Drew University

College of Liberal Arts

Effects of Essential Oils on Both Planktonic and Biofilm *Staphylococcus epidermidis* and *Escherichia coli* Persister Cells

A Thesis in Biochemistry

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Submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science

May 2025

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Abstract

Infections caused by antibiotic-resistant bacteria have led to a health crisis that worsens yearly in the United States. It is expected that death rates will continue to because development of bacterial resistance outpaces the discovery of new classes of antibiotic drugs. Major causes of the antibiotic-resistance crisis are persisters and bacterial biofilms. These persister cells account for a small portion of the bacterial population that remain hibernated. They do not respond to antibiotic stress, and they survive antibiotic treatment. Biofilm structures on internal medical devices create an environment suitable for persister cell survival. These biofilms provide encapsulation and protect persister cells from antibiotic treatment, leading to internal infection. The use of essential oils as a treatment method against these persisters and biofilm structures. was explored in this project. The treatment of planktonic persister cells in both *S. epidermidis* and *E. coli* bacteria with the essential oils lavender, geranium, and ylang-ylang results in complete eradication of persister cells at the concentrations of 0.5%, 1%, and 2.5% at various treatment times. The effect of essential oils on biofilm structures of *S. epidermidis* and *E. coli* bacteria was also examined. Geranium and ylang-ylang oil most effectively eliminated these biofilms at 4% and 8% concentrations at 24 and 48 h treatment time. Lavender essential oil still needs further investigation since the impact of biofilms was not conclusive.

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Chapter 1: Introduction

1.1 Historical Perspective of Drug Discovery and the Antibiotic Crisis

Antibiotics are antibacterial substances that suppress the growth of bacteria. These therapeutic agents can be natural products or synthetic and are used to treat microbial infections. The antibiotic crisis is an issue that has become significantly problematic over the last few years. [1] More and more bacteria are rapidly becoming antibiotic-resistant due to the misuse and over-prescription of antibiotic drugs. [1] A direct correlation exists between over-prescription and the generation of resistant bacterial strains. [2] Before antibiotic resistance emerged, the discovery of antibiotics as a treatment revolutionized the modern medical world.

The first antibiotic discovered was penicillin in 1928 by Alexander Fleming. However, penicillin did not become a commercially used drug until a decade after its discovery. In 1932, Gerhard Domagk discovered an antibiotic known as sulfonamide, which became the first commercially used antibiotic. Sulfonamide was used to kill *Streptococci* infections and was observed to selectively kill bacteria in the prontosil pathway in humans. [3] This discovery led to more antibacterial agents, such as sulfanilamide, sulfapyridine, and sulfacetamide. These agents were used from 1938 to 1942 to treat bacterial infections during World War II. [3] When tested on infected mice, Howard Florey determined that the fungus *Penicillium* could combat *Staphylococci* and other Gram-positive bacteria during this time. [4] The tested mice were infected with three species of bacteria: *Streptococcus pyogenes, Staphylococcus aureus,* and *Clostridium septicum.* Florey's experiments found that the mice injected with the bacterial strain, followed by the *Penicillium,* survived [4]. This experiment series led to the manufacture of penicillin by Howard Florey and Ernst Chain in the 1940s. This antibiotic was explicitly designed to treat various bacteria, leading to its classification as the first narrow-spectrum antibiotic. [5]

Following this breakthrough, Selman Waksal discovered the antibiotic streptomycin in the 1940s. [6] Historically, the prime time for antibiotic advancements occurred between 1950 and 1960 (Fig. 1). Streptomycin was developed to combat aerobic Gram-negative bacteria that cause tuberculosis and brucellosis. [7]

Research involving other antibiotics was also pursued during this same time. For instance, these antibiotics included streptomycin, chloramphenicol, tetracycline, erythromycin, vancomycin, methicillin, ampicillin, and cephalosporins (Fig. 1). These drugs were all discovered between the years 1943 and 1960 (Fig. 1). Ciprofloxacin was developed in the year 1978 (Fig. 1). Ofloxacin, a structurally related fluoroquinolone antibiotic, was discovered in 1979 (Fig. 1) by Hayakawa and is used to treat both Gram-negative and Gram-positive bacterial infections. The FDA approved ofloxacin as a drug that could help combat *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, all of which commonly cause ear infections. Ofloxacin also effectively treats corneal ulcers, skin irritation, and diseases caused by *Staphylococcus aureus* and *Streptococcus pyogenes* bacteria. These two antibiotics began to develop into a class of fluoroquinolones.

Since this period of rapid discovery, the pace of antibiotic development has stalled (Fig. 1). There has not been a significant advancement in a class of antibiotics. The lack of new antibiotic approvals is concerning from a public health perspective, as microbes continue to mutate in response to both evolutionary and man-made pressures. The overuse of antibiotics by physicians and the general misuse by individual patients contribute to the antibiotic-resistance problem. The more exposure bacteria have to an antibiotic, the more likely they are to become resistant. However, antibiotic resistance is merely one of the main reasons for the lack of drug

development and discovery. Another key factor influencing the development of antibiotic classes is financial responsibility.

