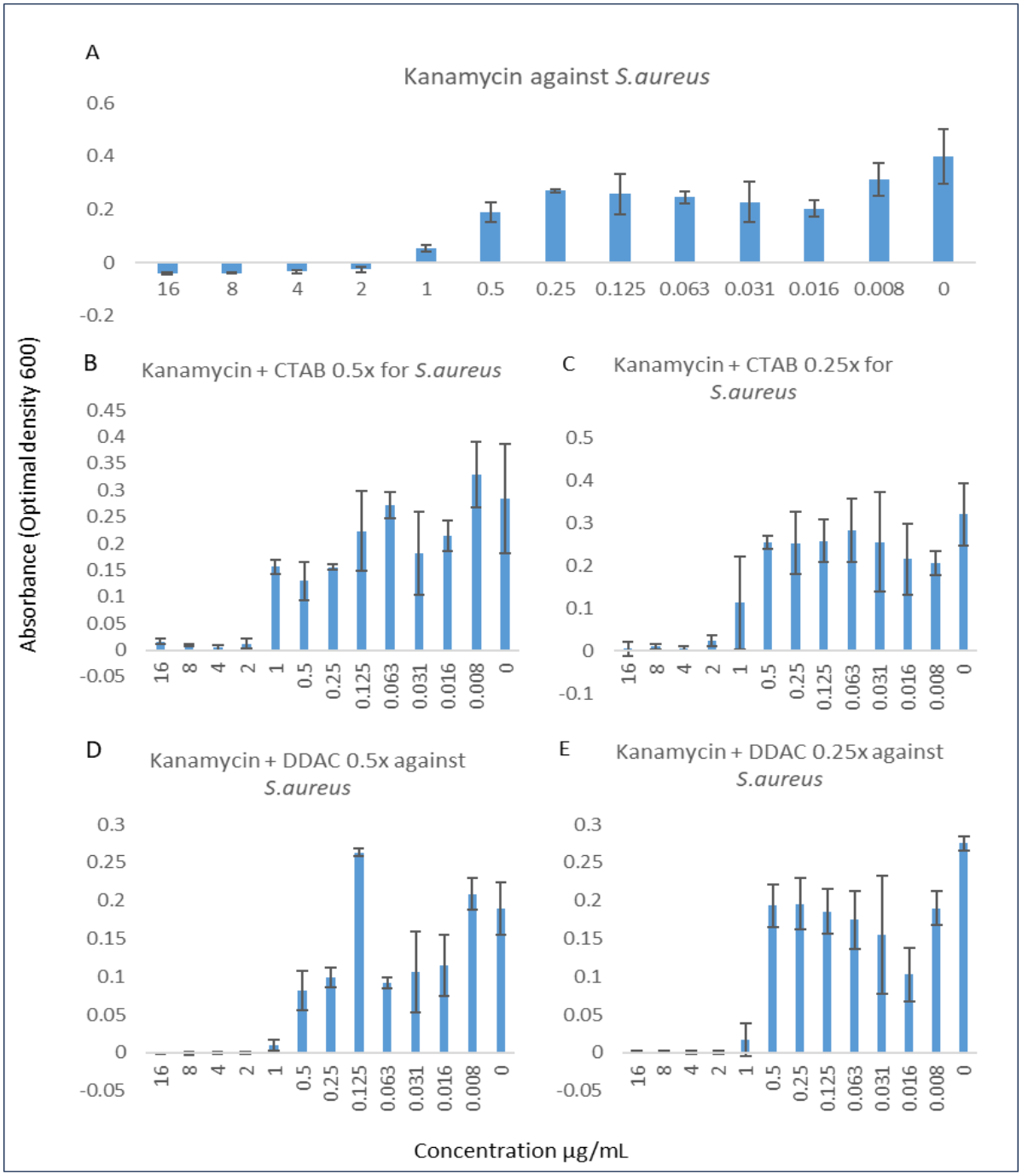


Figure 14: MIC of kanamycin against *S. aureus* with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm (A) kanamycin by itself against *S. aureus* (B) Combination of kanamycin and 0.5x CTAB MIC value (C) Combination of kanamycin and 0.25x CTAB MIC value (D) Combination of kanamycin and 0.5x DDAC MIC value (E) Combination of kanamycin and 0.25x DDAC MIC value

The MIC value of vancomycin by itself against *S. aureus* has been observed at concentration of 1µg/mL being the MIC, but the growth of the bacteria was visibly inhibited at a higher concentration of the antibiotic as demonstrated in **Figure 15A.** Moreover, the combination with 0.5x CTAB MIC value has shown a significant 2-fold reduction of 0.5µg/mL **(Figure 15B)** and with 0.25x CTAB MIC value have no significant difference of concentration of 1µg/mL **(Figure 15C)**. While the combination of 0.5x and 0.25x DDAC MIC value **(Figure 15D-E)** was observed having no significant difference of 1µg/mL.

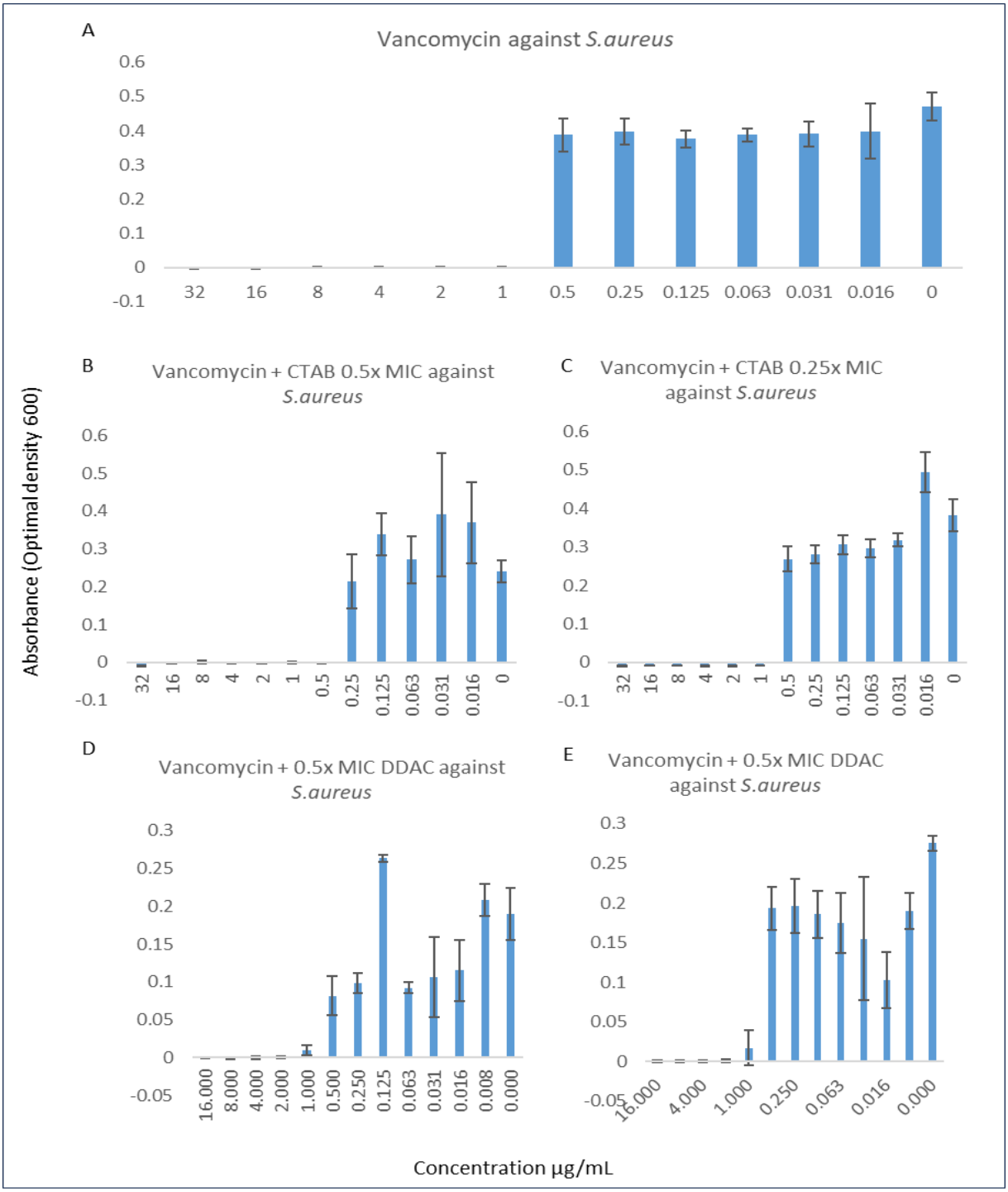
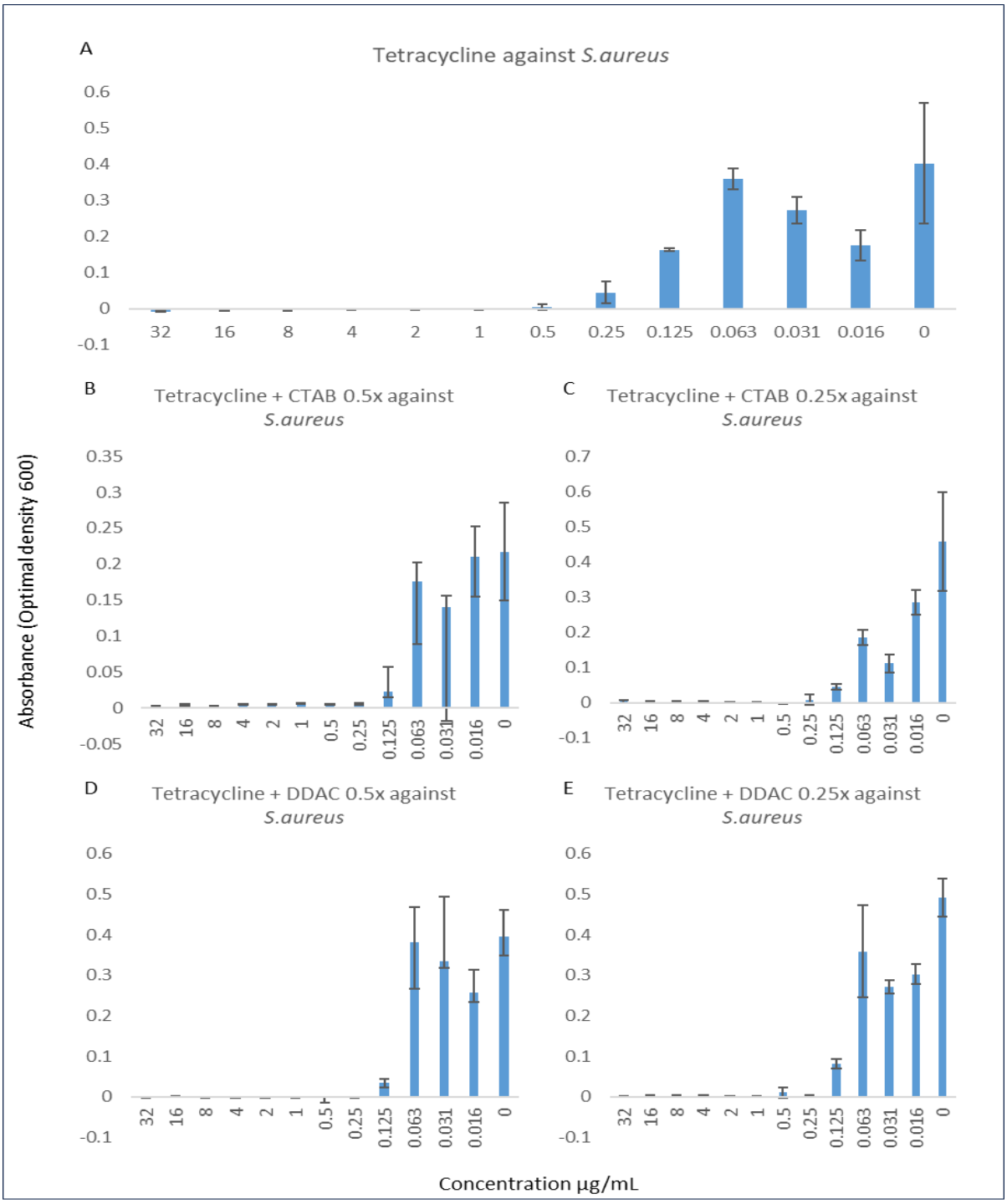


