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Pre-exposure of *Escherichia coli* and *Staphylococcus aureus* to sub-bactericidal concentrations of benzalkonium chloride and its effect on low-level antimicrobial resistance

By

Logan Fette

A Thesis Submitted in Fulfillment of the

Requirements for the Degree of

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This thesis paper has been examined and approved.

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Abstract

Antimicrobial resistance that develops in bacteria is a highly studied aspect of microbiology due to the concerns that it poses for public health. Antibiotics remain our primary care option when dealing with bacterial infections. The concept of tolerance of bacteria to disinfectants is similar and it also poses a significant health risk. Since use of disinfectants for sanitation is a primary manner in which we are able to prevent potential infections from occurring in the first place, it can be a helpful means to preventing development of antimicrobial resistance. However, what has not been explored to the same extent is whether an increase in tolerance to disinfectants due to their misuse can also influence tolerance to antimicrobials as well. Antimicrobial tolerance is different from antimicrobial resistance because the bacteria are still negatively affected when they are only able to tolerate an antimicrobial. The purpose of this study was to attempt to determine if there is an effect on antimicrobial tolerance, specifically an increase in antimicrobial tolerance, in bacteria when they are exposed to sub-lethal concentrations of a disinfectant. Sub-lethal disinfectant concentrations were used as an artificial selection pressure to attempt to force the bacteria to undergo adaptive mutation. If the adaptive response leading to an increased tolerance to the disinfectant is an example of cross-resistance that is linked with antimicrobial resistance, then an increase in antimicrobial tolerance was hypothesized to occur as well. There was no statistically significant effect observed on the tolerance levels to the antimicrobials used for the bacteria that had been exposed to the sub-lethal concentration of disinfectant. However, there were some trends observed that indicate that there is a difference in antimicrobial tolerance levels for tetracycline for both methicillin-

resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) that were promising. These trends suggest that there is an effect on the bacterial response to tetracycline following disinfectant treatment. This could present a significant public health dilemma as antimicrobial resistance is already a major problem. With these results indicating another potential method of adaption and development of antimicrobial resistance, it is concerning and merits future investigation for verification.

1. Introduction

Disinfectants include a wide spectrum of active ingredients and mechanisms of antimicrobial action, including but not limited to quaternary ammonium compounds (quats) such as benzalkonium chloride. Disinfectants are commonly used for the effective control of microbial growth and prevention of disease against a broad spectrum of bacteria. Using the example of quats mentioned above, this is achieved by using differences in the charge of the active ingredient of a quat relative to the membrane charge in order to interact with the bacterial cell membranes and destroy them which ultimately leads to the lysis of the cell and subsequent cell death (1, 2). One of the benefits of using quat-based detergents over some other disinfectants is that it is designed to be a continuously active product that retains its antimicrobial properties for an extended period (2). This will ensure that surfaces will remain clean and free of potential pathogens for an extended time and reduce the need to add more disinfectant to clean surfaces. Quats, of course, are not the only type of chemical disinfectants available, as there are other disinfectants such as alcohols and oxidizing agents that are also highly effective. However, for this research other disinfectants were used initially, but the focus was eventually narrowed to benzalkonium chloride, a very common example of a quat-based disinfectant that is widely available to both industry and consumers.

The economic and public health impacts of proper disinfectant use are substantial. Around the world, the cost and the case rate of infections/death attributed to infection from foodborne, healthcare-associated, and/or community-associated infections is high with extreme revenue loss in industry, especially the food industry, and a significant

amount of cases being reported due to pathogen transmission in healthcare settings as a consequence (3, 4). Another major concern is the development of tolerance to disinfectants due to improper usage, which typically occurs when using the wrong disinfectant or not preparing/applying the disinfectant properly, such as improperly or unnecessarily diluting the product (5, 6). Additionally, there is evidence that there may be a link between the tolerance to disinfectants and the tolerance/resistance to antimicrobials, highlighting the importance of developing new and more effective disinfectants as well as proper usage of disinfectants (5, 7).

The purpose of this study was to attempt to quantitate the level of bactericidal efficacy, in terms of a sub-bactericidal concentration, for the disinfectant that was used in this research. It was also intended to observe how successfully, or unsuccessfully, different bacteria can recover and develop tolerance to antimicrobials following sublethal disinfectant exposure. Relative bacterial susceptibility to antimicrobials was determined before and after sublethal disinfectant exposure to determine if prolonged exposure to disinfectants can indirectly increase tolerance levels to antimicrobials. These disinfectants and antimicrobials were applied against a pre-selected group of common but clinically relevant pathogens that can cause severe infection: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Bacillus cereus*, *and Salmonella typhimurium*. However, it is worth noting that all but *S. aureus* and *E. coli* were eventually removed from experimentation, the primary reasoning being that among clinical isolates, *S. aureus* and *E. coli* represent the greatest percentage of all clinical

isolates obtained and are therefore the most prevalent opportunistic pathogens of the group.

2. Hypotheses

The hypotheses for this project are as follows:

1. MRSA, MSSA, and *E. coli* will share similar adaptive responses to artificial selection via sub-lethal disinfectant exposure due to universal mechanisms of adaptive mutation.
2. Increased tolerance of the cultures to ampicillin and tetracycline will be directly influenced by the artificial selection via sub-lethal disinfectant exposure through adaptive mutation.

3. Literature Review

Background

The practice of actively using disinfection techniques throughout most of human history has focused primarily on the disinfection of wounds (8). Intriguingly, this long preceded the knowledge of microorganisms and their ability to cause infection, but people have been able to fight off potential pathogens for centuries regardless (1). However, there are examples of people using antimicrobial practices in other ways. For example, silver storage vessels or adding silver and copper coins, silver and copper being metals that are naturally antimicrobial, to their drinking water to extend its shelf life (1). Also, food preservation was crucial as well and people would use salt and spice mixtures to prevent the spoilage of their food by microorganisms (1). As time moved on, the methods of wound disinfection changed as well. As early as the 13th century, physicians of the time began using crude ointments and applying dressings to wounds to prevent the risk of infection (8). The concept of microbiology and the means of consistently controlling the infection they cause via disinfectants would truly begin with the observations of Antony van Leeuwenhoek. This being the true "discovery" of microorganisms and the start of microbiology as a scientific discipline.

However, the most significant advances in the concept of disinfection did not begin until the early 19th century. Wound infection was still the leading concern at the time, especially in terms of the medical community, and microbiologists put a lot of effort into figuring out how to rectify this issue. Louis Pasteur was able to confirm that

microorganisms are ubiquitous in the environment. Robert Koch was able to develop his postulates to verify that a microorganism is the cause of infection. Ignaz Semmelweis preached the importance of hygiene in medical practice to slow/halt transmission of infection. Lastly, Joseph Lister was able to apply a chemical disinfectant (phenol) to wounds in his surgeries which increased the survival rate of patients (8). There were also many advances in the development of sterilization practices in this time. These advances would carry forward into the 20th century and play an important role in controlling microbial growth and disease prevention.

There would be another shift in the concept of disinfection/sterilization practices in the 20th century. Previously, the focus was on disinfecting the wounds. However, the new idea was rather than disinfecting the wounds of the patient, it would be more prudent to develop aseptic techniques to sterilize any surfaces, equipment, etc. in a given setting in conjunction with wound care (8). Phenol, the same compound that Lister was able to use to disinfect surgical incisions and wounds, was among the first disinfectants used to clean medical surfaces and equipment (1). This concept of aseptic technique would no longer be exclusive to the medical field either and would be applied to other industries as well, especially the food industry.

The need for continuing to improve on our techniques and products used in disinfection is still of great importance to this day. Presently, now with the heightened awareness of sanitation due to the COVID-19 pandemic, it is commonplace for any surfaces/equipment that are used in the manufacturing of products for human use/consumption to be cleaned with chemical disinfectants. The medical industry and

food industry is still at the forefront of this issue, as they have been throughout history. For example, in the United Kingdom, it is estimated that foodborne pathogens and the resulting illness cause an annual loss of approximately £1.5 billion, which is equivalent to approximately $2 billion U.S. dollars (3). Similarly, the Center for Disease Control (CDC) in the United States estimates that 48 million people contract foodborne illnesses annually, with 3,000 of those cases resulting in death (3).

In medicine specifically, healthcare-associated infections (HAIs) in the United States occur in 7.1 million patients, with approximately 99,000 of those cases resulting in death. These infections are typically transmitted through the microbiota of patients and contact with medical staff (4). Many of these organisms are multidrug-resistant as well, such that it is estimated that 33,000 individuals in the European Union die each year resulting from multidrug-resistant bacteria contracted in hospital settings (9).

These statistics are recent and demonstrate that even in the technologically advanced modern world, the ineffective disinfection practices have very severe and often fatal consequences. Therefore, it is crucial to continue to improve upon our sterilization techniques and continue to develop and produce more effective disinfectants to control the microorganisms that can cause these serious infections.

Disinfectants

In this research, a wide spectrum of disinfectants were initially selected to analyze the difference in disinfectant efficiency, in terms of sub-minimum bactericidal concentration (MBC), among the differing disinfectant mechanisms. These disinfectants include Zoono® Microbe Shield (active ingredient is 3-trihydroxysilyl propyldimethyloctadecyl ammonium chloride), benzalkonium chloride, 3% hydrogen peroxide, 70% isopropyl alcohol, and chlorhexidine diacetate.

Zoono® Microbe Shield

The active ingredient in this disinfectant is 3-trihydroxysilyl propyldimethyloctadecyl ammonium chloride. This compound is an example of what is known as a quaternary ammonium compound and it is widely used in many industries, including but not limited to the medical and food industries (2). The advantage to using this in industry can be attributed to being non-toxic, environmentally friendly (approved for use by the EPA), and low cost with a high antimicrobial ability (10, 11). It also is advertised by the manufacturer as being able to remain continuously active for an extended period, typically up to 24 hours, on inanimate surfaces that are properly treated with the disinfectant (2). The mechanism of the surface adherence is through ionic and covalent bonding of the positively charged ammonium cation with the negatively charged surface it is applied to (12). This is what allows for the semi-permanent adherence of the molecule and allows it to remain an active disinfectant for an extended time.

The disinfectant mechanism, whether applied to a surface and allowed to dry or in aqueous form, is primarily through the presence of the alkyl chain associated with the quaternary ammonium compound. When bacteria encounter these molecular projections, the bacterial cell membrane will be punctured by the alkyl chain. The length of the alkyl chain or how many carbons are present in the chain has a direct effect on its ability as a disinfectant (1, 2). The more carbons present, the longer the alkyl chain and the more effective it is as a disinfectant (1, 2). Additionally, the longer the alkyl chain, the better the molecule is at adsorbing to the surface that is being treated with the disinfectant if allowed to dry on a surface (13). The ammonium cation of these types of disinfectants also have a crucial role in the mechanism of microbial control. They are attracted to the anionic charge of the cell and interact with the cell membrane, specifically the phosphate groups (1, 2). Thereby denaturing membrane proteins and disrupting other membrane structures as the cell is lysed (1, 2). Lastly, it is worth noting that this quaternary ammonium salt is in its inactive form until it is suspended in an aqueous solution (12).

Benzalkonium chloride

Benzalkonium chloride, more specifically alkyldimethylbenzyl ammonium chloride, is another example of a quaternary ammonium compound that is widely used in industry and is also very common for in-home use as a multi-surface disinfectant. The reasoning behind its wide use is because it has a broad spectrum of antimicrobial activity against bacteria but viruses and pathogenic fungi as well (14). It also has a relatively low toxicity to humans and a minimal effect on the environment (14). The spectrum of use for benzalkonium chlorides is much wider than most disinfectants as it is not solely

distributed as a disinfectant (14). Benzalkonium chlorides can also be used in agricultural equipment, soaps/personal hygiene products, laundry detergents, and more (14). Benzalkonium chlorides are especially common in the food industry as a means to prevent cross contamination. The reason it is so widely used in this industry is due to its robustness against changes in temperature and exposure to various organic matter compared to other common disinfectants (15). However, this widespread usage does provide the organisms with which it is employed against ample opportunity to adapt and develop tolerance to the compound (14).