The discovery of antibiotics is a slow and expensive process. Due to the high research costs and the potential for low returns, many pharmaceutical companies are hesitant to pursue research projects involving antimicrobials. However, one favorable development is that the zosurabalpin antibiotic was approved for clinical trials in 2024. It combats the bacteria *Acinetobacter baumannii* by preventing the LptB2FGC protein complex, a structure powered by ATP that contributes to the outer cell wall specific to Gram-negative bacteria. This complex helps synthesize and transport lipopolysaccharides, a unique feature of Gram-negative bacteria, quickly and efficiently. This drug also treats pneumonia and sepsis in disease-induced mice. However, this antibiotic has specificity for bacteria involving the LptB2FGC complex, which applies to *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*, as these are all Gram-negative bacterial strains.

Another antibiotic class that has been discovered is teixobactin, which was found to attack and destroy bacteria by destroying the cell wall (Fig. 1). [16] This antibiotic was found to eradicate bacteria such as methicillin-resistant bacterial strains, *Staphylococcus aureus, Streptococcus pneumoniae,* and vancomycin-resistant bacteria. [16] The teixobactin antibiotic class was discovered in 2015, but was not released and approved by the FDA until 2017. [17] This antibiotic class was developed to target the cellular wall of bacteria, specifically the production of peptidoglycan, thus targeting lipid II. This anchoring lipid helps the production of peptidoglycan in the bacterial cell wall. [16] Teixobactin and its attack mechanism targets lipid II, thus preventing the production of peptidoglycan, essential for forming prokaryotic cell membranes. [16] It is important to note that both Gram-negative and Gram-positive bacteria have

peptidoglycan in their membranes, thus making teixobactin class a broad-spectrum antibiotic. [16] Teixobactin has proved useful and is not toxic to eukaryotic cells. This is because teixobactin can identify a bacterial cell; lipid II is only present in bacterial cell membranes. [16] The discovery of this new antibiotic drug gives hope that a new class of broad-spectrum antibiotics will be developed soon. [14] The timeline of antibiotic discovery has been slow throughout history (Fig. 1); however, there is an increase in urgency, especially since the major crisis in the current century.

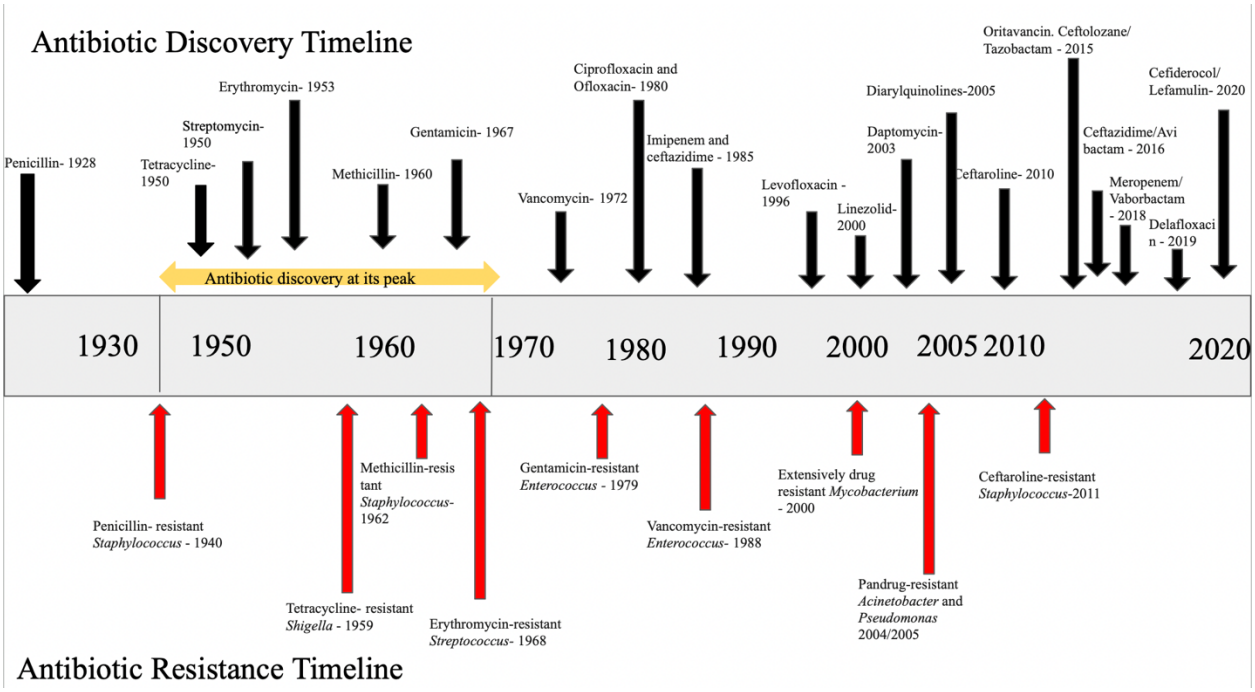

Figure 1: Timeline of Drug Discovery and Antibiotic resistance among different bacterial organisms. Adapted from refs. 8, 11,13,14, 15, and 16.