Figure 15: **MIC of vancomycin against *S. aureus* with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm** (A) vancomycin by itself against *S. aureus* (B) Combination of vancomycin and 0.5x CTAB MIC value (C) Combination of vancomycin and 0.25x CTAB MIC value (D) Combination of vancomycin and 0.5x DDAC MIC value (E) Combination of kanamycin and 0.25x DDAC MIC value



The MIC value of tetracycline by itself against *S. aureus* has been observed at concentration of 1µg/mL being the MIC as shown in **Figure 16A**, although the growth of the bacteria was considerably stopped at a higher concentration of the antibiotic. The combination with 0.5x and 0.25 CTAB MIC value has demonstrated a significant 2-fold reduction at concentration 0.25µg/mL (**Figure 16B-C**). The result with the combination of 0.5x and 0.25x MIC DDAC is the same with a 2-fold reduction of 0.25µg/mL (**Figure 16D-E**).

Figure 16: **MIC of tetracycline against *S. aureus* with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm** (A) tetracycline by itself against *S. aureus* (B) Combination of tetracycline and 0.5x CTAB MIC value (C) Combination of tetracycline and 0.25x CTAB MIC value (D) Combination of tetracycline and 0.5x DDAC MIC value (E) Combination of tetracycline and 0.25x DDAC MIC value

The MIC value of daptomycin by itself against *S. aureus* has been observed at concentration of 16µg/mL being the MIC as shown in **Figure 17A**, although the growth of the bacteria was considerably stopped at a higher concentration of the antibiotic. The combination with 0.5x and 0.25x CTAB MIC value has demonstrated a significant 2-fold reduction at concentration of 8µg/mL (**Figure 17B-C**). The result with the combination with 0.5x MIC DDAC is the same with a 2-fold reduction of 8µg/mL (**Figure 17D**), while the combination with 0.25x DDAC MIC did not have a significant difference as compared to daptomycin by itself (**Figure 17E**)

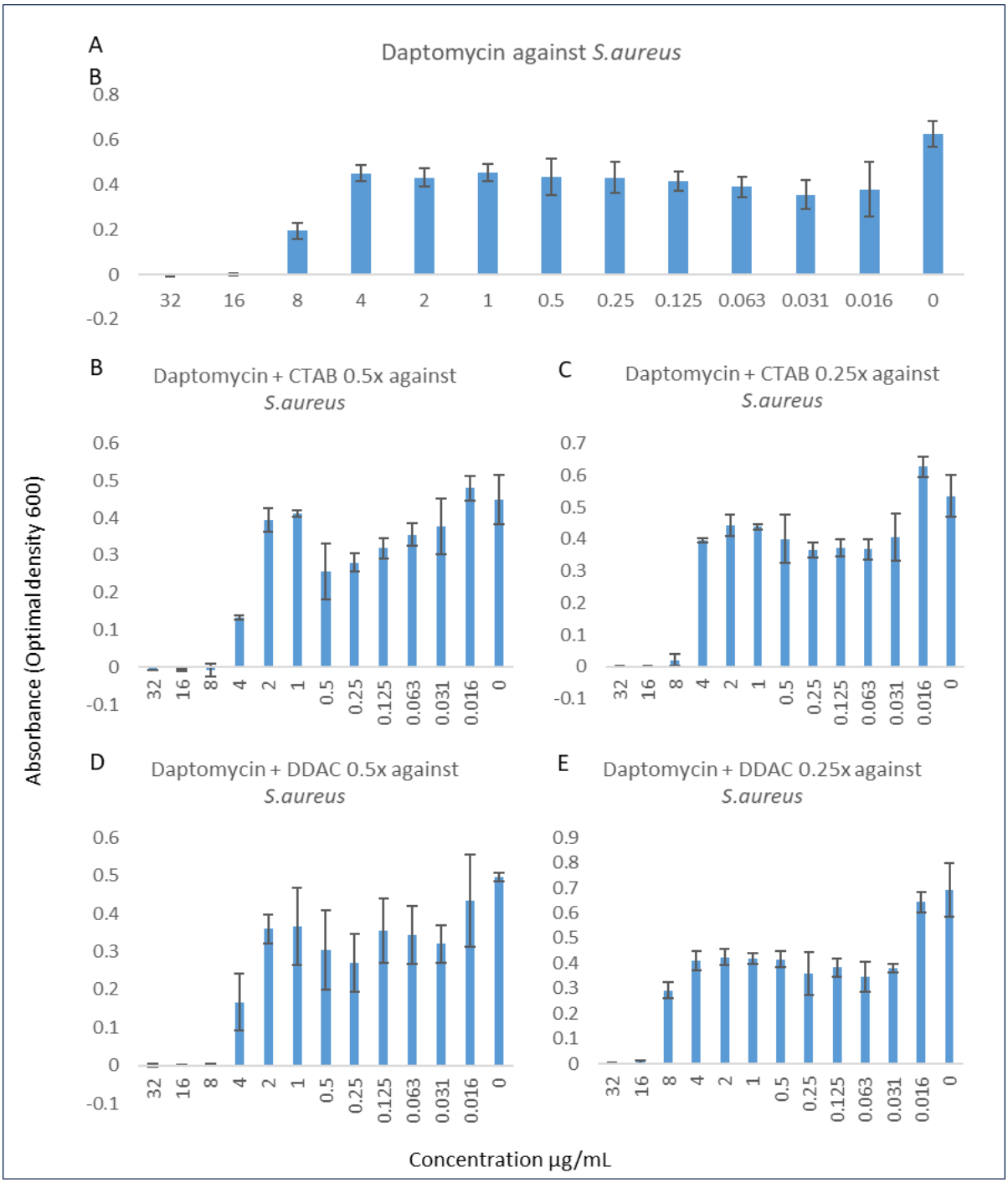


Figure 17: MIC of daptomycin against S. aureus with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm (A) Daptomycin by itself against S. aureus (B) Combination of daptomycin and 0.5x CTAB MIC value (C) Combination of daptomycin and 0.25x CTAB MIC value (D) Combination of daptomycin and 0.5x DDAC MIC value (E) Combination of daptomycin and 0.25x DDAC MIC value

The MIC value of colistin by itself against *S. aureus* has been observed at concentration of 32µg/mL being the MIC as shown in **Figure 18A**, although the growth of the bacteria has

considerably stopped at a higher concentration of the antibiotic. The combination with 0.5x, 0.25x CTAB and 0.25x DDAC MIC value has demonstrated no significant difference at concentration of 32µg/mL (**Figure 18B-C&E**). The result with the combination with 0.5x MIC DDAC has demonstrated a significant difference of 2-fold reduction having a concentration of 16µg/mL (**Figure 18D**).