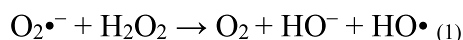
The disinfectant mechanism of benzalkonium chlorides, against bacteria specifically, is highly similar to the mechanism that was explained previously with the active ingredient present in Zoono® Microbe Shield because they are both examples of quaternary ammonium compounds. The mechanism being that the active ingredient, in this case benzalkonium chloride, will lyse the membrane of the organism via the alkyl chains and the charged ammonium cation will aid in the disruption of cell membrane structures as well (14).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) was first discovered in 1818 but, its usage as a disinfectant was not proposed until 1891 (5). In the present day, it is now one of the more widely used disinfectants with applications in the food, medical, industrial, and environmental (e.g. water treatment) settings (5). The reason it is so widely used is because it offers some strong advantages including a broad spectrum of antimicrobial

activity as a potent oxidizing agent, it is environmentally friendly/non-toxic, and it is biodegradable (5). It is especially useful in medical care settings as a surface disinfectant and for disinfecting medical equipment (5).

The antimicrobial mechanisms of H_2O_2 have not been extensively studied but there are some mechanisms that have been proposed and they both involve the production of highly reactive hydroxyl (HO•) radicals (5). These hydroxyl radicals are produced in two ways (5). The first is through the interaction between a superoxide ($O_2•^-$) radical and H_2O_2 (5). This reaction is known as the Haber-Weiss reaction (see Chemical Formula 1 below).

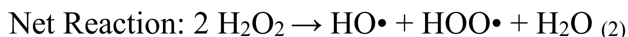


$$O_2•^- + H_2O_2 \rightarrow O_2 + HO^- + HO• \quad (1)$$

The second being the production of hydroxyl radicals in the presence of transition metal ions within the cell via the Fenton reaction (see Chemical Formula 2 below) (5). It is important to note that this reaction can also produce hydroperoxyl radicals (OOH•) as well with the net reaction resulting in the production of 1 HO• and 1 HOO• for every 2 H_2O_2 molecules present:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO•$$

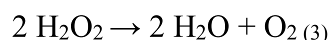
$$\underline{Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^+ + HOO•}$$



$$\text{Net Reaction: } 2\ H_2O_2 \rightarrow HO• + HOO• + H_2O \quad (2)$$

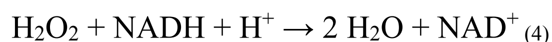
The hydroxyl radicals (and hydroperoxyl radicals) that are produced can act as potent oxidizing agents and interact with lipids, proteins/amino acids, and nucleic acids of a cell (5). However, the extent of these interactions and their role as antimicrobial mechanisms

are still not confirmed but some possibilities are through damage of bacterial DNA and through the disruption of the bacterial cell membrane (5). One potential drawback of using H_2O_2 is that some bacteria have the enzymes catalase and peroxidase. Catalase is an enzyme that catalyzes the decomposition reaction of H_2O_2 into water and oxygen gas and thereby prevents any potential oxidative damage to the bacterial cell (see Chemical Formula 3 below).



$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2 \quad (3)$$

Peroxidase is an enzyme that is similar to catalase, in that it can catalyze the decomposition reaction of H_2O_2 into water but without the release of oxygen (see Chemical Formula 4 below).



$$H_2O_2 + NADH + H^+ \rightarrow 2\ H_2O + NAD^+ \quad (4)$$

Isopropyl alcohol

As is the case with hydrogen peroxide, the usage of isopropyl alcohol (IPA) is widespread as it can be used in a variety of settings, such as healthcare, as a cheap and effective disinfectant (16). Ethanol (EtOH) is another example of a commonly used alcohol as a disinfectant as well as IPA because they share similar levels of efficacy and their mode of action is similar also (16). The reason alcohols are popular as disinfectants is because they have broad spectrum of activity that includes being bactericidal, fungicidal, tuberculocidal, and virucidal (16). However, they are not effective against spores of bacterial origin (16).

The mode of action of IPA and other alcohol-based disinfectants is through the denaturation of membrane proteins and the dissolution of lipids in the microorganism (16). One key factor in its efficacy is that it must be mixed with water because the presence of water aids in the rapidity of the protein denaturation than pure alcohol alone (16). It also prevents the rapid evaporation of the alcohol and allows it to remain on the surface long enough in order to kill any bacteria present. Therefore, it is crucial that the alcohol, such as IPA in this instance, should be in the concentration range of 60-90% (v/v) (16). This also varies among bacteria and other microbes, so its efficacy is highly dynamic and dependent on a range of variables (16).

Chlorhexidine diacetate

Chlorhexidine is a bisbiguanide disinfectant with a wide spectrum of antibacterial activity (17). It also has uses as an antiseptic or preservative (17). The three primary forms of chlorhexidine available for use are the acetate, gluconate, or the hydrochloride form (17). The use of chlorhexidine is also broad with applications in hand washes, creams/dressings, mouthwashes, general disinfectant, and in cleaning of instruments, primarily healthcare and veterinary equipment (17).

The mode of action for chlorhexidine is highly similar as the mode of action for many of the previously discussed disinfectants. It acts primarily by disrupting the cell membrane, causing the leakage of intracellular material from the cell (17). It can cause other structural and physiological damage as well such as the disruption of respiratory function of the cell (17). The concern with chlorhexidine is that only Gram-positive

bacteria are particularly susceptible to it whereas Gram-negative bacteria have an inherent tolerance to it (17). This is due to the presence of the outer membrane in Gram-negative bacteria which can impede chlorhexidine from reaching its target (17).

Antimicrobials

All of the following information regarding antimicrobials has been extensively reviewed in *Sherris Medical Microbiology* (18).

Beta-lactams (Ampicillin, Penicillin)

Penicillin, specifically penicillin G, which was used in this research is considered one of the original forms of this particular antimicrobial. Penicillin is a classic example of the class of antimicrobials known as the β-lactams and they are named based on their molecular structure containing a β-lactam ring. Even though it is older, penicillin is still an effective means of controlling microbial growth, however, its effectiveness is somewhat limited. It is mostly effective against Gram positive organisms and Gram negative cocci. Unfortunately, its effectiveness against Gram negative bacilli is limited to non-existent. This is due to the presence of the outer membrane not allowing the penicillin to reach its target and inhibit cell wall synthesis, its primary mode of action. Therefore, it is said that this antimicrobial has a narrower spectrum.

The other β-lactam that was utilized in this research is ampicillin. Ampicillin is an antimicrobial that was developed in response to the narrower spectrum of β-lactams, such as penicillin G. As suggested, ampicillin has a wider spectrum and is able to affect the same groups of bacteria that penicillin is effective against, but it is also able to be effective against Gram negative bacilli. It is able to do so by having the ability to pass the outer membrane and minimize the effectiveness β-lactamases found in these bacteria as well (18).

The way that β-lactams function is by interfering with the bacteria from conducting its transpeptidation reactions via what are commonly referred to as penicillin binding proteins (PBPs). This is done by not allowing them to perform their necessary function, the formation of the peptide cross-bridges that link the glycan strands.

Ciprofloxacin

Ciprofloxacin belongs to a class of antimicrobials known as the quinolones. Quinolones, in this specific case ciprofloxacin, which is a fluoroquinolone, are able to control microbial growth by interfering with the nucleic acid synthesis within the DNA of the bacterial genome. The targets of the quinolones and/or fluoroquinolones are two crucial enzymes related to DNA synthesis: DNA gyrase and topoisomerase IV. These two enzymes are needed to cut, negatively supercoil, and then finish by resealing the DNA during synthesis. By attacking two enzymes at once, the risk of resistance development via mutations is lowered. It is worth noting briefly that fluoroquinolones are now more commonly used than quinolones due to fluoroquinolones having a larger spectrum of bactericidal activity.

Streptomycin

Streptomycin, a member of the class of antimicrobials known as the aminoglycosides, are able to inhibit the protein synthesis functions of the bacterial ribosomes. This can be successfully done in various ways but, the most important factor in the efficacy of streptomycin is that the drug must be able to be taken in by the bacteria. The aminoglycosides are considered to have relatively wide spectrum of antibacterial

activity; however, they are limited in their use because this only applies to bacteria that are able translocate these compounds across their membrane. With that said, streptomycin and other aminoglycosides are highly effective and used often due to their broader spectrum and low rate of resistance development against them.

Sulfanilamide

Sulfanilamide is a member of the class of antimicrobials known as the sulfonamides, a class of drugs that function by inhibiting folate, also known as folic acid, synthesis. Folate is of high importance to bacteria because it has an important role as a coenzyme needed in the synthesis of nucleic acids and proteins for the microorganism. Using a folate inhibitor, such as sulfanilamide, will inhibit the synthesis of folate which will indirectly cause the inhibition of, as mentioned earlier, the synthesis of nucleic acids and proteins. These type of antimicrobials initially had a very broad spectrum, however, the downside to this type of antimicrobial is that bacteria are able to develop resistance to them rather easily.

Tetracycline

Tetracycline, fittingly, belongs to the class of antimicrobials known as the tetracyclines. Similar to the aminoglycosides, the tetracyclines function by entering the bacterial cell and inhibiting with ribosome function, specifically by binding the 30S subunit of the ribosome where the tRNA binds to the mRNA and prevent the synthesis of the protein. The downside between the tetracyclines and the aminoglycosides, however, is that the effect of tetracyclines is considered only bacteriostatic. This means that

treatment with a tetracycline will not kill the bacteria, it will only inhibit its growth. This does raise the question and real concern of whether bacteria can develop resistance more easily to tetracyclines than to aminoglycosides since tetracyclines do not kill the bacteria while the aminoglycosides are lethal to the bacteria.

Drawbacks

Despite the general effectiveness of disinfectants, there are potential issues with their usage. For example, the overuse or improper use of these products can result in the development of tolerance by bacteria (2). Improper use of disinfectants is often defined as improper dilutions of the product or the inactivation of the active ingredient through exposure to organic matter (9). Some other consequences of improper disinfectant use, specifically in medical care settings, can increase the risk of morbidity/mortality and the economic burden on the patient due to having to remain in the hospital longer because of infections potentially acquired during their stay (19).

Disinfectants also vary in their efficacy based on the physiology of the organism. Quaternary ammonium compounds have been shown to be less effective against Gram negative organisms compared to being highly effective against Gram positive organisms (6). On the other hand, hydrogen peroxide has a broader spectrum and is highly effective against both Gram positive and Gram-negative bacteria (6). The same is true against biofilms with hydrogen peroxide being more effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (20). Additionally, many commercial disinfectant producers claim the efficacy of their product, but these claims may not be accurate due to the inadequate testing of their product. Further, some disinfectants may be harmful to people as well (19). An example of this would be aldehyde-based disinfectants which have been linked to the development of asthma, eye irritation, dermatitis, and colitis in some instances (19). This demonstrates that not all disinfectants are equal, and they should be chosen accordingly depending on specific circumstances and needs.

Another drawback is that regular disinfection through chemical methods alone may not be enough to control pathogens (21). With the struggle to control healthcare-associated pathogens for example, there are concerns with maintaining sterile surfaces in medical settings, especially in areas such as intensive care units that require extended patient stays for individuals that may be more susceptible to infection. Even with the regular cleaning and use of disinfectants, hospital equipment such as hospital beds, are still at risk of carrying a bacterial load that is higher than the accepted levels (21). However, hospitals are attempting to combat this by incorporating other methods of disinfection such as physical disinfection through the use of ultraviolet light and also the use of naturally antimicrobial materials like copper metal for hospital surfaces (21).

Additionally, there are legitimate concerns that there may be a genetic link between the antimicrobial resistance and disinfectant tolerance of bacteria exposed to sublethal levels of disinfectants but, this has not yet been verified (9, 22). However, there is some evidence for bacteria that are less susceptible to disinfectants (compared to the wild-type counterparts) acting as a selective pressure for disinfectant tolerance (23).

These issues emphasize the importance of ensuring that the proper disinfectants, or combination of disinfectants/sanitation protocols, are used to control the growth of pathogenic microbes to ensure adequate disinfection of desired surfaces (9). This developing tolerance to quaternary ammonium disinfectants, as well as other disinfectants, is the driving force to the continuous production of new sanitation products and protocols.

Pathogens of Interest

Staphylococcus aureus

Staphylococcus aureus is a non-spore forming encapsulated Gram-positive cocci bacterium. It is known to be a commensal organism in humans with approximately 20% of U.S. adults considered carriers and this percentage is substantially higher in children (24). This is significant because *S. aureus*/MRSA is a serious human pathogen that can cause potentially life-threatening infections and is a major source of nosocomial infection (24–26). With infection by pathogenic organisms being the 2nd highest cause of death worldwide, the emergence of MRSA, a subtype of *S. aureus* that is highly resistant to antimicrobials, is now causing more public health concerns and emphasizes the continuous need for proper pathogen control (25). This is because environmental contamination plays a significant role in the transmission of MRSA, especially in healthcare settings (27). Surfaces with high human contact in these various settings, not just in healthcare, must be adequately disinfected to prevent surface to hand and subsequently person to person transmission of MRSA (27).