With the discovery of individual antibiotic drugs, an organization system has been developed to group these antibiotics based on standard killing mechanisms (Fig. 2). These groups are known as antibiotic classes. [18] [19] The fluoroquinolone antibiotic class targets DNA

gyrase, an enzyme required for bacterial DNA replication (Fig. 2). [18,19] β-lactams, another antibiotic class, specifically targets the cell wall as its mechanism of action (Fig. 2). [20] The lipopeptides antibiotic class targets different membrane functions within a bacterial cell (Fig. 2). [20] These membrane functions include molecular transport and maintaining structural integrity. [20] These cellular mechanisms include DNA synthesis, protein synthesis, and mitotic division (Fig. 2). These different antibiotic classes contain antibiotic treatments that target either vital cell mechanisms or cell structures such as the cell membrane or wall (Fig. 2). [21] Antibiotics are designed and categorized based on their ability and mechanism of destroying a given bacterial cell thus, stressing their importance in flighting infections disease (Fig 2).

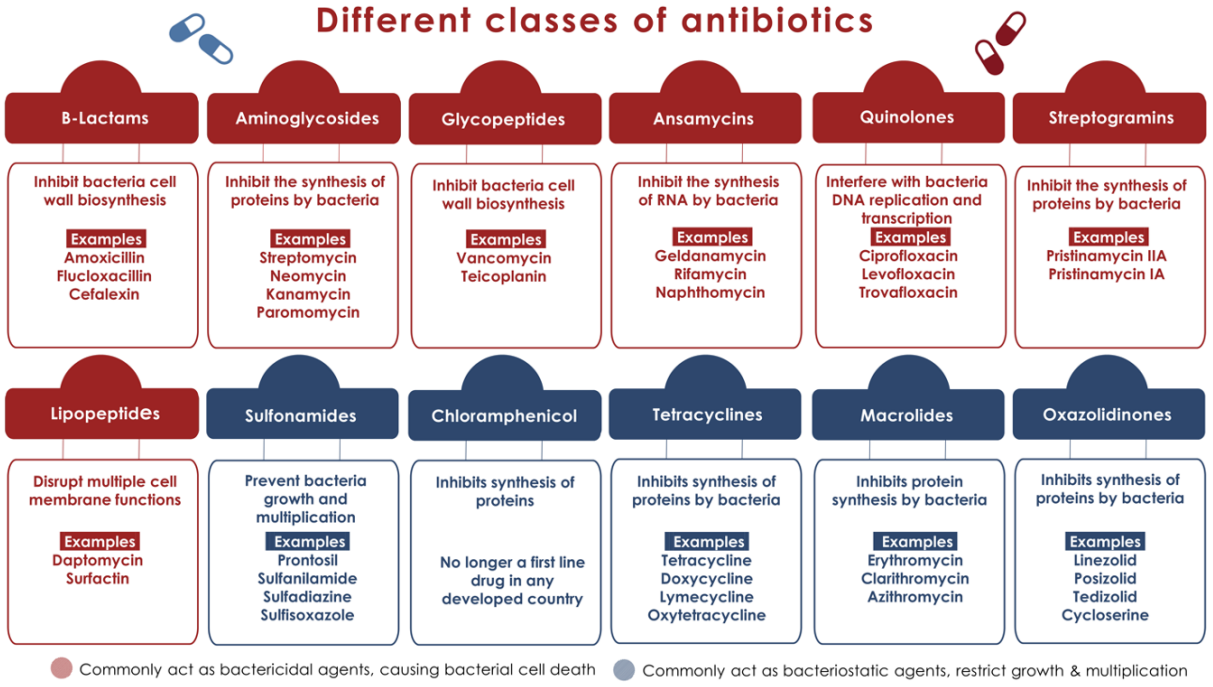

Figure 2: The different antibiotic classes and their mechanism of action. Figure from ref. 20.

1.2 Antibiotic/Multidrug-resistant bacteria (MDR)

Beginning in the late 1950s, antibiotic resistance emerged as a threat to human health. As a result of antibiotic-resistant bacteria, 700,000 people die each year worldwide. In 2019, the World Health Organization ranked antibiotic-resistant bacteria among the top 10 leading causes of human mortality. That same year, the World Health Organization assessed this issue by stating that, "the effects of the antibiotic crisis were responsible for killing 1.27 million people worldwide and were predicted to be the probable cause of nearly 5 million total deaths."