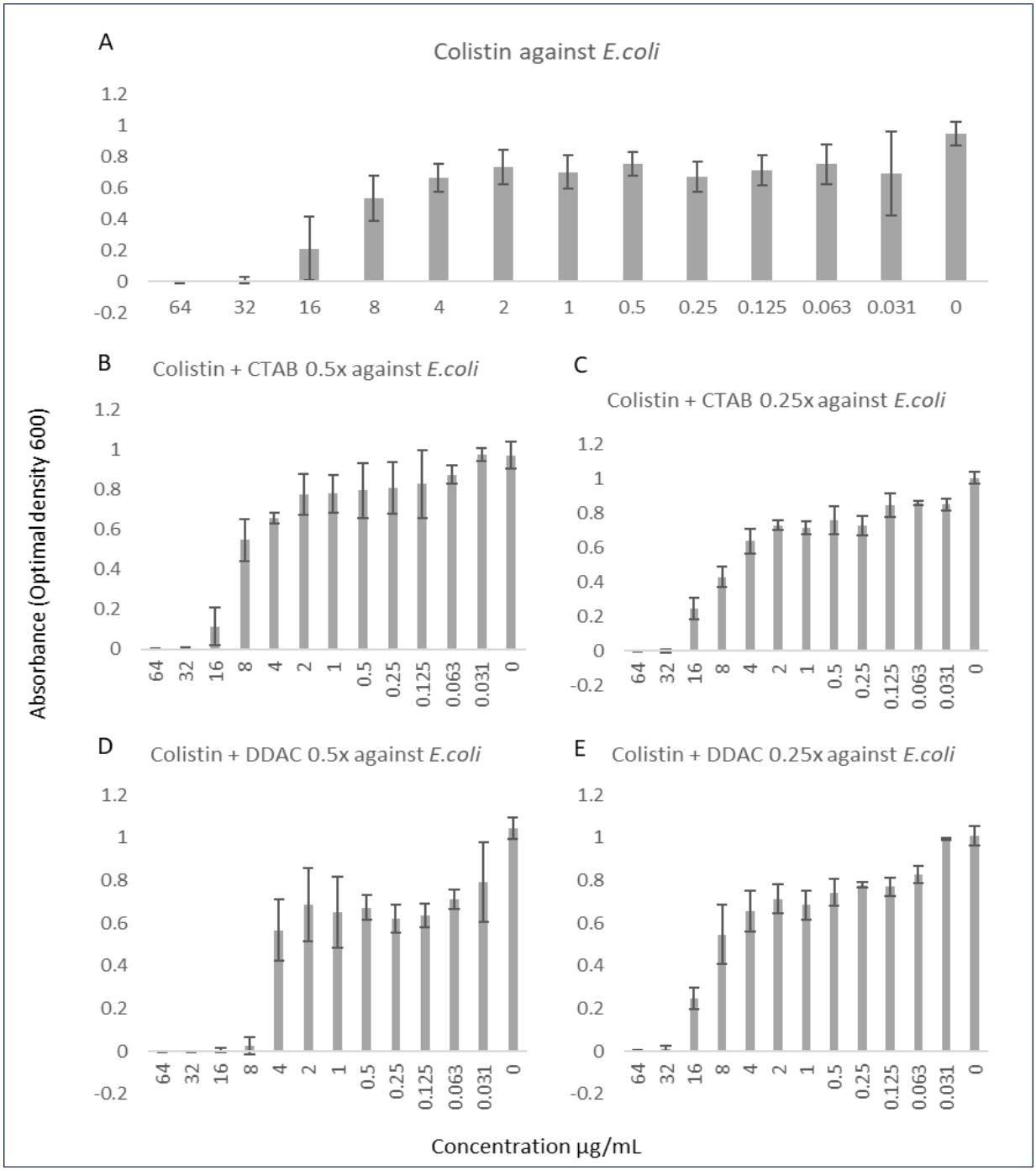


Figure 18: MIC of colistin against E. coli with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm (A) Colistin by itself against E. coli (B) Combination of colistin and 0.5x CTAB MIC value (C)

Combination of colistin and 0.25x CTAB MIC value (D) Combination of colistin and 0.5x DDAC MIC value (E) Combination of colistin and 0.25x DDAC MIC value

The MIC value of gentamicin by itself against *E. coli* has been observed at concentration of 1µg/mL being the MIC as shown in **Figure 19A**, although the growth of the bacteria was considerably stopped at a higher concentration of the antibiotic. The combination with 0.5x and 0.25x CTAB MIC value and 0.5x and 0.25x DDAC MIC has demonstrated no significant difference at concentration of 1µg/mL (**Figure 19B-E**).

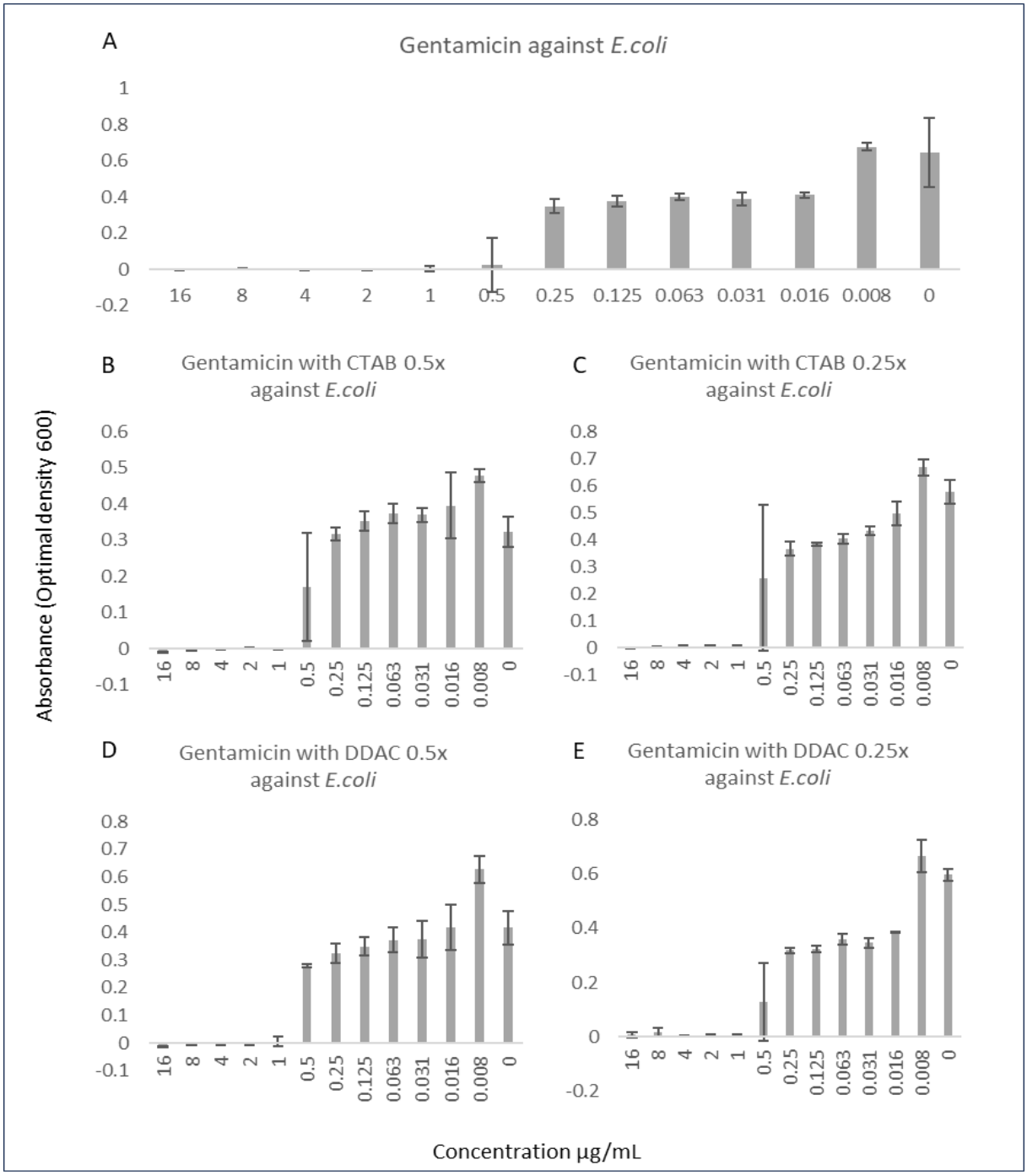


Figure 19: MIC of gentamicin against *E. coli* with 3 replicates, a positive control having just media and bacteria and a negative control with just media measured at 600 nm (A) Gentamicin by itself against *E. coli* (B) Combination of gentamicin and 0.5x CTAB MIC value (C) Combination of gentamicin and 0.25x CTAB MIC value (D) Combination of gentamicin and 0.5x DDAC MIC value (E) Combination of gentamicin and 0.25x DDAC MIC value

Cefazolin and vancomycin against *E. coli* **(Figure 20A&B)** and vancomycin against *Pseudomonas aeruginosa (P. aeruginosa)* **(Figure 20C)** have shown no evidence of complete inhibition of the bacteria. They were measured at OD 600 nm after the incubation period with different concentration of antibiotic.

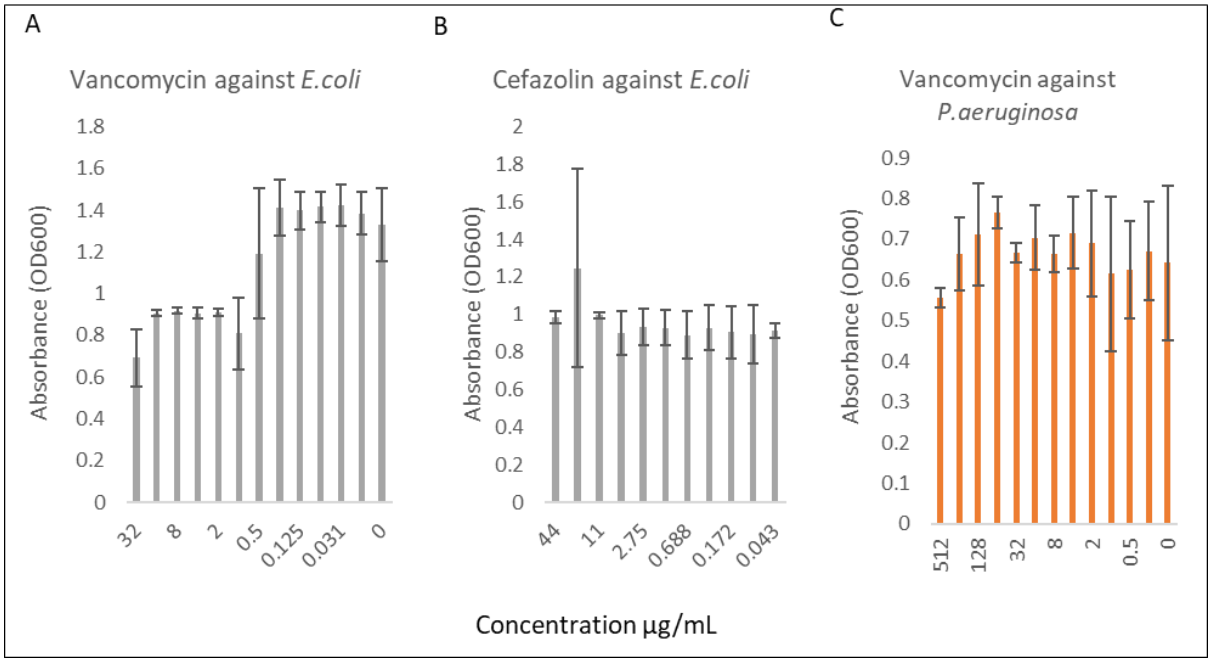


Figure 20: Antibiotics MIC assay against *E. coli* and *P.aeruginosa* in MHB having 3 replicates, a positive control having media and bacteria and a negative control of just media measured at OD 600 nm (A) cefazolin against *E. coli* (B) vancomycin against *E. coli* (C) vancomycin against *P. aeruginosa*