Escherichia coli

Escherichia coli is a non-spore forming encapsulated Gram-negative rod-shaped bacterium. This organism is commonly found in the gastrointestinal tract of most mammals, including humans, and is commonly transmitted through the fecal-oral route. Most outbreaks are usually attributed to contamination of food/food processing surfaces with animal feces (23, 28). There are different strains of *E. coli* that differ in their

pathogenicity ranging from relatively harmless common *E. coli* to the severe enterohemorrhagic *E. coli* (EHEC), the most commonly known strain being *E. coli* O157:H7 among others (28). EHEC is found to be commonly associated with foodborne illness outbreaks and these illnesses have the potential to be life threatening (28).

Bacillus cereus

Bacillus cereus is a non-encapsulated Gram-positive bacterium. Out of all the bacteria pre-selected in this study, *B. cereus* is unique because it is the only bacterium that can produce endospores. Endospores are a survival response by a bacterium when it is exposed to conditions that are potentially lethal, such as disinfectant exposure. It provides the organism a means to remain viable for an extended period until environmental conditions return to a preferred state that are conducive to survival. *B. cereus* is a common foodborne pathogen, especially problematic in the dairy industry due to ability to form robust biofilms on stainless steel surfaces, which are among the most used surface in this industry (29). Stainless steel is widely used in many other settings, such as restaurants and medical care facilities, which could be problematic as well.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a non-spore forming encapsulated Gram-negative bacterium which is widely found in the environment. The human gastrointestinal tract can be colonized by this organism through exposure to contaminated soil or water, including distilled water which is commonly used in medical settings (7). It is a significant opportunistic pathogen being associated with 10% of all hospital associated

infections. Among hospital associated infections; ventilator associated pneumonia, bloodstream infections, and UTIs are among the most common and/or severe (7, 26). For each of those three, *P. aeruginosa* is a significant and common cause for all of them (7, 26). This is especially problematic in healthcare settings responsible for the care of critically ill patients, immunocompromised patients, or patients with chronic respiratory diseases such as cystic fibrosis and obstructive pulmonary disease (7). This makes areas such as intensive care units and hospital operating rooms, and the equipment associated with these areas, high risk locations for the potential of *P. aeruginosa* infection in these patients (7).

Salmonella typhimurium

Salmonella typhimurium is a non-spore forming encapsulated Gram-negative bacterium that is a part of the non-typhoidal *Salmonella* serotype (30). Strains of *S. typhimurium*, among other non-typhoidal *Salmonella* serotypes, are prominent foodborne pathogens in the United States and worldwide (30, 31). The CDC estimates that in the United States alone, there are approximately 1 million cases of foodborne illness from *Salmonella* and that 19,000 of these cases required hospitalization (30). Additionally, just below 400 deaths annually can be attributed to *Salmonella* foodborne illness (30). Compounding the concern over *Salmonella* being so prevalent, its tolerance to disinfectant application has been increasing considerably over time (31). This has been especially the case with quaternary ammonium-based disinfectants (31).

Listeria monocytogenes

Listeria monocytogenes is a non-spore forming unencapsulated Gram positive bacterium which, similarly to *P. aeruginosa*, is widely found in the environment (32). It is an opportunistic human pathogen that can cause severe foodborne illness, typically in the form of listeriosis (32, 33). Listeriosis infections have a low rate of incidence, however, the fatality rate associated with listeriosis is rather high (33). Some reasons why *L. monocytogenes* poses significant challenges for the food industry is due to its ability to not only survive on but persist, due to biofilm forming ability, on food processing equipment surfaces for months to even years (32, 33). This is especially true in equipment that has hard to reach places that makes cleaning and disinfecting difficult (32). Also, its ability to form biofilms increases its tolerance towards the use of disinfectants which makes it difficult to eradicate the organism from food processing equipment (33). On top of all that, it is a psychrotrophic organism, which means that it can grow at low refrigeration temperatures (32). All of these survival abilities of *L. monocytogenes* are the primary reason why refrigerated and processed ready-to-eat foods, such as deli meats, are high risk products for *L. monocytogenes* contamination and transmission that can lead to foodborne illness (32, 33).

Summary

Throughout human history, people have been actively fighting against microbes, even before their knowledge of these microorganisms. They were coming up with methods to primarily treat/prevent infections and to prevent food spoilage as well. It would not be until the 19th century however for the significant advances in the concept of disinfection to become a reality and be common practice. Currently, the medical community was still facing a substantial challenge with patient mortality and the need for improving on preventing infections from occurring was paramount. Scientists of the time were able to demonstrate that microbes are ubiquitous and are the cause of these infections which led to the realization that disinfection of wounds was necessary. In the 20th century this was extended to entire areas being disinfected, such as operating rooms, and not just the wounds of patients to have further prevention of these infections from occurring. In the present day, these issues are still relevant unfortunately and the need to continue to improve upon sanitation practices and develop new disinfectants is extremely important.

Importance

The need for constant improvement in the means to control the growth of microbes is of utmost importance. Especially following the aftermath of the Sars-CoV-2 pandemic the inability to adequately control microbial growth and transmission of infection and/or disease is critical to public health, industry, and economies around the world. With the ease of international travel, the mass production of livestock for food that carry these organisms as part of their natural flora, high traffic/high risk environments such as hospitals and other public places, water treatment plants ensuring that water is adequately cleaned and safe to drink, the potential for bacteria to develop tolerance to disinfectants in the same way that many of them are acquiring antimicrobial resistance cause for concern. These all are examples of the ease of which that these pathogens can be transmitted. It also illustrates why it is important to continue to analyze disinfectant efficacy and continue to synthesize new chemical compounds for the chemical control of microbes and that chemical control alone is not always enough. Protocols should include some other methods, such as UV light mentioned earlier, in conjunction with chemical methods.

This research will benefit science by providing data that demonstrates which types of disinfectants are more effective against bacterial pathogens. Further studies can then be performed to further analyze the mechanisms of the successful disinfectants and improve upon them so that microbial growth can be controlled better and therefore prevent more infections from occurring. Additionally, by seeing which bacteria can recover following disinfectant exposure, future microbial physiology studies can be done

to determine why those specific organisms are more robust and can survive. There has also been proposed hypotheses recently that there may be a link between the development of resistance towards antimicrobials and development of tolerance towards disinfectants. The bacteria that are successful at tolerating disinfectant exposure may be more likely to develop resistance to antimicrobials as well. This can lead to many future studies that will allow us to continue to improve our understanding of bacterial growth, survival, and adaptability.

4. Methodology

Bacterial cultures

The seven bacterial cultures that were selected to be studied in this research included methicillin-resistant *S. aureus* (ATCC 43300), β-lactam sensitive *S. aureus* (ATCC 25923), *L. monocytogenes* serotype 4b (ATCC 19115), *P. aeruginosa* (ATCC 10145), *E. coli* (ATCC 25922), *S. typhimurium* (MNSU, Mankato collection), and *B. cereus* (MNSU, Mankato collection). *L. monocytogenes* serotype 4b (ATCC 19115), *P. aeruginosa* (ATCC 10145), *S. aureus* (ATCC 25923), and *E. coli* (ATCC 25922) cultures were purchased in freeze-dried form. Using a syringe and needle, nutrient broth (Hardy Diagnostics) was then added to the vials containing the freeze-dried cultures to revitalize the organisms. These vials were stored in the refrigerator for future use. Note that through the course of this project, the microorganisms including *L. monocytogenes*, *P. aeruginosa*, *B. cereus*, *S. typhimurium* were eliminated from all experimentation for various reasons which involved the MBC's of the disinfectants and/or antimicrobials either being too high or too low for these bacteria when the procedure for "sub-MBC determination" was followed for these bacteria.

For long-term storage of cultures, an 80% glycerol solution was prepared by adding 80 mL of glycerin (Fisher Scientific) to 20 mL of distilled water. For cryo-storage of "wild-type" cultures, 0.75 mL of 80% glycerol with an equal amount of bacterial culture in nutrient broth and added to a cryo-tube vial. This yielded a final concentration of 40% glycerol. These were mixed thoroughly. Multiple cryo-vials were prepared for each "wild-type" organism for storage at both -70℃ and at -80℃. Any mutant cultures

that demonstrated an altered level of tolerance to antimicrobials or disinfectants that developed during the course of this research were prepared and then stored as described above for future research. Any potential mutant cultures were prepared and stored in the same way. These mutant cultures were grown in brain heart infusion (BHI) broth instead of nutrient broth when storing these cultures to verify that the cause of cell death was not a result of nutrient deficiency.

Antimicrobials

The six antimicrobials used for this research included: ampicillin trihydrate (Amresco), penicillin G sodium salt (Acros Organics), streptomycin sulfate (Acros Organics), ciprofloxacin (Alfa Aesar), sulfanilamide (Mallinckrodt), and tetracycline (Ward's Science). These antimicrobials were purchased in powder form and stored at -20°C. Please note that through the course of this research that the antimicrobials including penicillin, streptomycin, ciprofloxacin, and sulfanilamide were eventually removed from experimentation in order to narrow the focus of the study.

Disinfectants

The five disinfectants that were selected for this research included: Zoono® Microbe Shield (Zoono USA, LLC.) with 3-trihydroxysilyl propyldimethyl ammonium chloride, Formula 409 (The Clorox Company) with benzalkonium chloride, Nolvasan® S (Zoetis) with chlorhexidine diacetate, 3% hydrogen peroxide (Walmart, Inc.), and 70% isopropyl alcohol (Walmart, Inc.). Please note that through the course of this experiment, the disinfectants including Zoono® Microbe Shield, Nolvasan® S, 3% hydrogen

peroxide, and 70% ethanol were removed from experimentation to narrow the focus of the study

Stock Solution Preparation

The stock solutions were prepared at the highest concentration that their solubilities in water allowed. For ampicillin trihydrate, penicillin G, streptomycin sulfate, ciprofloxacin, sulfanilamide and tetracycline, distilled water was used as the diluent and the solvent. Once the antimicrobial stock solutions had been made, they were partitioned into 1 mL aliquots and were stored at -20°C. The same is true for antimicrobial powder that is not placed in stock solution. Please refer to the Appendix section at the end of this document for more detailed instructions on stock solution preparation.

There was no preparation for disinfectant stock solutions. The disinfectants as purchased from the manufacturers served as the stock solutions. The labeling on the product packaging provided the necessary active ingredient concentrations needed. All working solutions for the disinfectants were prepared by diluting them in distilled water.

Sub-Minimum Bactericidal Concentration (Sub-MBC) Determination

The following procedure was executed under a previously sterilized hood with ultraviolet light and ethanol while wearing ethanol sterilized disposable gloves to prevent contamination and for safety. The frozen stock cultures were removed from the -80℃ storage freezer and immediately inoculated onto LB agar. I prepared plates by streaking for isolated colonies. Cultures were not allowed to thaw out during primary inoculation. These LB agar cultures were incubated overnight at 37℃, at which point, the LB agar cultures would be removed from the incubator and one colony from the LB agar culture would be collected and inoculated in BHI broth which was incubated overnight at 37℃. The BHI culture would be removed, and 1 mL of the inoculum was added to 1.5 mL microcentrifuge tubes and centrifuged at 15,000 x g for 60 seconds. The supernate was discarded in a waste beaker and the pellet was resuspended in 1 mL of the appropriate concentration of antimicrobial or disinfectant, depending upon what the sub-MBC was attempting to be determined for, was added to these 1.5 mL microcentrifuge tubes. After adding the antimicrobial or disinfectant and resuspending the cell pellet, they were left to incubate at room temperature for 60 minutes while the 96-well microplate was then prepared as follows. First, 200 µL of BHI broth was added and inoculated with bacteria that received neither antimicrobial nor disinfectant exposure. This served as a positive control with bacterial growth. Then 200 µL of uninoculated BHI broth was added and this served as a negative control. Once the controls had been added, 200 µL of sterile BHI broth was added to the remaining wells that were needed. Then the cultures that had been exposed to various concentrations of antimicrobials or disinfectants was used to inoculate

the uninoculated BHI broth in each designated well in the 96-well microplate. Once all the controls and cultures were added, the plate was placed in the incubator to incubate overnight at 37℃. After incubation, the plate was checked for the well that had growth at the highest concentration of antimicrobial or disinfectant for the sub-MBC, which we defined as the concentration a single two-fold dilution below the MBC. If no sub-MBC was apparent, the procedure was repeated at different concentrations of antimicrobials or disinfectants until the sub-MBC was determined. Please refer to the Appendix section at the end of this document for more detailed step-by-step instructions for this procedure.