These statistics are even more daunting when considering the impact of the Coronavirus pandemic. The Centers for Disease Control and Prevention states that from 2021 to 2022, the number of antibiotic-resistant bacteria drastically increased by 20% due to the pandemic caused by the Coronavirus. Due to the overabundance of patients being hospitalized and treated for this virus, a spike in antibiotic use has yielded more resistant bacterial strains. A model predicts that these strains could be the precursor to a "silent pandemic." This "silent pandemic" is expected to cause approximately 10 million annual deaths if this crisis is not resolved. Both genetic mutations of binding proteins and gene adaptations are also responsible for causing antibiotic resistance in these AMR bacteria.

Antibiotic-resistant bacteria (AMRs) can be classified into three subcategories based on their effect on the human body. They can be classified as either urgent, serious, or concerning. [22] The most dangerous AMRs include *Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus.* [23] These AMR bacteria are the most threatening as they are common in hospital settings and can be found on internal medical devices such as pacemakers and catheters.

[23] These infections travel from internal medical devices into a patient's bloodstream, leading to complications including sepsis and or death. [23]

AMRs can also be involved in causing repository infections. The AMR species bacteria responsible for some major respiratory illnesses include *Streptococcus pneumoniae*, *Haemophilus influenzae, Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. In 2005, Streptococcus pneumoniae caused 1.6 million deaths, and in 2023, it was responsible for 15% of total pneumonia cases reported in the United States. Penicillin resistance due to *Streptococcus pneumoniae* has resulted in hospitalizations. In addition, present-day *Streptococcus pneumoniae* has caused 60 deaths per 100,000 in the age bracket of 18-34, and 597 deaths per 100,000 in the age demographic of 65-74.

With the help of present-day modern medical advancements, such as vaccinations and other preventative measures, the death rates associated with AMR *Streptococcus pneumoniae* have lowered. Although these methods exclude the use of penicillin, they have evolved into other drug therapies such as β-lactam and macrolide dual therapies for non-critical patients in outpatient clinics. [25]

A bacterium can be resistant not only to one antibiotic but to multiple. These are multidrug-resistant (MDR) bacteria, including *Pseudomonas aeruginosa, E. coli,* and *Methicillin-resistant Staphylococcus aureus* (MRSA). [26] All these bacteria are responsible for causing a wide range of health complications. For example, *Pseudomonas aeruginosa* causes infections in the lower respiratory tract, specifically common in patients with asthma, Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD), and bronchiectasis. [27] Urinary Tract Infections (UTI) can be attributed to *E. coli* bacteria. [28] Post-surgical complications can be

attributed to the infection of *Methicillin-resistant Staphylococcus aureus* (MRSA), a bacterium spread through poor hygiene via direct skin-to-skin contact or contact with contaminated instruments. [26]All these AMR bacteria will cause major life-altering complications. [26]

S. epidermidis bacteria have also developed antibiotic resistance over time, especially to the antibiotic methicillin. [29] The methicillin resistance results from the genetic element known as chromosome *mec (*SCC*mec),* which contains the *mecA* gene, which encodes penicillin-binding proteins, PBP2a, and causes a decreased affinity for methicillin, leading to resistance. Currently, 10 different SCC*mec* structures have been identified in *S. epidermidis,* thus resulting in this bacterium has 10 different types of methicillin resistance.[29]

In addition to methicillin, *S. epidermidis* bacteria are resistant to rifamycin, fluoroquinolones, gentamicin, tetracycline, chloramphenicol, erythromycin, clindamycin, and sulfonamides. [29] The antibiotic resistance genes possessed by the bacterium have been plasmid-encoded; thus, the already established methicillin resistance leads to resistance to similar antibiotics.

S. aureus bacteria and *S. epidermidis* are in the same genus. Due to SCC*mec*, *S. aureus* is also resistant to methicillin and is known as community-associated methicillin-resistant *S. aureus* (CA-MRSA).[29] This process, also known as horizontal gene transfer, involves the sharing of genes, and the ability to pass the resistance off via association makes both bacterial species very dangerous. [29]

Treatment of *S. epidermidis* infections proves difficult with the rise of antibiotic resistance. [28,30] 80% of all *S. epidermidis* bacterial strains are resistant to methicillin. [30] Two antibiotic classes are known to be the most effective at helping to eradicate the bacteria *S.*

epidermidis: oxazolidinone and the quinupristin/dalfopristin group. [30] Another antibiotic not included in the methicillin group, vancomycin, was developed to be an end-all, be-all type of antibiotic, meaning that this strong antibiotic will help eradicate all multi-resistant bacteria. [30] However, a new antibiotic must be developed due to *S. epidermidis* and *S. aureus* bacteria becoming resistant to vancomycin. [30]