Table 3: Summary of MIC and MBC data from kanamycin, tetracycline, vancomycin, daptomycin and levofloxacin combined with CTAB against *S. aureus*

S. aureus			CTAB			
Antibiotics	MIC	MBC	0.5x MIC	MBC	0.25x MIC	MBC
Kanamycin	2	4	2	16	2	16
Tetracycline	0.5	16	0.125	> 0.5	0.125	> 0.5
Vancomycin	1	2	0.5	1	1	2
Daptomycin	16	32	8	8	8	8
Levofloxacin	0.0625	0.125	0.0625	0.125	0.0625	0.125
	QAC DOES change MIC of antibiotic			QAC does not change MIC of antibiotic		

Table 4: Summary of MIC and MBC data from kanamycin, tetracycline, vancomycin, daptomycin and levofloxacin combined with DDAC against *S. aureus*

S. aureus			DDAC			
Antibiotics	MIC	MBC	0.5x MIC	MBC	0.25x MIC	MBC
Kanamycin	2	4	1	4	1	4
Tetracycline	0.5	16	0.125	> 0.5	0.25	> 0.5
Vancomycin	1	2	1	2	1	2
Daptomycin	16	32	8	8	16	16
Levofloxacin	0.0625	0.125	0.0625	0.125	0.0625	0.125
	QAC DOES change MIC of antibiotic			QAC does not change MIC of antibiotic		

Table 5: Summary of MIC and MBC data from kanamycin, colistin, levofloxacin, cefazolin and gentamicin combined with CTAB against *E. coli*

E. coli			CTAB			
Antibiotics	MIC	MBC	0.5x MIC	MBC	0.25x MIC	MBC
Kanamycin	8	8	4	8	4	8
Colistin	32	32	32	32	32	32
Levofloxacin	0.0625	≅0.03125	<0.03125	≅0.03125	<0.03125	≅0.03125
Cefazolin	Not determined		Not determined		Not determined	
Gentamicin	0.5	1	1	1	1	1
	QAC DOES change MIC of antibiotic			QAC does not change MIC of antibiotic		QAC reduce the effect of antibiotic

Table 6: Summary of MIC and MBC data from kanamycin, colistin, levofloxacin, cefazolin and gentamicin combined with DDAC against *E. coli*

E. coli			DDAC			
Antibiotics	MIC	MBC	0.5x MIC	MBC	0.25x MIC	MBC
Kanamycin	8	8	8	8	8	8
Colistin	32	32	8	8	32	32
Levofloxacin	0.0625	≅0.03125	<0.03125	≅0.03125	0.015	<0.03125
Cefazolin	Not determined		Not determined		Not determined	
Gentamicin	0.5	1	1	1	1	1

	QAC DOES change MIC of antibiotic		QAC does not change MIC of antibiotic		QAC reduce the effect of antibiotic

3.2 Time-kill assay of kanamycin and tetracycline *E. coli* and *S. aureus* respectively

The time kill assay demonstrated that the combination of kanamycin and CTAB exhibited a synergistic effect against *E. coli*. The graph **Figure 21** evidently shown a rapid and sustained reduction in viable cell counts as compared to kanamycin by itself. In the control (MHB) and CTAB by itself, bacterial number continued to increase steadily over 24 hours reaching approximately 1 x 10^{10} cfu/mL.

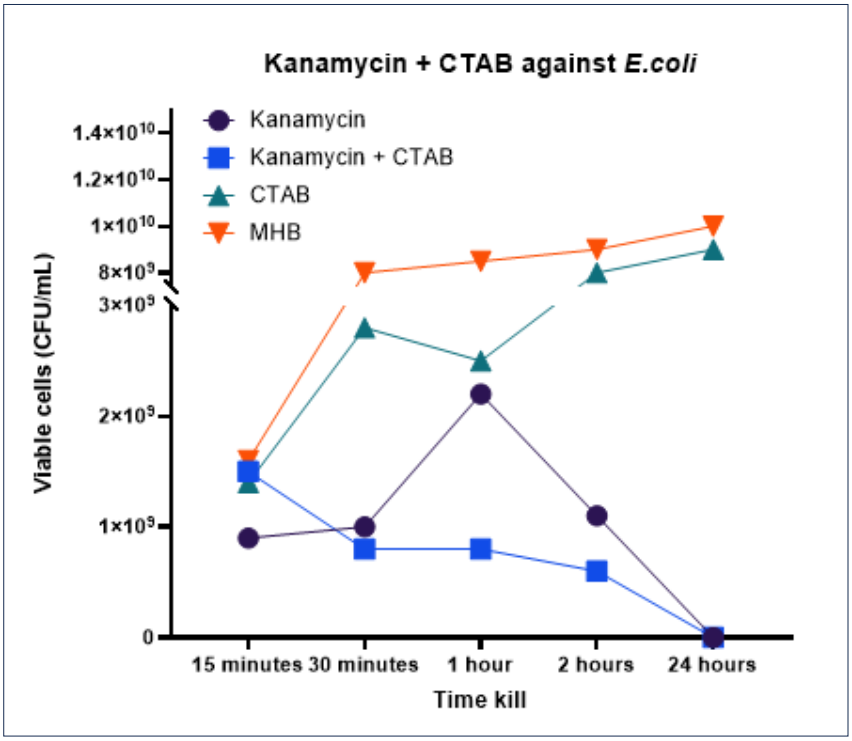


Figure 21: Time kill kinetics plot of the comparison of kanamycin by itself and in combination with CTAB against *E. coli* in a span of 15 minutes, 30 minutes, 1 hour, 2 hours and 24 hours with positive control having just CTAB with bacteria and negative control of media only

The time kill assay demonstrated that the combination of tetracycline and CTAB exhibited a synergistic effect against *S. aureus*. The graph **Figure 22** evidently shown a rapid and sustained reduction in viable cell counts as compared to tetracycline by itself. In the control (MHB) and CTAB by itself, bacterial number continued to increase steadily over 24 hours reaching approximately 1.1 x 10^{10} cfu/mL.

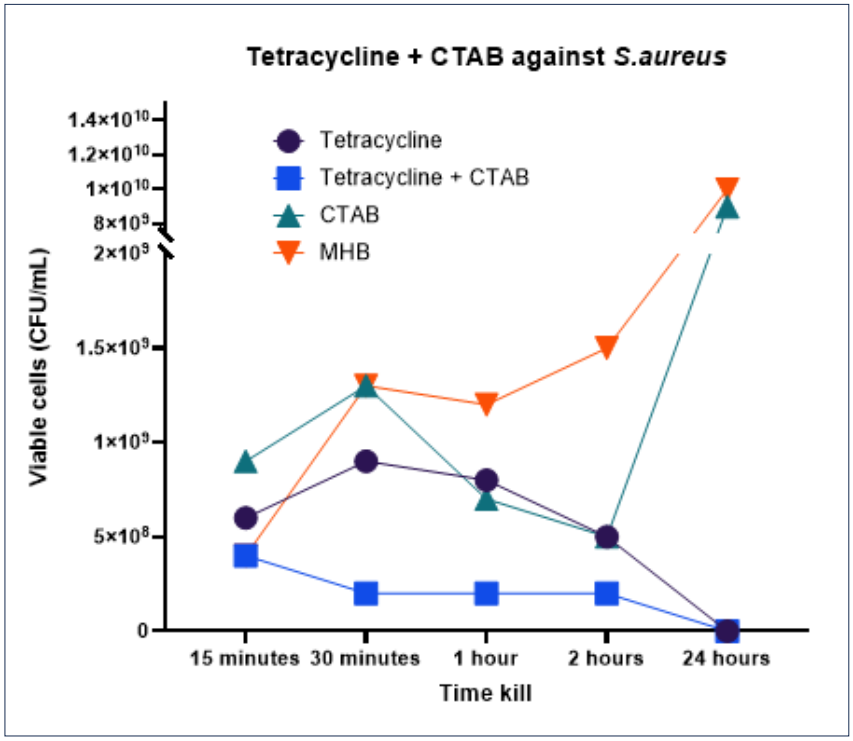


Figure 22: Time kill kinetics plot of the comparison of tetracycline by itself and in combination with CTAB against *S. aureus* in a span of 15 minutes, 30 minutes, 1 hour, 2 hours and 24 hours with positive control having just CTAB with bacteria and negative control of media only.