Selection of Antimicrobial Tolerance

The following procedure was executed as previously described in "sub-MBC determination." The supernate was discarded and the pellet resuspended in 1 mL of normal saline, to this 1 mL of the disinfectant diluted to the previously determined MBC was added to the saline. This diluted the disinfectant to the sub-MBC which is half the concentration of the MBC. This suspension was gently mixed by inversion daily to ensure that there is a consistent distribution of cells in solution for up to 5 days with the aim of selecting for bacteria with increased antimicrobial tolerance. During the 5-day pre-treatment with the sub-MBC of the disinfectant, the cultures were plated on LB agar after each day rather than after 5 full days. A placebo control culture (bacteria in 0.85% saline only) was incubated at room temperature alongside the disinfectant treatments. Any surviving culture collected after each day were stored at -80℃ in cryo-broth. If viable cells were present in culture, one colony from the "survivor" culture was collected to serve as a representative for the population. This was inoculated into BHI broth which was incubated overnight at 37℃. Following incubation, this was used to prepare cryo-broth for future study. Note that if no viable cells were obtained, this was repeated with a PBS wash in the event that disinfectant was still present. For future experiments, the cells that survived the pre-treatment the longest period of time for each culture of bacteria were used. For example, if MRSA and *E. coli* survived pre-treatment for 5 days but *S. aureus* survived for 3 days, then the 3 day cultures for all cultures would be used for future experiments. Please refer to the Appendix section at the end of this document for more detailed step-by-step instructions for this procedure can be found there.

Cell Injury, Death, and Viability Determination

The following procedure was executed under a previously sterilized hood with ultraviolet light and ethanol while wearing ethanol sterilized disposable gloves to prevent contamination and for safety. The frozen stock cultures including cultures that had survived the disinfectant treatment, saline only placebo treatment, and a control that had received no disinfectant pre-treatment were removed from the -80□ storage freezer and immediately inoculated onto LB agar. Plates were prepared by streaking for isolated colonies. Cultures were not allowed to thaw out during primary inoculation. These LB agar cultures were incubated overnight at 37□, at which point, the LB agar cultures would be removed from the incubator and one colony from the LB agar culture would be collected and inoculated in separate aliquots of BHI broth which was incubated overnight at 37□. The BHI culture would be removed, and 1 mL of the inoculum was added to 1.5 mL microcentrifuge tubes and centrifuged at 15,000 x g for 60 seconds. The supernate was discarded into a waste beaker and were then resuspended in the previously determined antimicrobial sub-MBC with respect to that organism. This was left to incubate for 60 minutes at room temperature after which the culture was centrifuged as before. The supernate was removed into a waste beaker and the cells were resuspended in fresh BHI broth. Cultures were then adjusted using sterile 0.85% saline buffer which was used to adjust the turbidity of the cultures to determine the initial concentration (CFU/mL) of the culture and to adjust all cultures to a #2 McFarland standard, which corresponds to a cell density of $6x10^8$ CFU/mL. Once the turbidity of each culture had been standardized. They were diluted to a final concentration of 10^{-4} with 0.85% saline.

10 μL of this suspension was added to a non-selective medium and a selective medium so that the spread plate method could be performed. For Gram negative bacteria, the non-selective medium was LB agar (Fisher Scientific) and EMB agar (acuMedia) was used as the selective medium. For the Gram-positive bacteria, LB agar (Fisher Scientific) was again used as the non-selective medium and mannitol salt agar (Hardy Diagnostics) was used as the selective medium. After spread plating, the plates were incubated overnight at 37□. These cultures were used to determine if the cells were killed, injured, or perfectly viable based on the number of colonies that were recovered. Viability for this experiment was defined as the presence of colonies on the plate indicating live cells capable of replication. Injured cells needed to be determined through calculation to figure out the proportion of the viable cells that were injured as a result of the treatment processes used. Death was defined as the lack of colony growth on the agar indicating that the cells inoculated had died through the treatment process and were unable to replicate. This described protocol will be repeated for each bacterium being studied in this research. Please refer to the Appendix section at the end of this document for more detailed step-by-step instructions for this procedure.

Statistical Analysis

The results of this experiment were analyzed using the statistical software GraphPad Prism. The data was analyzed using the graphs plotted to compare the difference for each culture in terms of their ability to increase their tolerance to antimicrobials due to disinfectant treatment compared to those cultures that received the saline control treatment. The data was tested for normality via both the Kolmogorov-Smirnov and Shapiro-Wilks normality tests. The two-way ANOVA test was used to determine standard error and to determine the statistical significance of the data. Results where $p < 0.05$ were considered statistically significant.

5. Results

Effect of exposure of bacteria to benzalkonium chloride and the sub-minimum bactericidal concentration determination

In order to determine the sub-minimum bactericidal concentration, the concentration defined as being the concentration that is half of the minimum bactericidal concentration, we first needed to determine the minimum bactericidal concentration. The active ingredient present in the disinfectant, benzalkonium chloride, was diluted by using serial two-fold dilutions and this range of concentrations was exposed to each of the three cultures of bacteria used in this study to determine the bactericidal threshold of this active ingredient against these cultures. We observed that the minimum bacterial concentration was noticeably higher for the Gram negative *E. coli* than it was for both MRSA and MSSA, both of which are Gram positive microorganisms (Table 1, Figure 1).

Counted from low to high concentrations, the first well that contained growth in the 96-well microplate was considered the sub-minimum bactericidal concentration for each of the three cultures. For example, in Figure 1 the well containing the minimum bactericidal concentration had no growth and the neighboring well that had bacterial growth was defined as the sub-minimum bactericidal concentration (Figure 1).

Effect of exposure of bacteria to ampicillin and tetracycline and the sub-minimum bactericidal concentration determination

In order to determine the sub-minimum bactericidal concentration, the concentration defined as being the concentration a single two-fold dilution below the minimum bactericidal concentration, we first needed to determine the minimum bactericidal concentration. Similar to the minimum bactericidal concentration/sub-minimum bactericidal concentration determination for the active ingredient benzalkonium chloride present in the disinfectant, the antimicrobial drugs ampicillin and tetracycline were diluted in a similar manner via two-fold dilutions. For ampicillin, since it belongs to the class of β-lactam antimicrobials, it was not surprising to see that it was ineffective at killing the MRSA culture because MRSA has a high level of β-lactam resistance (Table 1, Figure 2). Ampicillin was moderately effective against MSSA, with a minimum bactericidal concentration of 12.5×10^{-2} mg/ml ranking between MRSA and *E. coli* (Table 1, Figure 2).

The Gram negative *E. coli* appeared to be the most susceptible to the ampicillin exposure. (Table 1, Figure 2). For tetracycline, initially a minimum bactericidal concentration could not be observed initially, and testing was repeated at lower concentrations than used for both MRSA and MSSA (Figure 2 and 3). Among the three organisms, *E. coli* was the least affected by the exposure of tetracycline since it needed the highest concentration (3.13×10^{-2} mg/ml) in order to be killed, whereas MRSA and MSSA were more susceptible to tetracycline since lower concentrations (0.391×10^{-2} mg/ml and 1.56×10^{-2} mg/ml respectively) were effective (Table 1, Figures 2 and 3). Again, the well containing the minimum bactericidal concentration had no growth and it

neighbored a well that had bacterial growth which was the sub-minimum bactericidal concentration (Figure 1).

Effect of selective pressure pre-treatment of bacteria with sub-minimum bactericidal concentrations of benzalkonium chloride

In order to obtain viable cells that have undergone potential adaptive mutation and therefore have obtained a potential higher threshold of tolerance to antimicrobials, the cells received exposure to sub-lethal concentrations of the active ingredient benzalkonium chloride present in the disinfectant. Once the sub-minimum bactericidal concentration of the disinfectant against each of the bacteria was determined, this concentration was used to prepare a solution of the disinfectant at the sub-minimum bactericidal concentration along with cells suspended in saline in the same solution (Figure 4). This was considered the pre-treatment process to attempt to induce adaptive mutation via artificial selection. During the incubation process, it was observed that there were no viable *E. coli* cells present at any point during this process. The lack of viable *E. coli* cells was observed both in pre-treatment solution and on LB agar plate when culture was removed from solution and inoculated there (Figures 4 and 5).

However, this process was more successful for the MRSA and MSSA cultures as the solution tended to remain homogeneous rather than develop aggregation at the bottom of the test tube (Figure 4). It was also confirmed by removing the culture from solution and inoculating onto LB agar because we were able to observe some bacterial growth on the LB agar (Figure 5). Therefore, we were only able to continue with the MRSA and *S,aureus* cultures for the next phase of experimentation because only these two cultures displayed the desired result.

Effect of sub-minimum bactericidal antimicrobial exposure following selective pressure pre-treatment procedure

After cultures, MRSA and MSSA, had undergone and survived either the saline control pre-treatment or the sub-lethal disinfectant pre-treatment, these cells were then re-exposed to a sublethal dose of two antimicrobials: ampicillin and tetracycline. In doing so, we were able to determine the percentage of the population that had been killed by the antimicrobial exposure (relative to a culture that had undergone no pre-treatment of any sort) and what percentage of the surviving cells that had been injured through antimicrobial exposure within each culture by calculating based on colony counts from a non-selective media and a selective media (which should not allow injured cells to grow).

The results of MRSA exposure indicate that the effect of pre-treatment with a sub-lethal concentration of the disinfectant had no obvious effect on the tolerance levels of the microorganism against ampicillin, as the percentage of killed cells were quite similar. The percentage of cells that were injured for the disinfectant treated cells did increase when exposed to ampicillin which indicates it may be more sensitive to ampicillin (Figure 6). However, it can be seen that there was a slight decrease in the percentage of cells killed when exposed to tetracycline, indicating that there may be an effect on tetracycline tolerance when treated with a sub-lethal concentration of the disinfectant. Additionally, the percentage of cells that had been injured by tetracycline had also been decreased relative to the saline control culture, which further suggests that there may be an effect of disinfectant pre-treatment on tolerance levels of MRSA to tetracycline (Figure 6). Two-way ANOVA tests confirmed that none of the data were statistically significant ($p < 0.05$).

For the MSSA cultures, similar to what was observed for MRSA, there was no obvious difference between the saline control culture and the disinfectant pre-treated culture in terms of the percentage of cells killed when exposed to ampicillin. The difference with MSSA is that, unlike MRSA, there was no obvious difference between the saline culture and the disinfectant pre-treated culture in terms of the percentage of injured cells either (Figure 7). This indicates there may be no significant effect of disinfectant pre-treatment on the levels of ampicillin tolerance. However, when MSSA had been exposed to tetracycline, we observed the opposite result of what was observed in MRSA. For MSSA, we observed that a higher percentage of disinfectant pre-treated cells had been killed through tetracycline exposure than disinfectant pre-treated MRSA cells had been by tetracycline, even when it had a decrease in the percentage of injured cells similar to MRSA following tetracycline exposure (approximately a 10% increase in MSSA cells killed relative to MRSA) (Figure 7). Two-way ANOVA tests confirmed that none of the data were statistically significant ($p < 0.05$).