Some antibiotic-resistant bacteria have adopted the antibiotic resistance gene known as *Mobilized Colistin Resistance* (MCR-1). This gene causes even the last-ditch effort of antibiotic treatment to be ineffective. [24] The gene product provides a protective layer on the bacteria, preventing cell wall penetration of antimicrobial polypeptides. [24] Colistin, an antimicrobial peptide, binds to its target binding site, Lipid A, and causes a break in the cell membrane, resulting in cell death. [24] The MCR-1 gene allows Gram-negative bacteria to form a shield against colistin. [24] Polymyxin E recruits the phosphatidyl transferase enzyme, which is then carried to lipid A, and acts as a target site for antimicrobial peptides in antibiotics. [24] This phosphatidyl ethanolamine residue brought upon lipid A causes the binding affinity between colistin and Lipid A to decrease, creating an impermeable cell membrane and thus, an antimicrobial bacterium. [24 32] There have already been six variants of this gene in numerous antibiotic-resistant bacteria, including *E. coli, Salmonella, Shigella sonnei, K. pneumoniae, and E. aerogenes.* [32] The MCR-1 gene is spread via a plasmid, a circular DNA strand that can be easily transported between bacteria via modes of transformation, transfection, and horizontal conjugation. Due to the potential for rapid gene transfer, it is plausible that more bacteria will become Multidrug Resistant (MDR). The increase in bacterial populations becoming Multidrug resistant is a concern as the medical community considers the likelihood of a “silent pandemic”.

1.3 The Issues of Drug Discovery and Production

For the Federal Drug Administration (FDA) to approve and develop a drug, it was estimated that the total cost in 2017 was about 1.5 billion dollars. For a drug to receive FDA approval, a considerable amount of time, money, and preapproval testing is required before the drug is released into the market. With the FDA and drug development being so expensive, the amount of money pharmaceutical companies recoup from developing a new antibiotic was estimated in 2017 to be about $46 million. Not only does a significant amount of money go into inventing a new drug, but the motivation for these companies to develop such drugs is also low. This is due to big pharma companies' limited profit in return. To further complicate this issue, when big pharma companies do develop a new antibiotic that appears to be the best available, it often does not become the first choice for bacterial treatment. The more an antibiotic is exposed to bacteria, the more likely resistance will develop. Because of this risk, new effective antibiotics are not prescribed as the first line of defense due to their exposure risk. As a result, quality antibiotics are more likely to remain on the shelves and only be used as a last resort treatment method.

The challenges of making a new drug are extensive, requiring lots of preplanning and thoughtful consideration. Big pharma companies need to synthesize and test a new drug via clinical trials to find a successful, non-toxic concentration in the body. [13] However, with such a low return, the motivation for big pharma companies to continue development is becoming lower. It is also becoming a massive problem with how fast bacteria are becoming resistant to antibiotics. This rapid resistance makes it challenging to produce an antibiotic, as bacteria become resistant faster. To make matters worse, even when an antibiotic drug is created, it must be reformulated to combat the antibiotic resistance that eventually occurs. [13]

1.4 Bacteria Staphylococcus epidermidis and Escherichia coli

In this work, three different essential oils are examined as a method of treating planktonic culture and biofilm structures of Gram-negative and Gram-positive bacteria, *Staphylococcus epidermidis* and *Escherichia coli.* Bacteria are generally classified into two classes based on their membrane components; this is very useful in developing antibiotic treatments for bacteria. Treating Gram-negative bacteria is useful; however, what it means to be a broad-spectrum antibiotic is that both Gram-negative and Gram-positive bacteria are eradicated by treatment. Both *Staphylococcus epidermidis* and *Escherichia coli* have unique features that make them ideal for experimentation in the antibiotic resistance field.

Staphylococcus epidermidis

As previously mentioned, the AMR bacteria species responsible for causing nosocomial infections is the bacterial strain *Staphylococcus epidermidis*. *S. epidermidis* is an antibiotic-resistant bacterium that forms biofilms on medical devices as a protective mechanism. Consequently, it has become resistant to antibiotic treatment. Molecular studies have found that *S. epidermidis* may cause chronic disease due to its ability to invade the immune system.

S. epidermidis belongs to the bacteria group known as coagulase-negative *staphylococci*, which is different from *Staphylococcus aureus,* a coagulase-positive *staphylococci*.[34] *S. epidermidis* is found on mucus membranes and lines the skin[29]. The difference is that *S. epidermidis* lacks the enzyme coagulase compared to *S. aureus,* which has the enzyme coagulase. [34] This distinction is vital because the coagulase enzyme helps with clotting, specifically causing fibrinogen to be converted into fibrin. [34] *S. aureus* with this enzyme can

more easily adhere to tissues within the body, thus providing entry into the bloodstream, which causes infection. [34] Studies indicate that the bacterial group, coagulase-negative *staphylococci* (CoNS), *S. epidermidis* bacteria, causes most non-specific infections reported. At least 22% of bloodstream infections in the ICU are directly related to the bacteria *S. epidermidis*. [29]