3.3 Synergy checkerboard assay of kanamycin and CTAB against *E. coli* and tetracycline and CTAB against *S. aureus*

Kanamycin combined with CTAB against *E. coli* exhibited a 2-fold reduction as compared to kanamycin by itself. The result of this checkerboard here **(Figure 23)** confirms that the MIC of CTAB and kanamycin against *E. coli* is 16µg/mL and 8µg/mL respectively, while the combination of kanamycin with CTAB and CTAB with kanamycin resulted at concentration 0.125µg/mL and 1µg/mL respectively. Using the formula provided in section

2.5, the combination resulted at a FICI value of 0.078125 which means synergistic or improved antimicrobial activity as described in **Table 1**.

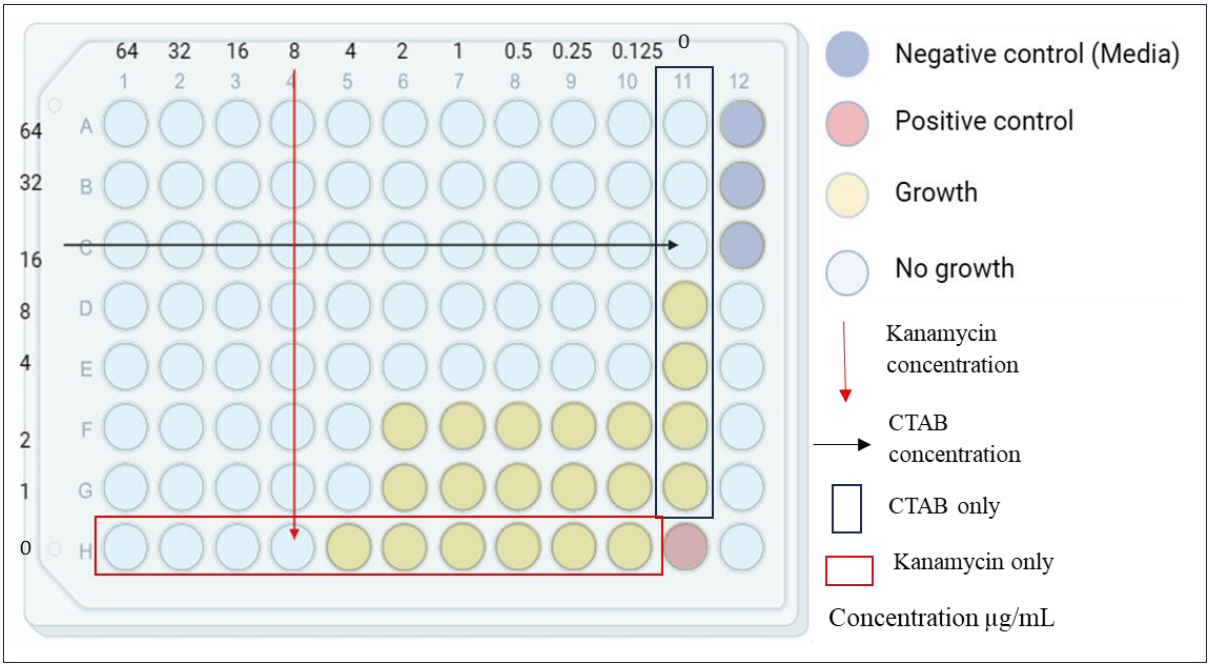


Figure 23: Checkerboard assay result for kanamycin and CTAB against *E. coli*. Row H with a red box being kanamycin by itself or referred to as *Compound A*, to confirm its MIC against *E. coli*, while Column 11 with black box is CTAB by itself or referred to as *Compound B* to confirm its MIC against *E. coli*. The dark blue colour represents the negative control having just media, red represents the positive control, yellow indicates growth and light blue indicates no growth. The numbers with a horizontal red arrow is the concentration of compound A, while the numbers with a vertical black arrow is the concentration of compound B.

Tetracycline combined with CTAB against *S. aureus* exhibited a 2-fold reduction as compared to tetracycline by itself. The result of this checkerboard here **(Figure 24)** confirms that the MIC of CTAB and tetracycline against *S. aureus* is 1µg/mL and 0.5µg/mL respectively, while the combination of tetracycline with CTAB and CTAB with tetracycline resulted at concentration 0.062µg/mL and 0.125µg/mL respectively. Using the formula provided in section 2.5, the combination resulted at FICI value of 0.375 which means synergistic or improved antimicrobial activity as described in **Table 1**.

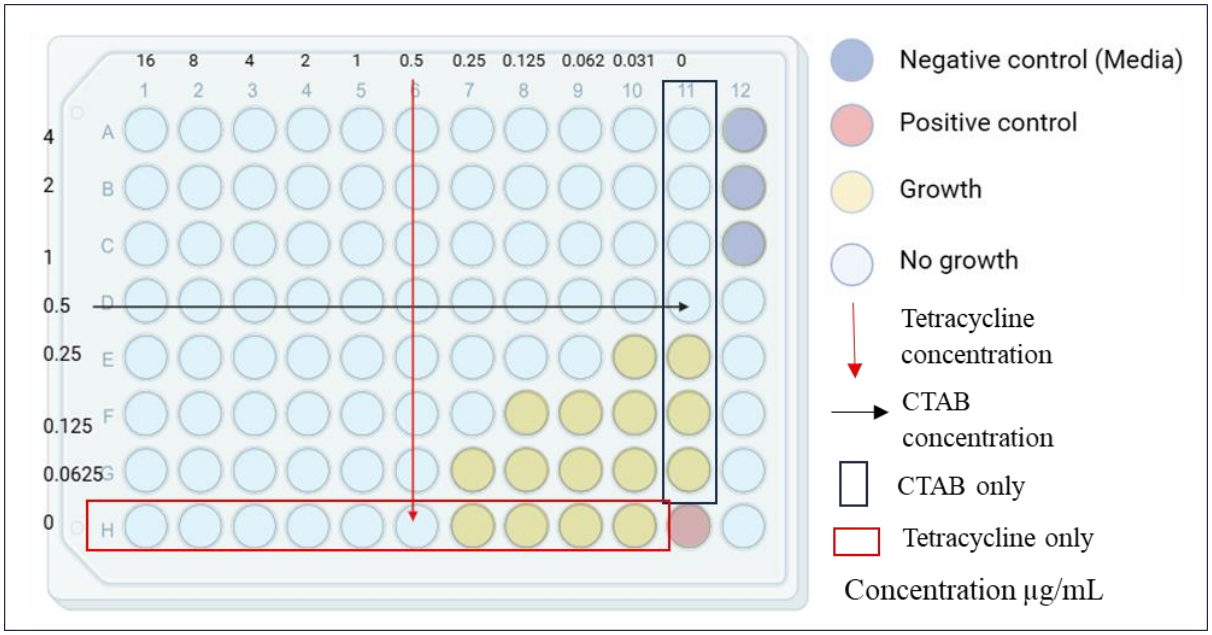


Figure 24: Checkerboard assay result for tetracycline and CTAB against *S. aureus*. Row H with a red box being tetracycline by itself or referred to as *Compound A*, to confirm its MIC against *S. aureus*, while Column 11 with black box is CTAB by itself or referred to as *Compound B* to confirm its MIC against *S. aureus*. The dark blue colour represents the negative control having just media, red represents the positive control, yellow indicates growth and light blue indicates no growth. The number with a horizontal red arrow is the concentration of compound A, while the numbers with a vertical black arrow is the concentration of compound B.

3.4 Flow cytometric analysis of *S. aureus* and *E. coli* before and after QAC treatment

The permeability of the membrane of *E. coli* cells with the CTAB and DDAC treatment was assessed using propidium iodine (PI) staining via flow cytometry. PI penetrates and attached to the DNA cells of the permeabilized membrane emitting fluorescence and was excluded by the intact viable cell membranes. The unstained *E. coli* **(Figure 25A)** has shown a minimal PI uptake of 1.94% permeable cells. Upon staining with PI by itself, the percentage of the permeable cells elevated to 11.38% **(Figure 25B)**, that could be interpreted as it is more likely a portion of cells that are in the process of division, because they must remodel their cell envelope and therefore may be more prone to up taking PI in their environment. CTAB treatment at different concentration (1x, 0.5x, 0.25x MIC values) resulted in 95.55%, 97.70% and 94.35% **(Figure 25CDE)** permeable cells respectively. While DDAC treatments at

different concentrations (1x, 0.5x, 0.25x MIC values) resulted in 79.89%, 45.80% and 16.44% respectively **(Figure 25FGH)**.