Table 1. Diagram of 96-well microplate layout for sub-MBC determination of the active ingredient in the disinfectant, benzalkonium chloride (this table corresponds with Figure 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Positive control**	**$0.06x10^{-2}$%**	**X**	**X**	**Positive control**	**$0.06x10^{-2}$%**	**X**	**X**	**Positive control**	**$0.06x10^{-2}$%**	**X**	**X**
B	**Negative control**	**$0.12x10^{-2}$%**	**X**	**X**	**Negative control**	**$0.12x10^{-2}$%**	**X**	**X**	**Negative control**	**$0.12x10^{-2}$%**	**X**	**X**
C	**X**	**$0.23x10^{-2}$%**	**X**	**X**	**X**	**$0.23x10^{-2}$%**	**X**	**X**	**X**	**$0.23x10^{-2}$%**	**X**	**X**
D	**X**	**$0.47x10^{-2}$%**	**X**	**X**	**X**	**$0.47x10^{-2}$%**	**X**	**X**	**X**	**$0.47x10^{-2}$%**	**X**	**X**
E	**X**	**$0.94x10^{-2}$%**	**X**	**X**	**X**	**$0.94x10^{-2}$%**	**X**	**X**	**X**	**$0.94x10^{-2}$%**	**X**	**X**
F	**X**	**$1.88x10^{-2}$%**	**X**	**X**	**X**	**$1.88x10^{-2}$%**	**X**	**X**	**X**	**$1.88x10^{-2}$%**	**X**	**X**
G	**X**	**$3.75x10^{-2}$%**	**X**	**X**	**X**	**$3.75x10^{-2}$%**	**X**	**X**	**X**	**$3.75x10^{-2}$%**	**X**	**X**
H	**X**	**$7.50x10^{-2}$%**	**X**	**X**	**X**	**$7.50x10^{-2}$%**	**X**	**X**	**X**	**$7.50x10^{-2}$%**	**X**	**X**
I	**X**	**$15.0x10^{-2}$%**	**X**	**X**	**X**	**$15.0x10^{-2}$%**	**X**	**X**	**X**	**$15.0x10^{-2}$%**	**X**	**X**

***Key:**

X = empty wells on the 96-well microplate

Columns 1 & 2 = *E. coli*

Columns 5 & 6 = MRSA

Columns 9 & 10 = MSSA

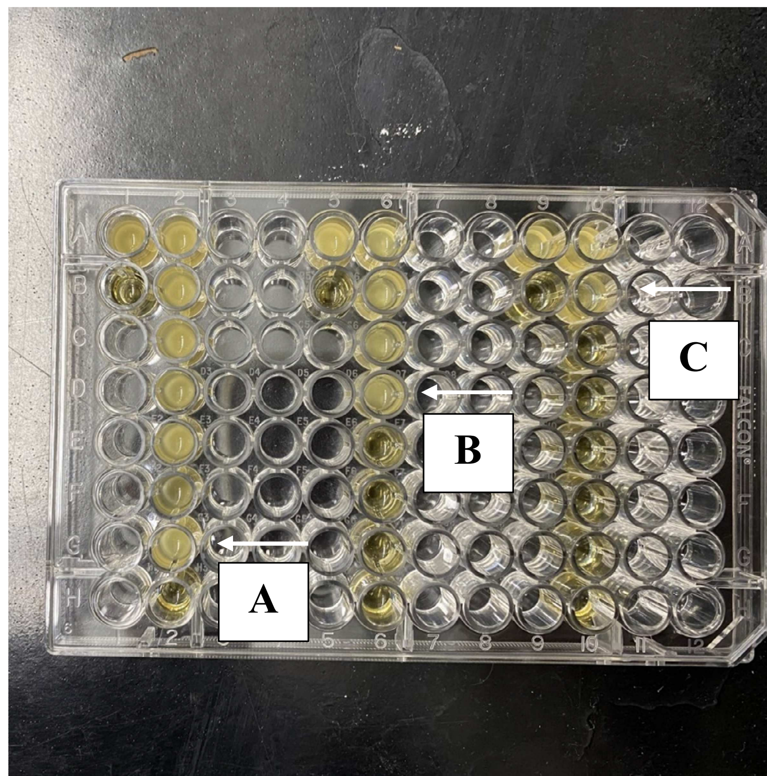


Figure 1. A photograph of a 96-well microplate containing *E. coli* (left), MRSA (middle), and MSSA (right) cultures when exposed to a range of concentrations of the disinfectant containing the active ingredient: benzalkonium chloride. The concentration of the disinfectant was consistent for all three organisms and the highest concentration is the final well at the bottom and the lowest concentration is at the top of the plate. The first well that contains no bacterial growth is the minimum bactericidal concentration (MBC). The first well after the MBC was considered the sub-MBC and was the concentration used for additional experimentation. Each culture has two wells next to the disinfectant cultures to serve as a positive control with bacterial growth and a negative control with no bacterial growth. Arrow A is pointing to the sub-MBC (7.50×10^{-2} %) for *E. coli*, arrow B is pointing to the sub-MBC (0.20×10^{-2} %) against MRSA, and arrow C is pointing to the sub-MBC (1.00×10^{-2} %) against MSSA.

Table 2. Generalized diagram of 96-well microplate layout for sub-MBC determination of the antimicrobials, ampicillin and tetracycline. (This table corresponds with Figure 2).

	1*	2	3**	4	5	6**	7**	8	9	10**	11**	12
A	Positive control	3.13×10^{-2} mg/mL	#2	X	Positive control	#2	#2	X	Positive control	#2	#2	X
B	Negative control	6.25×10^{-2} mg/mL	#2	X	Negative control	#2	#2	X	Negative control	#2	#2	X
C	X	12.5×10^{-2} mg/mL	#2	X	X	#2	#2	X	X	#2	#2	X
D	X	25.0×10^{-2} mg/mL	#2	X	X	#2	#2	X	X	#2	#2	X
E	X	50.0×10^{-2} mg/mL	#2	X	X	#2	#2	X	X	#2	#2	X
F	X	X	X	X	X	X	X	X	X	X	X	X
G	X	X	X	X	X	X	X	X	X	X	X	X
H	X	X	X	X	X	X	X	X	X	X	X	X
I	X	X	X	X	X	X	X	X	X	X	X	X

***Key:**

X = empty wells on the 96-well microplate

Column 1 (*E. coli* controls), Column 2 (*E. coli* with ampicillin), Column 3 (*E. coli* with tetracycline)

Column 5 (MRSA controls), Column 6 (MRSA with ampicillin), Column 7 (MRSA with tetracycline)

Column 9 (MSSA controls), Column 10 (MSSA with ampicillin), Column 11 (MSSA with tetracycline)

**Columns 3, 6, 7, 10, and 11 used the same concentrations and layout as Column 2

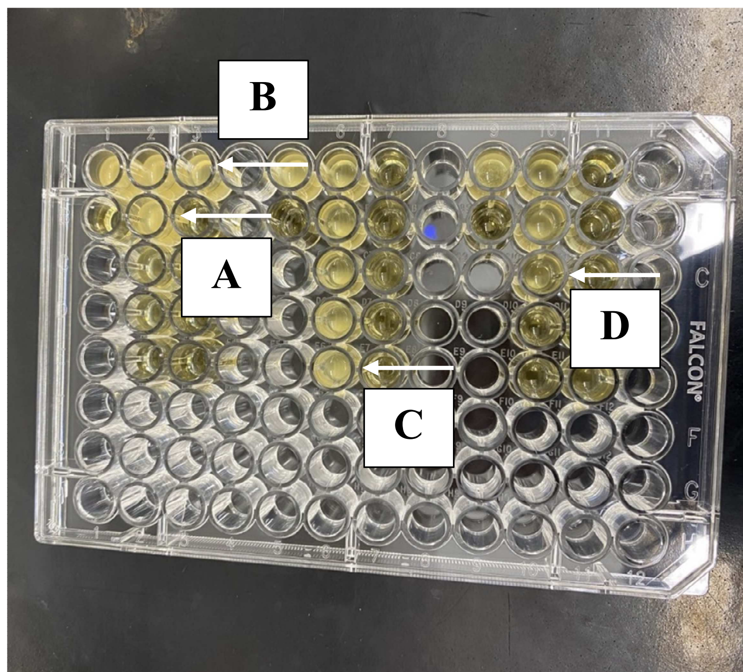


Figure 2. A photograph of a 96-well microplate containing *E. coli* (left), MRSA (middle), and MSSA (right) cultures when exposed to a range of concentrations of the antimicrobials: ampicillin and tetracycline with ampicillin being the middle column for each bacteria and tetracycline being the rightmost column for each bacteria. The concentration of the antimicrobials were consistent for all three organisms and the highest concentration is the final well at the bottom and the lowest concentration is at the top of the plate. The first well that contains no bacterial growth is the minimum bactericidal concentration (MBC). The first well after the MBC was considered the sub-MBC and was the concentration used for additional experimentation. Each culture has two wells next to the antimicrobial cultures to serve as a positive control with bacterial growth and a negative control with no bacterial growth. In this first attempt, only MBC's for ampicillin could be determined against *E. coli* and MSSA. A tetracycline MBC was only determined for *E. coli* in this first attempt. As expected, ampicillin was ineffective against MRSA. Arrow A is pointing to the sub-MBC (6.25×10^{-2} mg/ml) for *E. coli* against ampicillin, arrow B is pointing to the sub-MBC (3.13×10^{-2} mg/ml) for *E. coli* against tetracycline, arrow C is pointing to the sub-MBC (50.0×10^{-2} mg/ml) for MRSA against ampicillin, and arrow D is pointing to the sub-MBC (12.5×10^{-2} mg/ml) for MSSA against ampicillin.

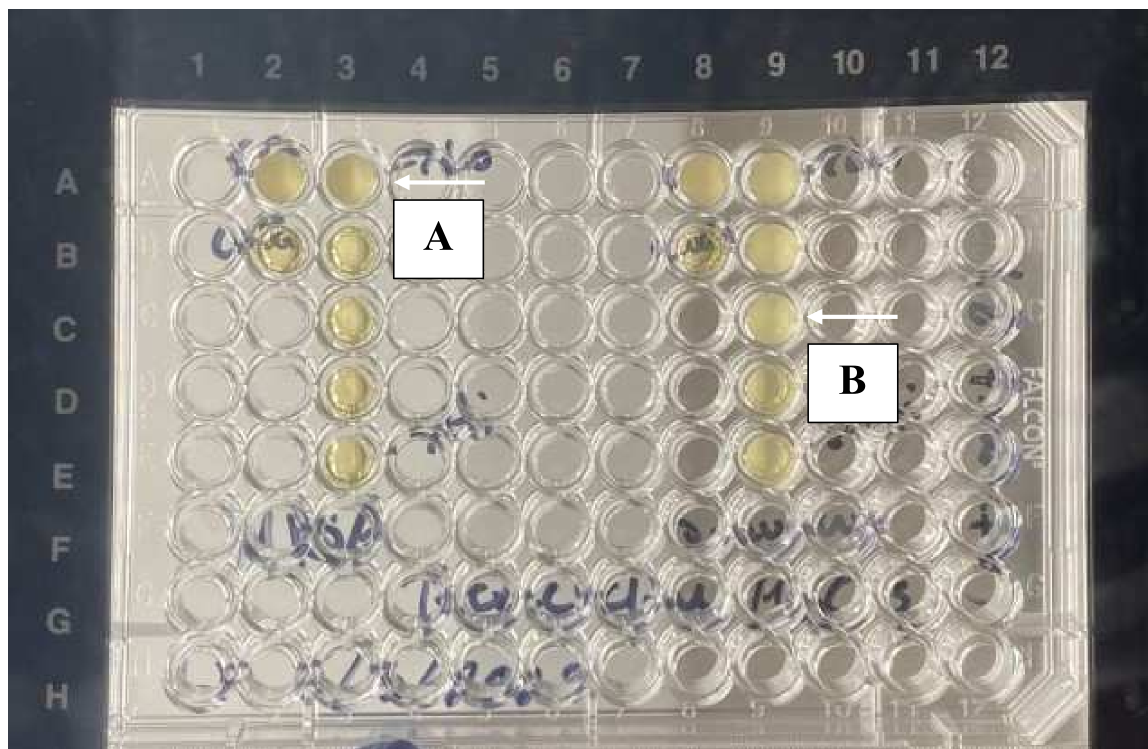


Figure 3. A photograph of a 96-well microplate containing MRSA (left), and MSSA (right) cultures when exposed to a range of concentrations of the antimicrobial: tetracycline with tetracycline being the righthand column for each bacteria. The concentration of the antimicrobials was consistent for both organisms and the highest concentration is the final well at the bottom and the lowest concentration is at the top of the plate. The first well that contains no bacterial growth is the minimum bactericidal concentration (MBC). The first well after the MBC was considered the sub-MBC and was the concentration used for additional experimentation. Each culture has two wells next to the antimicrobial cultures to serve as a positive control with bacterial growth and a negative control with no bacterial growth. In this second attempt, an MBC could be determined for both MRSA and MSSA when exposed to tetracycline. Arrow A is pointing to the sub-MBC (0.391×10^{-2} mg/ml) for MRSA against tetracycline and arrow B is pointing to the sub-MBC (1.56×10^{-2} mg/ml) for MSSA against tetracycline.

Table 3. Sub-minimum bactericidal concentrations of the antimicrobial drugs; ampicillin and tetracycline, and of the active ingredient in the disinfectant; benzalkonium chloride.