S. epidermidis bacteria have 74 different genomic sequences. The IS256 sequence and the *ica* gene allow *S. epidermidis* to become invasive. Two *S. epidermidis* strains have been identified genetically, one known as biofilm-negative ATCC12228 and biofilm-positive RP62A, an isolated strain derived clinically. [29]

This bacterium is also unique because of its ability to create colonies of antibiotic-resistant bacteria, which have been defined as biofilms. [33] These biofilms occur on foreign surfaces and can become a breeding ground for the *S. epidermidis* bacteria. Biofilms create a protective structural layer due to the production of poly-γ-glutamic acid, an exopolymer. [33] The formation of these biofilms protects from the microbes' exotoxins and endotoxins and results in a pathogenic response inside the host. [33] The current rate at which medical devices get contaminated seems to be increasing more and more, and the fact is that *S. epidermidis,* along with other bacteria, are getting harder and harder to treat due to the antibiotic-resistant crisis. [29]

Escherichia coli

E. coli is a gram-negative bacterium that can cause various symptoms and illnesses once it becomes pathogenic in the human body. [35] Some *E. coli* strains are non-pathogenic and are common in the human body, specifically in the intestinal system. [35] When pathogenic, *E. coli* can cause infections and illnesses that can become life-threatening due to renal failure and septic shock.

E. coli is an opportunistic pathogen in hospitals and long-term care facilities. [35] As a result, medical staff are concerned about the possibility of E. *coli* outbreaks. [35] When transmitted into the body via external means such as a pacemaker, catheter, or other internal medical devices, *E. coli* can potentially cause nosocomial infections. *E. coli* is responsible for causing Urinary Tract Infections in patients with internal catheters and ventilator-associated infections. [35]

The strand of *E. coli* known as enterotoxigenic *Escherichia coli* (ETEC) causes infection in the digestive tract and results in diarrhea and stomach distress. [35] Infection outbreaks are due to inadequate food and water sanitation. [35] The bacterial strain enterohemorrhagic *Escherichia coli* (EHEC) is responsible for causing intestinal distress via contact from person to person, often found in young children. The other strain of *E. coli* includes shiga toxin, which is responsible for food poisoning because of uncooked meats and raw and unpasteurized dairy products. This type of *E. coli* strain is linked to food poisoning outbreaks and presents symptoms of vomiting, diarrhea, and the occasional fever.

Depending on the strain of *E. coli* bacteria will determine the method of infection that persists. The ETEC pathogen will be able to become pathogenic by attaching itself to the intestinal wall and expressing a toxin known as heat-labile toxin (LT). [35] The toxin causes the villi in the intestines to absorb sodium chloride and cause free water secretion into the lumen of the intestinal wall, thus producing diarrhea. The other toxin that ETEC strain produces is heat-stable toxin (ST), which causes the body to increase cyclic guanine monophosphate (cGMP), resulting in chloride secretion and sodium chloride absorption by the intestinal microvilli, producing watery diarrhea.

The *E. coli* strain famously characterized as one that causes food poisoning in uncooked chicken and food products is EHEC/STEC. [35] This strand will become pathogenic inside the

body by producing and expressing a toxin known as Stx1 or Stx2, which is known as shiga toxin one and shiga toxin two. These AB protein toxins target eukaryotic ribosomes, ultimately leading to enterocyte cell death and inducing an inflammatory response in the colon. The EHEC/STEC bacteria also encode intimin, which expresses a pore-forming toxin known as EHEC-hemolysin. [35] This will allow Stx toxins to enter the host cells and target epithelial cells, promoting damage. These bacteria may induce hemolytic uremic syndrome (HUS), which is characterized by a triad of microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency.

E. coli bacteria are facultatively anaerobic and can ferment lactose. This bacterium is catalase positive, which means that the bacterium is a facultative anaerobe that produces oxygen. Because *E. coli* is a Gram-negative bacterium, it contains features such as a cell envelope, outer and inner cell membranes, and a peptidoglycan cell wall (Fig. 3). These key features make Gram-negative bacteria harmful and very difficult to treat with regular antibiotics as there cell wall and membrane has so many layer (Fig. 3). [36] The outer membrane comprises the lipid bilayer, and lipopolysaccharides that will release toxins if the cell membrane is destroyed. [35]

The main difference between Gram-negative and Gram-positive bacteria is the components of the cell wall (Fig. 3) [37]. A Gram-positive bacterium has a thick peptidoglycan layer. In contrast, a Gram-negative bacterium has a thin layer (Fig. 3) [37]. The Gram-negative bacteria has many layers to their cell wall, containing an inner and outer cell membrane (Fig. 3) [37]. This is different from a Gram-positive bacterium, which only contains one cell membrane (Fig. 3) [37]. Another key difference between the Gram-positive and Gram-negative bacteria membranes is that Gram-negative bacteria contain lipopolysaccharides (LPS) compared to Gram-positive bacteria, which do not [37]. This could explain why Gram-negative bacteria are more complex to treat with antibiotics compared to Gram-positive bacteria [37].