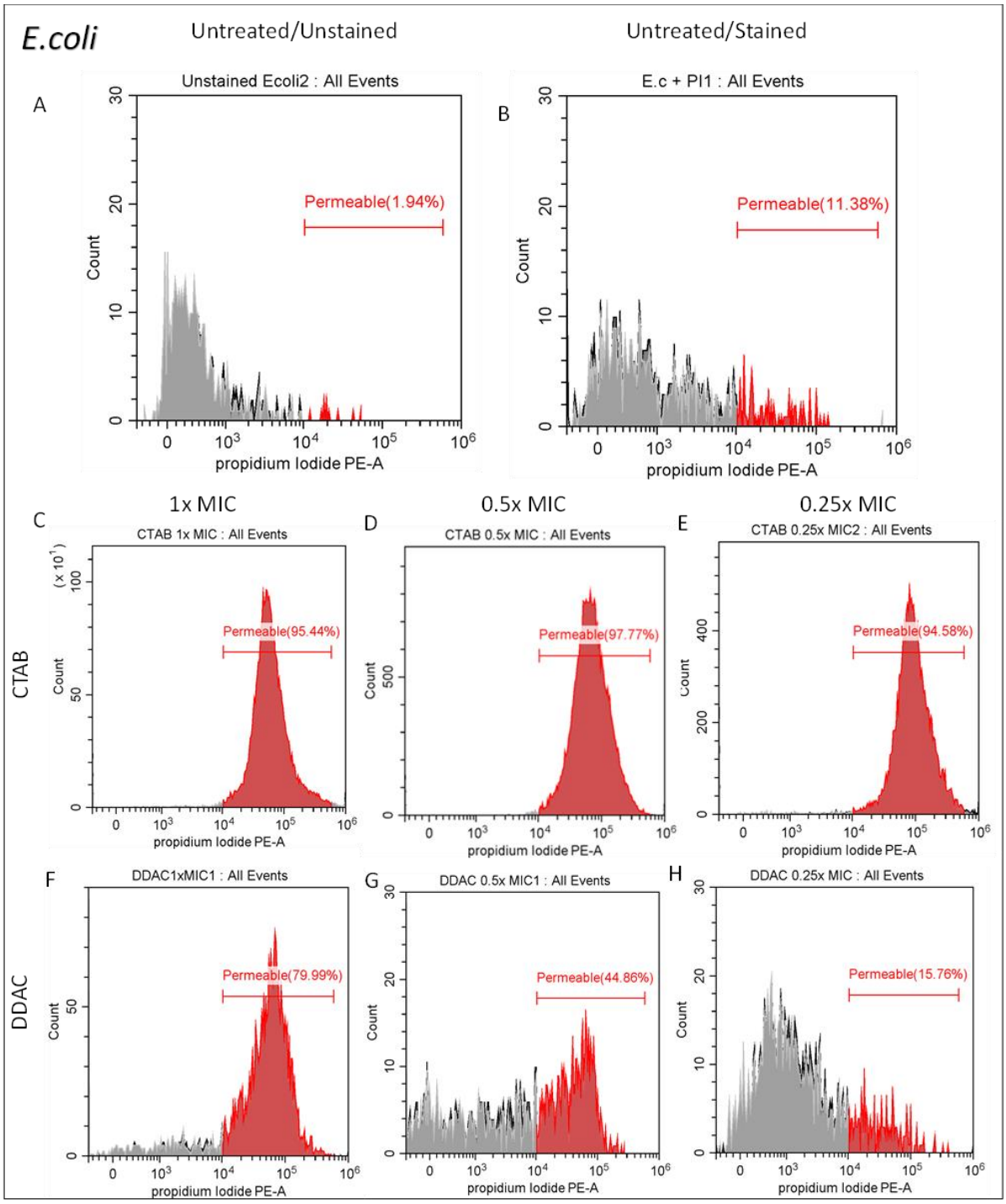


Figure 25: Flow cytometric plots representing how the individual population looks like after the treatments of *E. coli* permeability treated with CTAB and DDAC (A) Plot of *E. coli* untreated & unstained (B) *E. coli* untreated & stained with PI (C) *E. coli* after 1x CTAB MIC value treatment (D)) *E. coli* after 0.5x CTAB MIC value treatment (E) *E. coli* after 0.25x CTAB

MIC value treatment (F) *E. coli* after 1x DDAC MIC value treatment (G) *E. coli* after 0.5x DDAC MIC value treatment (H) *E. coli* after 0.25x DDAC MIC value treatment.

The PI percentage of *E. coli* was determined after the exposure of the treatments (DDAC and CTAB at 1x, 0.5x and 0.25x of its MIC value). As shown in **Figure 26,** untreated control and PI only resulted with a slight permeabilization having approximately 2% and 10% respectively. DDAC at 1x MIC value which is 4µg/mL significantly elevated the membrane permeability as compared to the PI-only stained control ($P < 0.00001$) with an average of approximately 80%. Moreover, an evident concentration-dependent decrease in permeabilization was observed at half (0.5x) with approximately 45% permeability while there is no significant difference at 0.25x concentration as compared to PI-only, which also means DDAC 0.25x did nothing towards permeabilization. On the other hand, all the concentration of CTAB (1x, 0.5x and 0.25x) have shown an extremely elevated membrane permeabilization at approximately 94%, with no significant differences between each concentration. These findings conclude that permeabilization of CTAB subinhibitory concentration was significantly higher than that of DDAC).

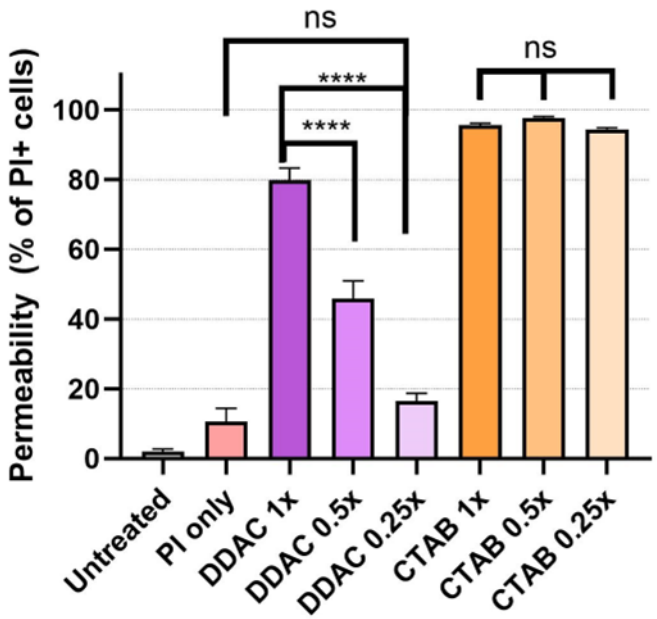


Figure 26: Permeabilization of *E. coli* following subinhibitory QAC treatment where "ns" is equivalent to not significant and "*" indicates significance (more *= more significant).

PI penetrates and attached to the DNA cells of the permeabilized membrane emitting fluorescence and was excluded by the intact viable cell membranes. The unstained *S. aureus* **(Figure 27A)** has shown a minimal PI uptake of 1.35% permeable cells. Upon staining with PI

by itself, the percentage of the permeable cells elevated to 10.74% **(Figure 27B)**, that could be interpreted as it is more likely a portion of cells that are in the process of division, because they must remodel their cell envelope and therefore may be more prone to up taking PI in their environment. CTAB treatment at different concentration (1x, 0.5x, 0.25x MIC values) resulted in 5.81%, 7.81% and 8.30% **(Figure 27CDE)** permeable cells respectively. While DDAC treatments at different concentrations (1x, 0.5x, 0.25x MIC values) resulted in 30.77%, 24.75% and 12.87% respectively **(Figure 27FGH)**.

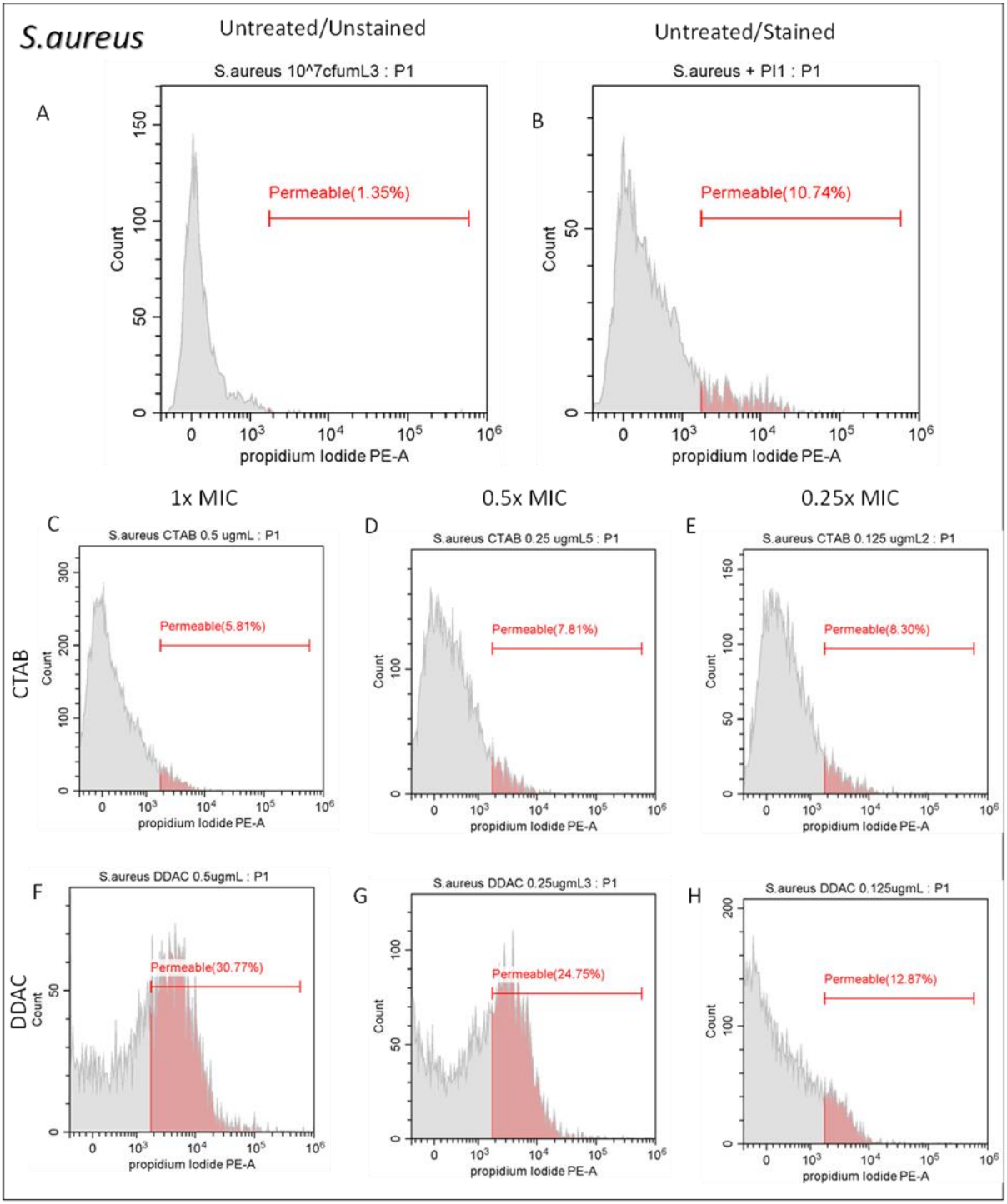


Figure 27: Flow cytometric analysis of *S. aureus* permeability treated with CTAB and DDAC (A) Plot of *S. aureus* untreated & unstained (B) S. aureus untreated & stained with PI (C) *S. aureus* after 1x CTAB MIC value treatment (D)) *S. aureus* after 0.5x CTAB MIC value treatment (E) *S. aureus* after 0.25x CTAB MIC value treatment (F) *S. aureus* after 1x DDAC MIC value treatment (G) *S. aureus* after 0.5x DDAC MIC value treatment (H) *S. aureus* after 0.25x DDAC MIC value treatment.