	BC*	A	Te
E. coli (ATCC 25922)	**$7.50x10^{-2}$ %**	**$6.25x10^{-2}$ mg/ml**	**$3.13x10^{-2}$ mg/ml**
MSSA *(ATCC 25923)*	**$0.20x10^{-2}$ %**	**$12.5x10^{-2}$ mg/ml**	**$1.56x10^{-2}$ mg/ml**
MRSA *(ATCC 43300)*	**$1.00x10^{-2}$ %**	**$50.0x10^{-2}$ mg/ml****	**$0.39x10^{-2}$ mg/ml**

***Key:**

BC – benzalkonium chloride

A – ampicillin

Te – tetracycline

** - Indicates that no MBC was present, used concentration a single two-fold dilution below stock concentration of 1 mg/ml as a substitute

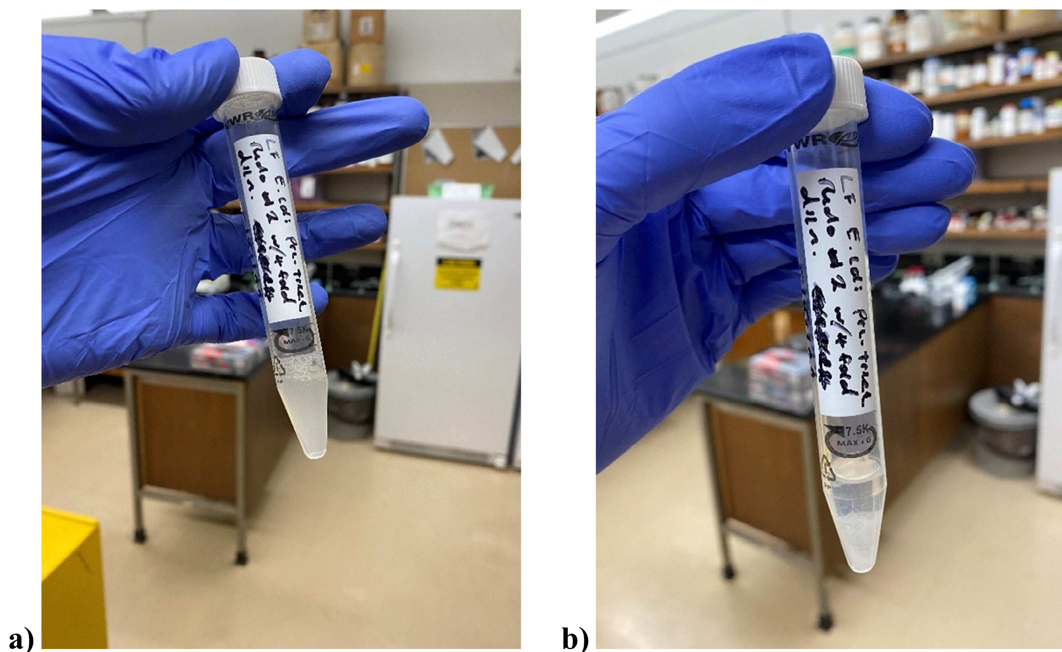


Figure 4. Representative photographs depicting the set-up and results for the artificial selection pre-treatment of the bacteria. The same treatment process was used for *E. coli* (pictured here), MRSA, and MSSA. The photo on the left is showing the bacterial cells suspended in saline and a sub-MBC dose of the disinfectant containing the active ingredient: benzalkonium chloride at the beginning of the treatment process. Note that the solution is homogenous. The photo on the right is showing an *E. coli* culture that had an aggregation of cells at the bottom of the tube following a 24 hour incubation in solution at room temperature. This was not observed in MRSA or MSSA after 24 hours, cultures of which still resembled the photo on the left. The time frame of 24 hours is relevant being that it was the maximum time that yielded surviving cells for all bacteria, excluding *E. coli* as it never yielded viable cells which lead to its removal from further experimentation.

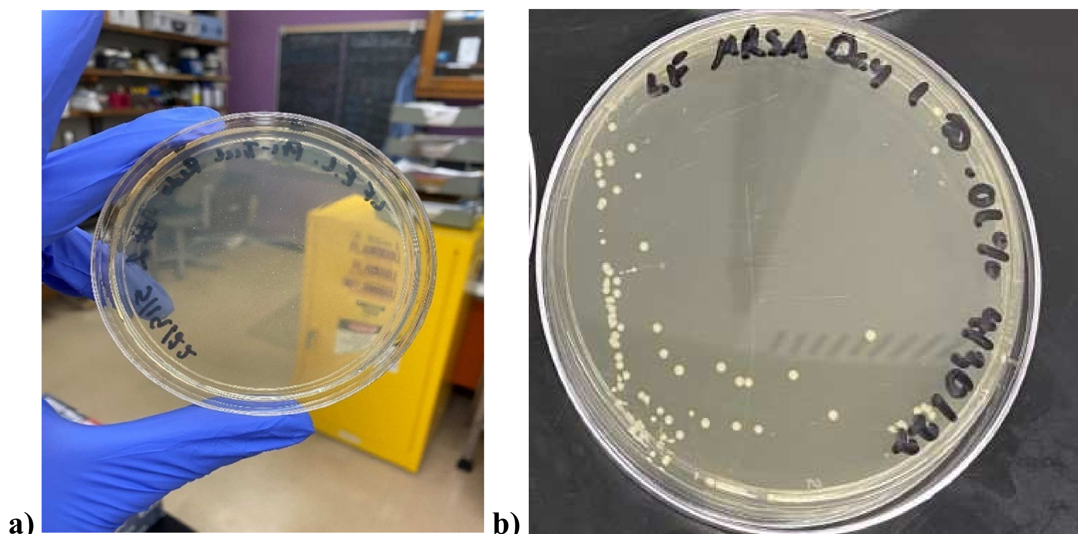


Figure 5. Representative photographs of LB agar plates inoculated with *E. coli* (left) and MRSA (right). Note that there is no photograph of MSSA included as its results were similar to MRSA. The photo on the left is the result of *E. coli* pre-treatment with a sub-lethal concentration of disinfectant following 24 hours of incubation at room temperature. Inoculum was obtained from disinfectant treatment solution (see Figure 4). There are no colonies visible on the plate indicating there were no viable cells present; this was repeated with the addition of multiple washing steps with 1x phosphate buffered saline (PBS), at lower concentrations of disinfectant, or both. The same result was obtained. On the right is the result of MRSA pre-treatment with the disinfectant following 24 hours of incubation at room temperature. Again, the inoculum was obtained from a disinfectant treatment solution. The PBS washing was not required here as viable cells were obtained without it for both MRSA as well as MSSA following 24 hours in the treatment conditions. However, washing step was attempted later in treatment process for MRSA and MSSA as well to see if there were any viable cells to be recovered and only *S. aureus* yielded viable cells. Therefore, only cultures from 24 hour incubation were used.

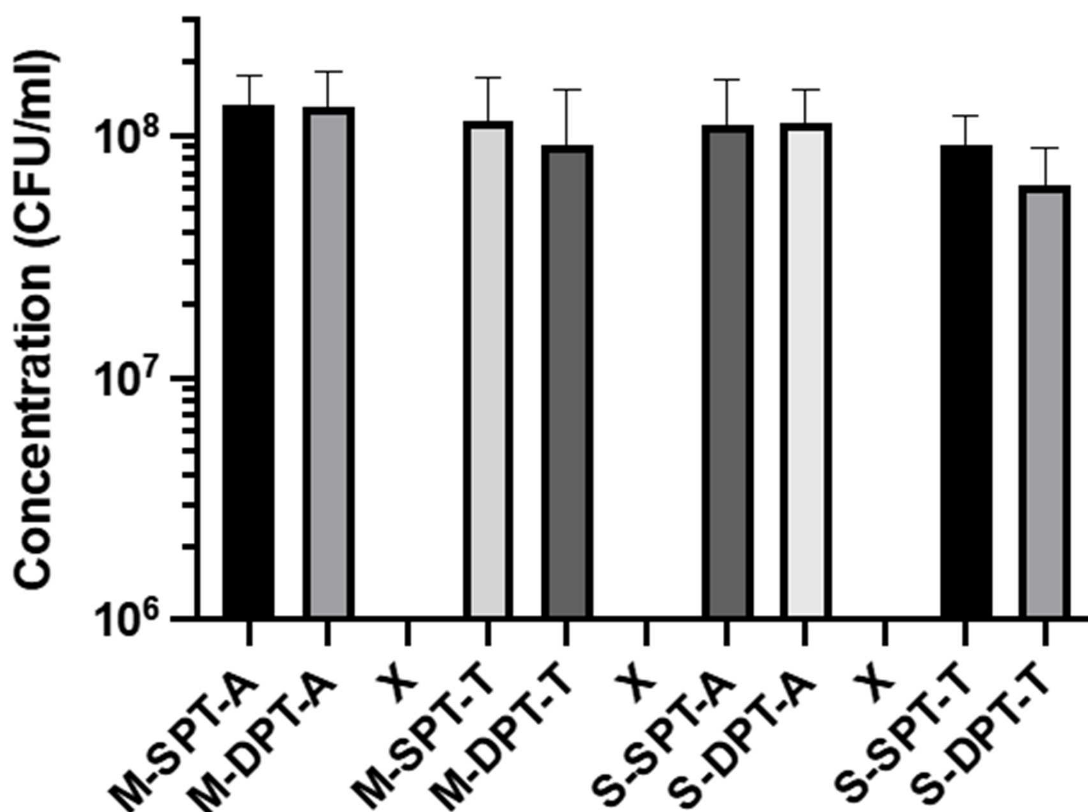


Figure 6. The result of antimicrobial exposure in terms of the concentration of cells (CFU/mL) that had remained viable for both MRSA and MSSA cultures. The MRSA cultures had a saline pre-treatment (M-SPT) which serves as a control and the second MRSA culture had a sub-lethal disinfectant pre-treatment (M-DPT) which serves as the experimental culture. The MSSA cultures followed a similar format to the MRSA cultures (S-SPT and S-DPT). The antimicrobials used were ampicillin (A) and tetracycline (T). Standard error and statistical significance/insignificance was calculated using the two-way ANOVA test with a value of $p < 0.05$.

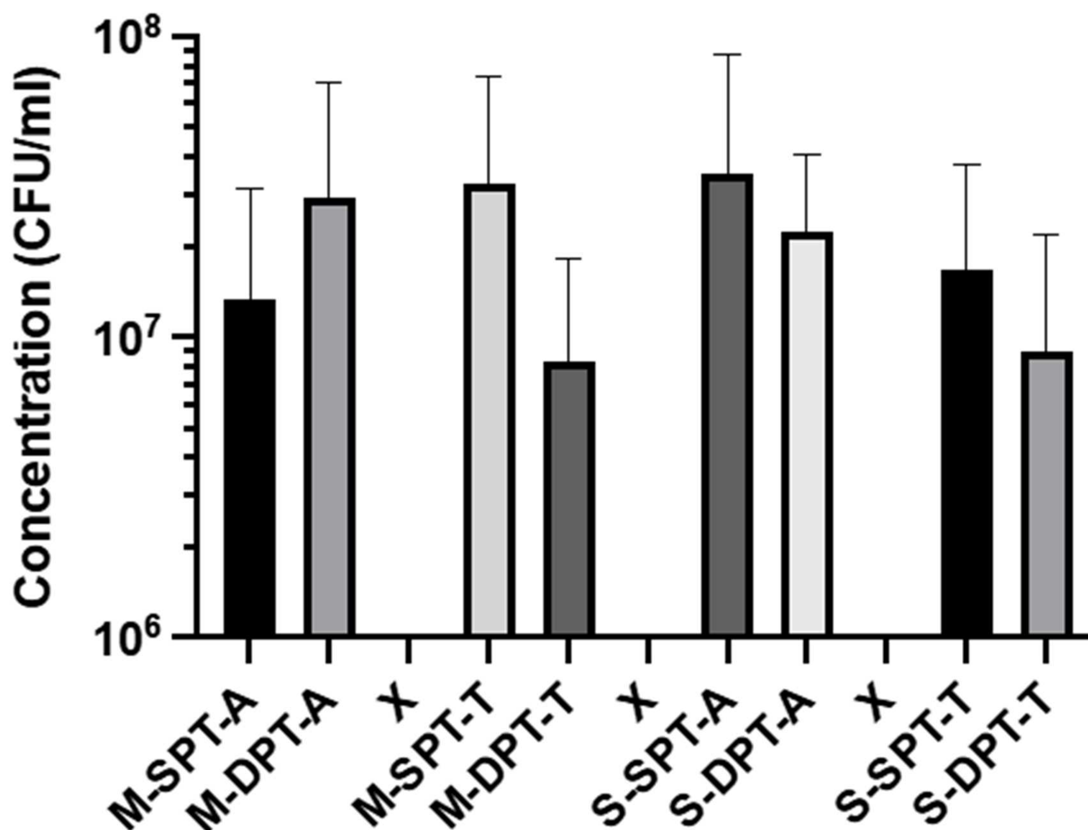


Figure 7. The result of antimicrobial exposure in terms of the concentration of cells (CFU/mL) that had been injured for both MRSA and MSSA cultures. The MRSA culture had a saline pre-treatment (M-SPT) which serves as a control and the second MSSA culture had a sub-lethal disinfectant pre-treatment (M-DPT) which serves as the experimental culture. The MSSA cultures followed a similar format to the MRSA cultures (S-SPT and S-DPT). The antimicrobials used were ampicillin (A) and tetracycline (T). Standard error and statistical significance/insignificance was calculated using the two-way ANOVA test with a value of $p < 0.05$.