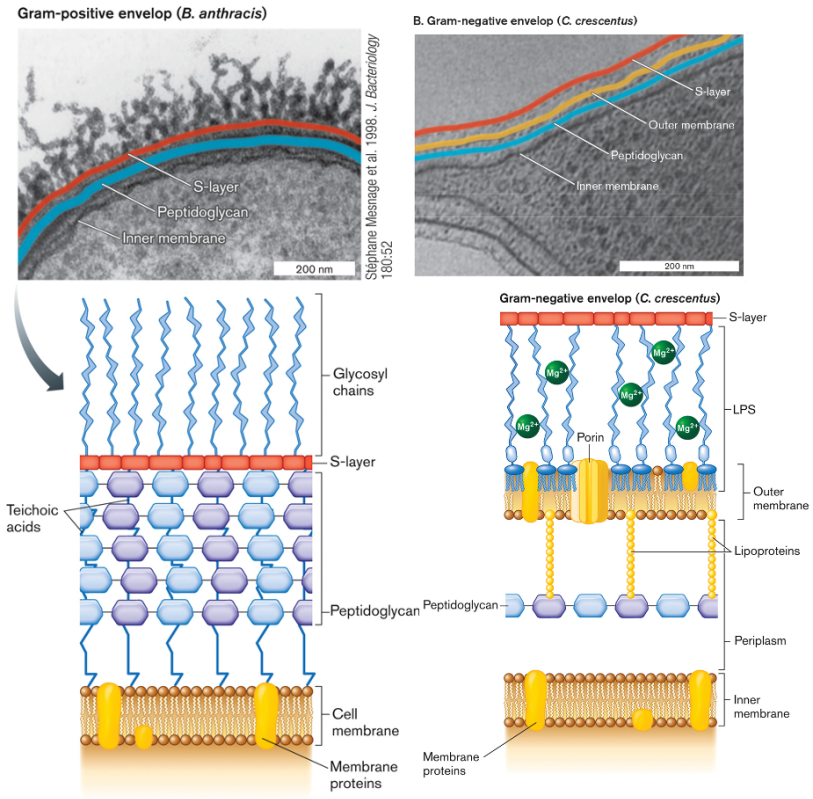

Figure 3: This figure represents the differences between a Gram-negative and Gram-positive cell wall in a cartoon depiction under a 200 nm microscope. Figure from ref 37.

1.5 Persister cells

This antibiotic-resistant crisis has been linked to a subpopulation of cells known as persisters that remain in a dormant state. Persister cells stay in metabolic dormancy or "hibernation" after the antibiotic therapeutics kill off all susceptible or non-tolerant cells. [38] These cells account for less <1% of the remaining bacterial population after antibiotic treatment.[38] Persister cells were first discovered by Hobby (1942) through studies involving *Staphylococcus aureus.* [39] The researchers observed that even after the antibiotic penicillin was added, a small fraction of bacterial cells remained unaffected by the antibiotic treatment. [39] The number of cells that survived the antibiotic treatment was quantified as small, approximately 1%

of the remaining bacterial population. In 1944, Bigger formally classified these cells and named them persister cells. [39]

In addition, to persister cells bacteria can also be described by the terms' antibiotic resistant and antibiotic tolerant. Bacteria can establish a tolerance to an antibiotic treatment, [38] which takes longer than usual, but all die off when enough antibiotic and exposure time is applied.[36] Antibiotic resistance, however, refers to the condition where a bacterium has changed its genetic information and will instead grow due to a genetic change, even in the presence of antibiotics.[36]

Persister, tolerant, and resistant bacteria can best be described by examining a graph known as a biphasic killing curve (Fig. 4). This biphasic curve measures the survival of bacteria after being treated with antibiotics. The persister cells are dormant and have not died with antibiotics, and they are represented with a flat line.[38] At the beginning, when an antibiotic is added to a bacterium, all the susceptible cells die, as indicated by the line with the steepest slope (Fig. 4). Following a slowly decreasing line indicates tolerant bacteria, and they exhibit slow cell death over time. Resistant bacteria increase in survival as indicated by the increasing line. Due to genetic changes, resistant bacteria grow in the presence of antibiotics.[38] This is compared to persister bacteria where a flat line indicates a plateau of growth (Fig. 4).