As shown in **Figure 28,** untreated control and PI only resulted with a slight permeabilization having approximately 1.5% and 10% respectively. DDAC at 1x MIC value resulted at approximately 39% permeability and again, showed a dose-dependent effect, similar to the *E. coli* plot, however, the permeabilization is much lower, ranging from ~30% to ~15%. Again, DDAC 0.25x does not significantly improve permeability versus PI only control. Moreover, CTAB for the second time showed no dose-dependent nature in the measured range of 0.25x to 1x MIC, and even at 1x MIC CTAB did not improve permeability versus the PI only control, this implies that for any antibiotic with improved activity with CTAB, permeabilization is absolutely not the cause. As a general observation, DDAC is more effective at permeabilization compared to CTAB against *S. aureus* specifically. This is the reverse of the trend for *E. coli*, where CTAB was much greater at permeabilization than DDAC.

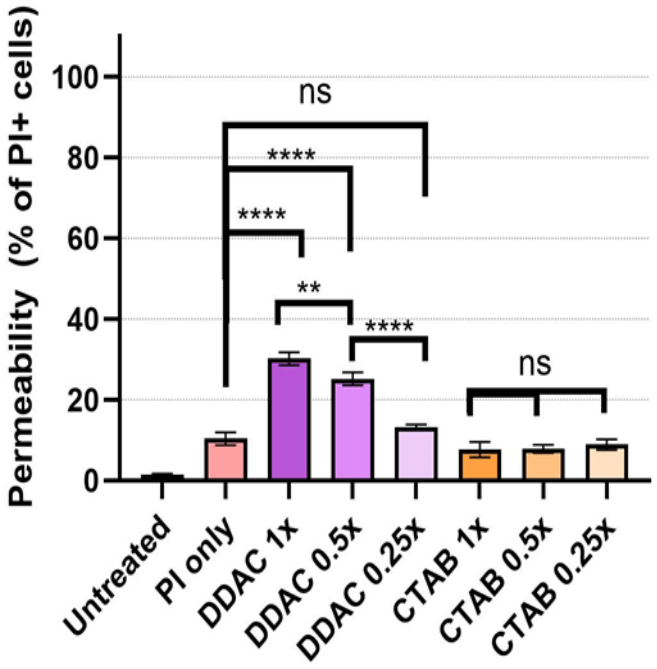


Figure 28: Permeabilization of *S. aureus* following subinhibitory QAC treatment where "ns" is equivalent to not significant and "*" indicates significance (more *= more significant).

CHAPTER 4: DISCUSSION

4.1 Overview

The purpose of this study is to assess whether a subinhibitory concentration of quaternary ammonium compounds (QAC) particularly cetyltrimethylammonium bromide (CTAB) and didecyldimethylammonium chloride (DDAC), can potentiate the activity of traditional antibiotics against Gram-negative bacteria S. aureus and Gram-negative bacteria E. coli. The research utilized minimum inhibitory concentration (MIC) assay, time kill kinetics assay, checkerboard synergy assay, and membrane permeability determination via flow cytometry to show that QACs can increase antimicrobial activity of some traditional antibiotics.

4.2 Potentiation of Antibiotic activity by QACs against *S. aureus*

The results of antibiotics and QAC MIC assays provided evidence of significant potentiation of several antibiotics when combined with QACs subinhibitory concentration against *S. aureus*. Particularly for CTAB, a significant 2-fold reduction in the MIC was seen for tetracycline which had an MIC of 0.5µg/mL when used alone, or 0.25µg/mL when combined with CTAB (at 0.25 and 0.5X MIC) **(Figure 17).** This agrees with previous studies by Farrag and colleagues indicating that membrane permeabilizers can allow for increase uptake into bacteria of ribosome-targeting drugs such as tetracycline through their promoted dispersion through bacterial cell membranes (Farrag et al., 2019). Moreover, a decrease in MIC value has also been observed with vancomycin, an antibiotic specifically active on peptidoglycan synthesis, resulted a notable 2-fold reduction in MIC with a concentration of 1µg/mL to 0.5µg/mL after combining with 0.5x and 0.25x CTAB MIC value **(Figure 16)**. Hence, this is interesting knowing *S. aureus* doesn't have an outer membrane that has to be bypassed and vancomycin mode of action which inhibits synthesis of peptidoglycan suggests that the increase in antimicrobial activity caused by CTAB is not because of membrane permeabilization but rather other things like amplified levels of oxidative stress or the level of reactive oxidative species (ROS) inside the bacteria has elevated above normal stress levels (Song et al., 2018). Furthermore, vancomycin has been shown to decrease the expression of *katA*, a gene which codes for catalase. Catalase is an enzyme that protects *S. aureus* from oxidative stress, by breaking down hydrogen peroxide (Wang et al., 2015). Thus, the effects of any increased in ROS generation caused by CTAB would likely be enhanced by vancomycin due to its ability to prevent *S. aureus* from defending itself against oxidative stress. In line with

this, a relevant report also showed the potentiation of vancomycin using palmitoleic acid (PA). related studies also potentiate vancomycin using palmitoleic acid (PA) (Sidders et al., 2023). The structure of PA is very similar to a QAC's structure, both having long alkyl chain and an ionic head.

Furthermore, the lipopeptide antibiotic daptomycin which has previously been established for its membrane disruption effect (Huang, 2020) also has demonstrated a 2-fold reduction in MIC with concentration from 16µg/mL to 8µg/mL with CTAB 0.5x and 0.25x MIC **(Figure 18).** This supports a synergy between different membrane active compounds has been shown in a study by Lambert, which reports a synergy between various QACs and ethylenediaminetetraacetic acid (EDTA) (Lambert et al., 2004). On the other hand, kanamycin and levofloxacin combined with CTAB did not result a significant decrease or change in their MIC values against *S. aureus* **(Table 3).**

Moving forward with DDAC, similar decrease in MIC after the combination results have been observed. Tetracycline in combination with DDAC at 0.5x MIC resulted to 4-fold reduction from 0.5µg/mL to 0.125µg/mL and 2-fold reduction at 0.25x DDAC MIC **(Figure 17).** This aligns from the QAC's role in enhancing the protein synthesis inhibitors effectiveness (Crncevic et al., 2025). Also, the MIC of kanamycin exhibited a 2-fold reduction with a concentration of 2µg/mL to 1µg/mL, when combined with DDAC 0.5x and 0.25x MIC value **(Figure 14),** this indicates the potential of DDAC to sensitize *S. aureus* for aminoglycoside antibiotics (Garza-Cervantes et al., 2020). The decrease in MIC could be because of structural variations among the QACs; that DDAC with two alkyl chains should facilitate more extensive membrane disruption than single-chain CTAB (Tezel & Pavlostathis, 2015). In accordance with observations by Baudrion and team that multiple or longer hydrophobic chains increase membrane perturbation (Baudrion et al., 2000). With that, the results suggest that increase permeability is not the driving factor for improved activity of kanamycin against *S. aureus.* In line with this, a 2-fold reduction in MIC value has also been observed with daptomycin from 16µg/mL to 8µg/mL after combining with DDAC 0.5x MIC **(Figure 18)** affirming further synergistic interactions. However, vancomycin and levofloxacin did not exhibit a significant changes in their MIC upon combination with DDAC **(Table 4).**

To accurately describe the nature of observed potentiation via MIC assay, synergy checkerboard assays was done and factional inhibitory concentration index (FICI) values has been calculated using the formula provided in section 2.5. The FICI provides a quantitative

evaluation of the interaction between two antimicrobial agents as synergistic, which indicates improved antimicrobial activity, additive, which means slight improvement but not true synergy, indifferent, and antagonistic which decrease the antimicrobial activity **(Table 1).** Specifically, for *S. aureus* tetracycline in combination with CTAB was tested using the checkerboard assay. The combination has shown an FICI value of 0.375 **(Figure 25),** based on the FICI interpretation scale **(Table 1)** an FICI value of ≤ 0.5 indicates a synergistic interaction, which also interpreted as a pronounced increased in antimicrobial activity (Pharma, 2023). This matters as this kind of synergistic effect is highly attractive in clinical applications because it has multiple benefits. To begin with, such synergistic interactions can result in lower antibiotic effective doses, subsequently decreasing potential toxicity and limiting adverse side effect hazards for the patient, also because synergistic pairs attack bacteria along various pathways at a time, it confers a reduction in antibiotic-resistant strain development (Martins da Silva Filho et al., 2023).

Flow cytometry analysis using propidium iodine (PI) was used in order acquire crucial information relative to the mechanism of QAC for potentiation by measuring the membrane permeability of the bacteria. PI is a fluorochrome dye that is normally excluded from intact cell membranes but penetrates to the cells whose membranes are disrupted, then binding to the DNA and fluorescing (Adan et al., 2017; Ma et al., 2020). This was use to directly measure the integrity of the cell population after the treating with CTAB and DDAC. The *S. aureus* outcome showed a highly significant result to CTAB. As a general observation of the influence of these two different QACs on membrane permeability, we observed that DDAC acts in a dose-dependent manner (with permeability percentage decreasing as a function of DDAC concentration), while CTAB did not display such a trend. When *S. aureus* treated with CTAB 1x MIC, 0.5X MIC and 0.25X MIC, the permeable cell population was not significantly different from the untreated 'PI-only' **(Figure 29).** However, CTAB clearly showed significant potentiating activity in combination with tetracycline, vancomycin and daptomycin against *S. aureus.* With regards to the increase in activity of tetracycline, the effect is clearly not generated by an increase in cell permeability (as evidenced by the PI uptake results). This is strong evidence that CTAB exerts an influence, other than cell permeabilization, on the *S. aureus* population that acts to promote antibiotic activity. This interpretation is further supported by the increase in activity of vancomycin, which acts on the peptidoglycan layer, and does not need to pass through a membrane layer to be active. A plausible candidate for the mechanism

of antibiotic potentiation is the induced generation of reactive oxygen species elicited by (Wang et al., 2020).