6. Discussion

The continued development of antimicrobial resistance in multiple species of bacteria continues to pose a significant concern and risk to public health globally. Antimicrobials remain a primary care option in treating bacterial infections but with the increase in usage comes the risk of over-prescription of these medications by medical personnel, their misuse by the patient when prescribed, or both. Disinfectant tolerance is also a matter of public health concern in that the use of disinfectants in sanitation is a primary method used to prevent infections from occurring. This provides a useful manner in which to combat the development of antimicrobial resistance. If a bacterial infection is prevented, then no antibiotics need to be prescribed in order to cure that infection. However, it is not clear if or to what extent the development of tolerance towards disinfectants by bacteria may influence the development of tolerance to antimicrobials. The goal of this research was to evaluate whether disinfectant tolerance had a noticeable effect to antimicrobial tolerance levels based on disinfectant tolerance developed through adaptive mutation.

In our first hypothesis we posited that the response among the three organisms used in this study (*E. coli*, MSSA, and MRSA) would share similar results, i.e. they all were going to exhibit an increased tolerance to antimicrobials following disinfectant treatment than control cultures that received no disinfectant treatment. We reasoned that the mechanisms for adaptive mutation would be similar in all three species.

However, the results we observed did not fully support this hypothesis. Initial data obtained in the determination of the MBCs of these bacteria to both the disinfectant and antimicrobials yielded divergent results. Evidence suggests that different disinfectants do not all share the same spectrum of efficacy when dealing with different genera and/or species of bacteria (34). This can occur within the same class of disinfectants as well. For example, different quaternary ammonium compounds have different molecular structures and this can influence their efficacy against different bacteria (35). Other factors may also affect disinfectant efficacy such as the presence of proteins, varying levels of water hardness, etc. (35). Therefore, it is not surprising to see these divergent results among *E. coli*, MRSA, and MSSA. These initial data also suggest that these bacteria may have intrinsically different levels of tolerance to these antimicrobials at the onset of this study. In terms of ampicillin and tetracycline, these are considered broad spectrum antimicrobials, so they work against a larger variety of bacteria. However, it is accepted that not all bacterial species respond to antimicrobial treatment in the same manner (36). This provides more support to the data obtained in this study illustrating the divergence in response to benzalkonium chloride, ampicillin, and tetracycline between the three bacteria in this study.

When the cultures were treated with sub-lethal concentrations of the disinfectant, this likewise revealed that each of the bacteria responded differently. Both MSSA and MRSA were able to withstand a 24 hour incubation period in a sub-lethal concentration of the disinfectant. However, *E. coli* was unable to survive these same conditions; even through repeated attempts with incorporating additional washing steps, no viable *E. coli*

cells could be obtained. As a result, *E. coli* was removed from further experimentation. How *E. coli* responds to antimicrobial exposure following sub-lethal disinfectant treatment exposure remains to be determined. MSSA and MRSA cultures were subjected to exposure to both ampicillin and tetracycline following sub-lethal disinfectant treatment. The results obtained were not consistent with our first hypothesis. First and foremost, when compared with controls, those cultures that were pre-treated with disinfectant were more susceptible to ampicillin and tetracycline than those cultures that weren't. This suggests that the pre-treatment had the opposite effect of what was hypothesized, and the cultures became more susceptible to the antimicrobials rather than less susceptible. This may be due to that those cells that had survived disinfectant treatment were injured and that injury made it easier for the antimicrobials to enter those cells.

There was a significant decrease in viability between saline treated cultures and disinfectant treated cultures when compared to cultures that had undergone no treatment (control). This result was expected. When looking at the saline cultures and disinfectant treated cultures, there were similarities between these two cultures with respect to the antimicrobials used. The proportion of bacteria that were viable following ampicillin treatment in these cultures was similar for both MSSA cultures. No substantial differences between the saline controls and the disinfectant treated cultures were observed, but there was a difference in the proportion of cells that was injured. MRSA showed a decrease in injured cells in the saline control cultures but similar values in the disinfectant treated cultures when compared to MSSA cultures in the same conditions.

This was interesting because MRSA is known to be resistant to β-lactam class antimicrobials and the MSSA used was considered β-lactam sensitive. This may have been a consequence of disinfectant treatment. It may be that the disinfectant which targets the cell wall of bacteria had made the organism more susceptible to injury by the β-lactam, which also targets the cell wall. This may also have been a consequence of the MRSA culture being repeatedly subcultured in a lab prior to our having received it. Repeated subculturing ultimately weakens the organism through artificial selection, causing them to be less robust when encountering harsh conditions. A similar observation was seen in a MSSA culture obtained from the same lab in that, similar to *E. coli*, MSSA was unable to survive the sub-lethal disinfectant treatment. However, once we purchased a fresh MSSA culture from ATCC, which was also the source of the *E. coli* culture, the new MSSA culture then was able to survive the sub-lethal disinfectant treatment. Repeated subculturing is known to have an effect on both the genotype and phenotype of bacteria (37). These effects may positively or negatively affect the bacteria. In regard to this study, this indicates that repeated subculturing "weakened" the bacteria due to greatly reduced selective pressure as compared with normal host-microbe interactions.

When comparing the results between the MRSA and MSSA cultures following tetracycline exposure, the differences between the two cultures were more pronounced than they had been for the ampicillin exposure. The proportion that remained viable after tetracycline treatment following disinfectant pre-treatment had decreased relative to saline controls and was noticeably lower than that observed for MSSA. Regardless, there was no significant difference with respect to cell injury following exposure of both to

tetracycline among saline and disinfectant treated cultures. These results suggest that disinfectant pre-treatment seems to have increased the tolerance of MRSA towards tetracycline. The opposite can be stated for MSSA; it appears to have become more susceptible rather than more tolerant to tetracycline.

Therefore, our second hypothesis, which states that the response of the bacteria towards developing tolerance to ampicillin and tetracycline was going to be directly influenced by the disinfectant pre-treatment is only partially supported. In MRSA and MSSA, we did not observe that the disinfectant had a significant direct effect on these cultures. For MRSA, there was no difference between the number of cells killed between the saline controls and those killed following disinfectant treatment. This suggests that the cells actually became more susceptible rather than more tolerant. For MSSA, there were no changes in the number of cells killed or injured in the disinfectant treated cultures relative to the saline controls which indicated that the disinfectant had no effect.

However, for tetracycline, as mentioned above, MRSA did have fewer cells that were injured when MRSA was treated with disinfectant. Thus, it appears that the disinfectant had some effect on the ability of MRSA to resist tetracycline. While MSSA culture did also show a reduction in injured cells among those surviving disinfectant treatment, more disinfectant treated MSSA were killed with exposure to tetracycline. This indicates that the disinfectant treatment had an effect opposite what was predicted by our hypothesis, mainly, making MSSA more susceptible to tetracycline. When analyzing these results, it is important to note that according to the ATCC website, the MRSA strain having the designation of ATCC 43300 is not considered to be resistant to

tetracycline. However, as with many antimicrobials, bacteria often contain genes or can acquire genes necessary to become resistant to an antimicrobial and tetracycline is no exception. Therefore, it is possible that the artificial selection with a sub-lethal treatment of a disinfectant did provide the conditions needed for MRSA to become more tolerant to tetracycline as the data suggests.

Another aspect to consider is the differences in the cell walls between MRSA and MSSA. There is some evidence that suggests that MRSA does have a thicker cell wall than MSSA (38). It is not clear if this feature extends to all strains of MRSA or if it is unique to certain strains or only those cultures that experience specific conditions conducive to this adaptation. There are multiple mechanisms hypothesized to responsible for the thickening of the cell wall in MRSA with the most notable for this research being when the organism is in the presence of tetracycline (38). Tetracycline, a protein synthesis inhibitor, is able to, as the name suggests, inhibit the synthesis of many proteins. The inhibition of protein synthesis can cause a reduced amount or complete absence of kinases and phosphatases which can cause deformity in the peptidoglycan resulting in a thicker cell wall (38). It is possible that the sub-lethal disinfectant treatment given to the MRSA culture in this research may have also caused thickening of the cell wall and therefore increased the tolerance of MRSA to tetracycline.

To translate this research into further investigation, one should begin by reviewing the protocols used in this research and critically evaluate how they can be improved. One particular aspect under consideration is how to get a broader perspective of the population investigated. In this project, we had repeatedly tested a single colony

that descended was derived from the same parent culture. Selecting one colony means that we assumed all members of the population will respond the same way. However, if adaptive mutation is the mechanism underlying our observations, each member is likely to try to solve the "problem" differently compared to all others. This means that each individual may reach the same endpoint, i.e. increased tolerance, via different paths taken to reach that endpoint.

The utmost care was taken to ensure that the same concentration of cells was used during each independent trial. However, by using colony counts as the basis of retrieving my data, there are sources of error including human error, as well as inconsistencies from batch to batch in medium that can't be ignored. Although, care was also taken to make the media as consistently as possible, any variations in the media formulations may have had an effect on the bacterial cultures as well. For future experiments, it may be wise to complement the results of this research by repeating the same procedures but instead of using colony counts to determine the proportion of the population that had been killed or injured, flow cytometry and live/dead staining may be substituted instead, or through other physiological means. This may provide a more accurate representation of the population and also give some indication in to the degree of the injury to the cells as well.

Lastly, it would be intriguing to do genetic analysis on the colonies that are able to survive sub-lethal disinfectant exposure and sub-lethal antimicrobial exposure. It would provide some insight as to how, for example, the gene expression may have been altered in a culture that had received sub-lethal disinfectant exposure relative to a control culture that had received no disinfectant exposure. Then once that has been observed,

then expose the cultures to sub-lethal doses of the antimicrobials as well and do additional genetic analysis and determine if there have been any significant changes in these cultures.

In making these provisions in future experiments, a better understanding may be obtained regarding the effect of disinfectant tolerance levels on survival. Additionally, it could provide valuable insight into their ability to develop tolerance to antimicrobials through adaptive mutation under these specific conditions. For example, if the process of adaptive mutation is entirely random or is there a strict hierarchical mutational pathway that is followed to ensure the most beneficial mutation is the result.

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Appendices

Appendix A: Media and Stock Solution Preparation

LB agar

Peptone	10 g
Yeast Extract	5 g
Sodium chloride	10 g
Agar	12 g
dH_2O	1000 mL

Mix components together, check pH (pH ~ 7.4) and then autoclave. Agar can be stored either at room temperature or in the refrigerator (~ 5□).

Hardy Diagnostics Brain Heart Infusion (BHI) broth

Casein Peptone	14.5 g
Brain Heart Infusion from Solids	10.0 g
Animal Tissue Peptone	5.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Dextrose	2.0 g
dH_2O	1000 mL

Thoroughly mix the pre-mixed powder formula in water, check pH (pH ~ 7.4) and then autoclave. Broth media stored at room temperature.

acuMedia Eosin Methylene Blue (EMB), Levine Agar

Enzymatic Digest of Gelatin	10 g
Lactose	10 g
Dipotassium phosphate	2 g
Eosin Y	0.4 g
Methylene Blue	0.065 g
Agar	15 g
Sucrose	5 g
dH_2O	1000 mL

All ingredients, except for sucrose, is in pre-mixed powder form. Sucrose and pre-mixed powder formula added to water and mixed thoroughly. Check pH (pH ~ 7.1). Autoclave and then store either at room temperature or in the refrigerator (~5□).

0.85% Saline Stock Solution

Component	Amount
Sodium chloride	8.5 g
dH_2O	1000 mL

Add 8.5 g NaCl to dH_2O, mix thoroughly, check pH (pH ~ 7.0) and then autoclave. Store at room temperature.

80% Glycerol Freezing Stock Solution

Component	Amount
Glycerin	80 mL
dH_2O	20 mL

Add components together, mix thoroughly, and autoclave. Store in the refrigerator at ~5□.

Hardy Diagnostics Mannitol Salt Agar

Component	Amount
Agar	15.0 g
Beef Extract	1.0 g
Mannitol	10.0 g
Sodium chloride	75.0 g
Phenol Red	25.0 mg
Proteose Peptone	10.0 g
dH_2O	1000 mL

Add 116.75 g of pre-mixed powder formula to 1000 mL of dH_2O. Mix thoroughly and check pH (pH ~ 7.4) . Autoclave. Stored at room temperature or refrigerate (~5□).

Antimicrobial Stock Solutions

Antimicrobials
(Ampicillin Trihydrate, Penicillin G, Streptomycin Sulfate, Tetracycline, Sulfanilamide, and Ciprofloxacin)
dH_2O

Begin by filtering dH_2O and while working under laminar flow hood, add the appropriate amount (mg) of antimicrobial to the appropriate amount of water for a final concentration of 1 mg/mL and mix thoroughly. After mixing, quickly ration the stock solution for each antimicrobial in to 1 mL aliquots and place in the freezer (-20□).

Appendix B: Experiment Protocols

Sub-Minimum Bactericidal Concentration (sub-MBC) Determination (Antimicrobial/Disinfectant)

1. All following steps are performed under sterile conditions of HEPA-filtered laminar flow hood. Laminar flow hood sterilized before and after by wiping work surface with 70% ethanol followed by 15 minutes of UV light.
2. Remove bacteria of interest in nutrient broth/40% glycerol solution from -80°C storage and streak for isolated colonies on a plate of LB agar. Do not allow cultures to thaw out when inoculating. Incubate overnight at 37°C.
3. Remove from incubator and use a single isolated colony from LB agar to inoculate BHI broth. Incubate overnight at 37°C.
4. Remove BHI broth culture from incubator and 1 mL to sterile 1.5 mL microcentrifuge tube.
5. Spin culture(s) at 15,000 x g in centrifuge for 60 seconds to pellet the cells.
6. Remove the supernate into a waste beaker and add 1 mL of antimicrobial or disinfectant (at various concentrations) to the pelleted cell cultures. Note: when working with antimicrobials, keep them on ice as much as possible until ready to add to cultures.
7. Resuspend cells and incubate for 60 minutes at room temperature in disinfectant/antimicrobial solution.
8. Set up 96-well microplate by adding 200 uL of BHI inoculum that received no antimicrobial or disinfectant exposure as a control. Add 200 uL of uninoculated BHI broth to serve as a blank. Then add 200 uL of sterile BHI broth and inoculate with bacteria exposed to the various antimicrobial or disinfectant concentrations.
9. Cover the 96-well microplate to prevent airborne contamination and incubate overnight at 37□.
10. After incubation, remove from the incubator and look for the well that contains the growth at the highest concentration of antimicrobial or disinfectant for the sub-MBC. The sub-MBC is defined as the highest concentration that has allowed bacterial growth.
11. If no sub-MBC is apparent, repeat at different concentrations until sub-MBC can be determined.

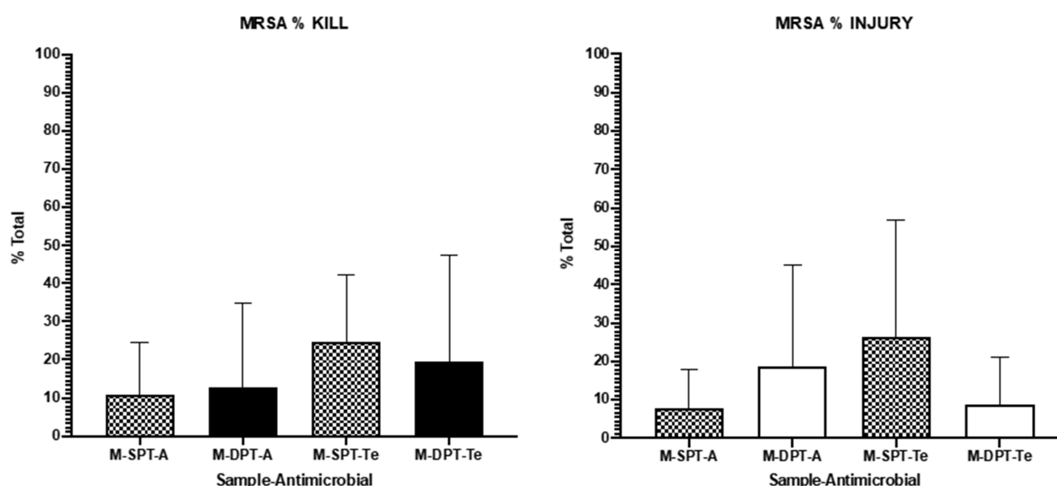
Cell Death, Injury, and Viability Determination (Perform Following Selection Protocol)

1. All following steps are performed under sterile conditions of HEPA-filtered laminar flow hood. Laminar flow hood sterilized before and after by wiping work surface with 70% ethanol followed by 15 minutes of UV light.
2. Remove BHI broth/40% glycerol cultures from -80℃ storage. (cultures include a control with no pre-treatment, placebo that received saline only pre-treatment, and cultures that survived disinfectant pre-treatment)
3. Inoculate the cultures on LB agar, streak for isolated colonies. Do not allow the cultures to thaw out when inoculating. Incubate overnight at 37℃.
4. Remove from incubator, take one colony from each plate and inoculate BHI broth for each culture. Incubate overnight at 37℃.
5. Remove BHI cultures from the incubator.
6. Add 1 mL of each culture to a 1.5 mL microcentrifuge tube and centrifuge at 15,000 x g for 60 seconds to pellet cells. Remove supernate.
7. Remove antimicrobials from -20°C and add 1 mL of the previously determined antimicrobial sub-MBCs to each culture (when working with antimicrobials, keep on ice as much as possible until ready to add to cultures). Incubate cultures with antimicrobials for 60 minutes at room temperature.
8. Centrifuge cultures at 15,000 x g for 60 seconds to pellet cells. Remove supernate.
9. Add 1 mL of fresh BHI broth to each culture and resuspend cells.
10. Adjust cell density via turbidity using 0.85% saline buffer to determine the initial approximate cell concentration and adjust cell concentration using McFarland standards as a reference. All cultures were adjusted to a #2 McFarland standard ($6x10^8$ CFU/mL).
11. Perform serial 100-fold dilutions of the cultures, using 0.85% saline buffer, to a final dilution factor of 10^{-4}.
12. Add 10 uL of 10^{-4} dilutions to properly labeled non-selective (LB agar) and selective media (EMB agar or mannitol salt agar depending on bacteria) for spread plating.
13. Incubate the spread plates overnight at 37℃.
14. Count the colonies, calculate CFU/mL, and use these values to determine via calculation the % viable, % killed, and % injured of the bacterial cultures.

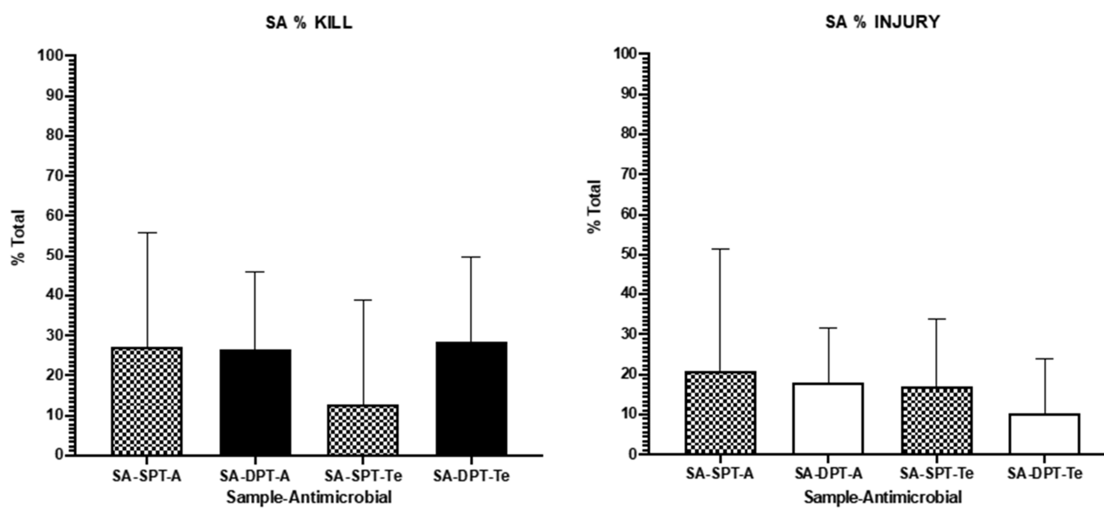
Selection of Antimicrobial Tolerance

1. Preparation of cultures performed under sterile environment of HEPA-filtered laminar flow hood. Laminar flow hood sterilized before and after by wiping work surface with 70% alcohol (ethanol or isopropanol) followed by 15 minutes of UV light exposure.
2. Remove bacteria of interest in nutrient broth/40% glycerol solution from -80°C storage and streak for isolated colonies on a plate of LB agar. Do not allow cultures to thaw out when inoculating. Incubate for overnight at 37°C.
3. Remove from incubator and use isolated colony from LB agar to inoculate BHI broth. Incubate again overnight at 37°C.
4. Prepare two-fold disinfectant dilutions (1 mL disinfectant to 1 mL 0.85% saline) for each disinfectant. Dilute the disinfectant to the concentration that matches the previously determined MBC for that organism. This will be done for each disinfectant.
5. Add 1 mL of BHI culture to empty and appropriately labelled 1.5 mL microcentrifuge tubes. Centrifuge cultures at 15,000 x g for 1 minute to pellet the cells. Remove supernate in waste beaker.
6. Add 1 mL of 0.85% saline to each of the microcentrifuge tubes containing the cell pellets. Vortex to resuspend cells and then transfer entire solution to a sterile 15 mL centrifuge tube.
7. Add the MBC of each disinfectant solution to the appropriately labelled 15 mL centrifuge tube that now contains 1 mL of bacterial cells resuspended in 0.85% saline. This serves as the final two-fold dilution to get the disinfectant concentration a single two-fold dilution lower than the previously determined MBC for each disinfectant.
8. Gently mix thoroughly by inversion and then leave cultures to sit for a maximum of 5 days at room temperature. Repeat gentle inversion each day so that cells are constantly being resuspended in the solution.
9. During the 5-day pre-treatment process with a sub-MBC of the disinfectant, cultures will be inoculated after each day for the entire pre-treatment to keep track of cell viability. If no viable cells are being obtained with repeated attempts, the addition of a washing step with 1x phosphate buffered saline (PBS) may be needed.
10. If viable cells are present, collect one colony from "survivor" culture to serve as representative culture of the population and inoculate into BHI broth and incubate overnight at 37□. After incubation, use 80% glycerol stock solution to prepare 40% glycerol solution (0.75 mL 80% glycerol + 0.75 mL BHI culture) and mix in sterile cryotube. Tubes now are ready for long-term storage at -80°C for use in future experiments. Use the cells from each culture that survive the longest for future experiments (i.e. if MRSA and *E. coli* survive 5 days but *S. aureus* survives 3 days, use the Day 3 cultures from each culture).

Appendix C: Supplemental Figures



Supplemental Figure 1. The result of antimicrobial exposure in terms of the proportion of cells that had been killed and of those cells that were not killed, the proportion of cells that were injured for two MRSA cultures. The first MRSA culture had a saline pre-treatment (M-SPT) which serves as a control and the second MRSA culture had a sub-lethal disinfectant pre-treatment (M-DPT) which serves as the experimental culture. The antimicrobials used were ampicillin (A) and tetracycline (Te). Note that there was a third culture of MRSA which received no disinfectant pre-treatment (M-NPT) that was exposed to each antimicrobial as well, and this served as a control for both the M-SPT and M-DPT cultures when calculating the percentage of killed cells. The values for M-NPT were not included in the graphs above because the percentage of kill could not be calculated for this culture because it already was serving as the negative control with which the other cultures were based on for these values. The percentage of injured cells was calculated within each culture using growth values from a non-selective media and selective media for each.



Supplemental Figure 2. The result of antimicrobial exposure in terms of the proportion of cells that had been killed and of those cells that were not killed, the proportion of cells that were injured for two MSSA cultures, labeled as "SA" in graph titles. The first MSSA culture had a saline pre-treatment (SA-SPT) which serves as a control and the second MSSA culture had a sub-lethal disinfectant pre-treatment (SA-DPT) which serves as the experimental culture. The antimicrobials used were ampicillin (A) and tetracycline (Te). Note that there was a third culture of MSSA which received no pre-treatment (SA-NPT) that was exposed to each antimicrobial as well, and this served as a control for both the SA-SPT and SA-DPT cultures when calculating the percentage of killed cells. The values for SA-NPT were not included in the graphs above because the percentage of kill could not be calculated for this culture because it already was serving as the negative control with which the other cultures were based on for these values. The percentage of injured cells was calculated within each culture using growth values from a non-selective media and selective media for each.