Furthermore, time-kill kinetics confirmed the enhance antimicrobial activity of tetracycline with CTAB. Expectedly, tetracycline alone due to its bactericidal properties kill the bacteria after 24, but as seen in **Figure 23** at 2 hours point of time, it shows that growth of *S. aureus* with combination of CTAB and tetracycline with CTAB alone, has slowed down resulted to approximately $2x10^8$ cfu/mL as compared to tetracycline alone with $5x10^8$ cfu/mL. It is also evident that the bacteria continued growing after 24-hours with bacteria having CTAB alone, while the bacteria were eliminated with tetracycline + CTAB after 24 hours **(Figure 23)**. However, we did not conclude that the combination of tetracycline and CTAB actually increases the rate of killing because the time point between 2-24 hours has not been investigated, so further and deep investigation of the potentiation activity is suggested using time kill kinetics.

This study clearly showed how subinhibitory concentration of CTAB and DDAC could possibly enhance the activity of antibiotics against *S. aureus*. A synergistic interaction between tetracycline and CTAB was confirmed using synergy checkerboard assay, flow cytometry showed the increased percentage of the membrane permeability after the QAC treatments, and time kill confirmed the enhance antibiotic activity in combination with QAC.

4.3 Potentiation of Antibiotic activity by QACs against *E. coli*

The potentiating action of the QACs was most notable against *E. coli*, a Gram-negative bacteria naturally more resistant to antimicrobial agents because of its extra outer membrane. Particularly, CTAB demonstrated a significantly enhanced activity for both kanamycin and levofloxacin with a 2-fold decreased MIC value of 4µg/mL from 8µg/mL and 0.031µg/mL from 0.062µg/mL respectively. Since *E. coli's* natural resistance by outer membrane impermeability as well as by active efflux (Chan et al., 2021) was already in place, these findings highlight the effectiveness of membrane-disruptive agents such as CTAB in increasing antibiotic accumulation inside cells, and increased permeability should have allowed greater uptake of antibiotics, lowering the MIC in a manner (MacNair & Brown, 2020). While DDAC 0.25x did not change the antibiotic activity, 0.5x MIC had potentiated the activity of colistin having a 4-fold reduction in MIC value after the combination with concentration from 32µg/mL to 8µg/mL, affirming membrane-targeting antibiotic-membrane-disrupting adjuvant synergy. The pattern of such synergy is also supported by related literature showing that membrane

permeabilizers improve colistin's binding to lipopolysaccharides (Jennings et al., 2015). Interestingly, when either QAC was combined with gentamicin, its activity was decreased, as evidence in an increase in MIC **(Figure 20)**, a possible reason for this observation could be because gentamicin and QACs competes for binding sites on bacterial membrane based on charged, disrupting a proton motive force needing for aminoglycoside uptake or by binding within micellar structure (Hancock, 1984). On the other hand, vancomycin plus QAC did not sensitize *E. coli*, a Gram-negative bacteria, therefore rejecting the hypothesis b.

Quantitative interaction between kanamycin and *E. coli* was measured by synergistic checkerboard assay. Specifically, kanamycin was combined with CTAB, resulting in a FICI value of 0.078125 **(Figure 24)** which is well within the range determined to indicate synergistic interaction. Moreover, flow cytometry revealed that CTAB induced 95% membrane permeability at concentrations equivalent to 1X MIC, 0.5X MIC and 0.25X MIC. This is interesting because it suggests that, in the case of *E. coli*, the membrane permeabilization triggered by CTAB is not the primary factor in inhibiting cell division. This further indicates that QACs influence other physiological processes beside membrane integrity. In contrast, DDAC has shown a dose-dependent effect where each decrease in concentration results in a decrease in permeability reaction having an approximately 79% membrane permeability at 1x MIC value, 45% and 16% at 0.5x and 0.25x respectively. This follows a similar, but more pronounced trend to what was previously shown for *S. aureus*. Despite these permeability observations, no clear trend has emerged between membrane integrity and antibiotic activity. This further suggests that the antibiotic potentiation we have reported is not primarily a cause of membrane disruption and likely involves other factors that are yet to be investigated.

Another way to confirm the potentiation activity is by time kill assay, particularly kanamycin plus CTAB against *E. coli* has been assessed. Kanamycin alone killed the bacteria after 24 this is expected due to its bactericidal properties, but as seen in **Figure 22** at 2 hours point of time, it shows that growth of *E. coli* in combination of CTAB and kanamycin has dramatically slowed down resulted to $6x10^8$ cfu/mL as compared to kanamycin alone with $1x10^9$ cfu/mL. It is also evident that the bacteria continued growing after 24-hours with bacteria having CTAB alone, while the bacteria were eliminated with tetracycline + CTAB after 24 hours (Figure 23) although this study cannot conclude whether the combination of kanamycin and CTAB against *E. coli* can actually increases the death rate of bacteria as the time point between 2-24 hours has not been done, so further and deep investigation of the potentiation activity is encouraged using time kill kinetics.

CONCLUSIONS

In this project, we set out to determine whether the activity of conventional antibiotics could be enhanced by their strategic combination with quaternary ammonium compounds (QACs). Our hypothesis was that QAC-mediated permeabilization of bacteria will enable greater antibiotic influx, leading to a heightened antibacterial activity. To investigate this, we set a broad scope of combinations, involving two different QAC compounds (CTAB having either 1 hydrophobic alkyl chains, and DDAC, having 2 hydrophobic alkyl chains), and antibiotics from different mechanism classes. Further, we investigated this combination strategy against both Gram-negative pathogens (represented by *E. coli*), and Gram-positive pathogens (represented by *S. aureus*). We firstly established the MIC of each QAC against each bacterial species and subsequently used sub-inhibitory concentrations of QACs to combine with antibiotics. Within this broad scope of combinations, we found multiple QAC/antibiotic combinations that produced enhanced antibiotic activity against both *E. coli* and *S. aureus*. To determine whether the QAC/antibiotic interactions were synergistic or additive, we selected one combination for each bacterial pathogen and performed checkerboard assays to quantify fractional inhibitory concentration indices (FICI). For *E. coli* we used kanamycin/CTAB and reported a FICI of 0.08, while for *S. aureus* we used tetracycline/CTAB and reported a FICI of 0.375. Both values fall within the 'synergistic' category (FICI $\leq$ 0.5). We further investigated this synergy by evaluating the time-kill kinetics of the combined treatment over 24 h by measuring colony forming units (CFUs) at timepoints of 15, 30, 60, 120 mins and 24 h. For both combinations, in the periods up to 120 mins, we observed significantly fewer CFUs when combination treatments were applied, in comparison to antibiotics alone. To ensure this was a result of the combination, we also included a CTAB-only control, which did not produce any inhibition (which was expected and intended, due to using sub-inhibitory concentrations). By 24 h, the entire bacterial population was eradicated by both the combined treatments, as well as the antibiotic-only groups. Unfortunately, we were not able to determine whether the combined treatment results in an accelerated time-kill profile, because the time at which all cells died was somewhere between the measured timepoints of 2 and 24 h. As our hypothesis stated that increased antibiotic activity would be mediated by QAC-dependent membrane permeabilization, we set out to determine whether a relationship could be drawn between QAC-mediated membrane permeabilization and antibiotic activity. To do this, we used flow cytometry coupled with the fluorescent probe propidium iodide to quantify proportions of membrane-permeable cells. While we were able to reliably quantify membrane permeability at

QAC concentrations ranging from 1X MIC to 0.25X MIC, we were not able to draw a meaningful relationship between this and antibiotic activity. Specifically, we observed multiple instances where antibiotic activity was enhanced even in the absence of increased permeability (such as in the case where *S. aureus* was exposed to CTAB (all concentrations) and DDAC (0.25X MIC). In these instances, QAC-mediated effects other than cell permeability must be involved. A plausible candidate for this mechanism may involve elevated oxidative stress.

LIMITATIONS AND FUTURE DIRECTION

As for the limitations, we only measured activity vs two bacterial species, with *E. coli* representing the Gram-negative pathogens, and *S. aureus* representing Gram-positive pathogens. While the results we got were encouraging, we cannot yet state whether this strategy would be broadly applicable, because microbial pathogens have a significant diversity in their envelope composition and arrangement and metabolic processes. Future studies will cast a broader net to determine the spectrum of activity of this strategy. Regarding the different outcomes between CTAB and DDAC, we cannot determine whether these differences are due to alkyl chain number (1 vs 2), or chain length (10 for DDAC and 16 for CTAB), or a combination of both. Future investigations may benefit from decoupling these factors, by comparing QACs with different chain number and equal chain length, or different chain length and equal chain numbers. Also, we do not know whether QAC resistance mechanisms would be a barrier to the efficacy of this strategy. It is tempting to assume that QAC resistance would negate our strategy, we cannot be certain until further investigation is conducted. Specifically, this is because QAC resistance is driven by export pumps (Buffet-Bataillon et al., 2016), but this mechanism does not prevent QACs from interacting with the outer surface of the cell. Since we do not yet know the exact mechanism for increased activity in our study, it is possible that the effect is driven by the interaction between QACs and the cell surface, and its downstream influences like oxidative stress or decrease membrane polarization. Lastly, we have not yet determined whether this strategy can be achieved at QAC concentrations that are biocompatible. However, even if cytotoxicity presents an issue with our QAC-based strategy, there is still value in understanding the mechanisms of the combination strategy, because we may then find other biocompatible compounds that can produce a similar effect like the study conducted by Sidders and colleagues. (Sidders et al., 2023).

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