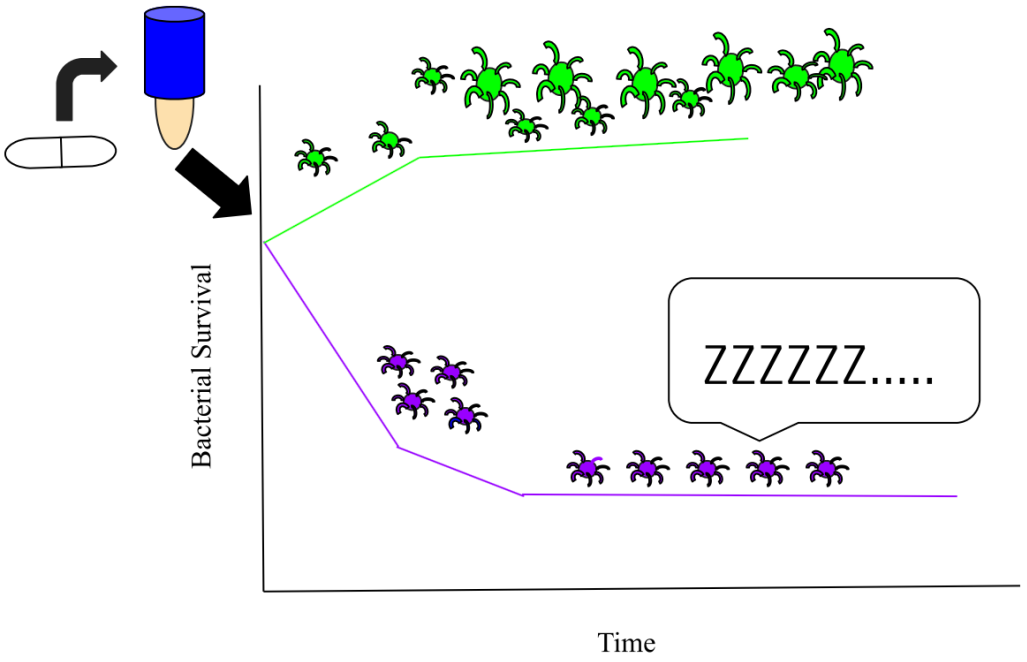

Figure 4: Biphasic killing curve relating bacterial survival to time after antibiotic administration. The green line represents antibiotic-resistant bacteria; this increasing line shows that resistant bacteria will grow even in antibiotic treatment. The purple line, starting with the steep slope down, represents rapid cell death. These bacteria are compared to the line whose slope decreases but decreases more slowly. This line represents tolerant bacteria where they are still dying cells; however, their death is not as drastic. The plateau line represents the dormant bacteria, thus, the persister cells. Adapted from ref 12.

Persister cells are found in bacterial species such as *E. coli, P. ananases,* and *S aureus*. [40] Persister cells are a bacterial phenotype that causes bacterial cells to become metabolically inactive due to stress, specifically in the stationary phase. [41] Persister cells are categorized into two classes based on how they are formed. [40] Type I persisters form via a triggered persistent mechanism in response to a triggered stress signal, such as starvation, acid stress, or immune stresses.[40] These stress signals cause the bacteria to become persistent, usually during the stationary phase, where resources are limited. [42] Type II persisters, on the other hand, are persisters that form via a spontaneous signal that occurs in response to no kind of outside

environmental signal. [40] These types of persisters occur in the late exponential and early to late stationary phase of bacterial growth, where there are more stresses due to a lack of nutrients and means of survival. [40]

Antibiotic treatment has been shown to eradicate bacterial cells, but has been less effective on bacterial persister cells. [2] This is surprising because susceptible, tolerant, and persister cells all have the same genetic makeup. The main difference, however, is that persister are metabolically inactive because of a phenotypic change. As a result, persister cells do not grow or die when treated with an antibiotic. Persister cells are challenging to treat with antibiotic measures because they have developed a phenotypic change by turning off and on cellular pathways involved with metabolic inactivity. The phenotype change allows these cells to control growth and instead remain dormant in the presence of antibiotics. When the antibiotics have finished their cycle, persister cells can turn their metabolism back on and continue to grow. In addition to genetic mutations from damaged DNA, persister cells can also arise from environmental stressors such as a lack of oxygen or nutrients. Stressors cause the cell to activate a stress response, effectively shutting down its metabolism to adapt to the conditions and survive.[1]

Persister cells result from the stressors in the bacterial stationary phase of growth. Bacteria grow in multiple phases, starting at the log phase, then grow exponentially during the exponential phase, where nutrients and resources are in short supply. Then, after the rapid bacterial growth, bacterial growth will slow as nutrients and resources become limited, thus entering the stationary phase. The exponential phase of the bacterial growth curve is also known as the logarithmic phase. This is the stage of bacterial growth where the environment is

favorable, and bacteria proliferate. Orman and Brymildsen experimentally demonstrated that *E. coli* survived the treatment of a lethal amount of ofloxacin during the early exponential phase. [43] Umetani at. el showed that when bacteria were treated with 200ug/mL of AMP during the exponential phase of bacterial growth, persister formation had occurred. Although these bacteria were in the non-dormant state, 12 persister cell lineages remained after the treatment with AMP.[43] They also determined that persisters in the dormant state are primarily located in the stationary phase of the cell growth cycle. In the exponential phase, *E. coli* treated with AMP increased the number of dormant persisters present. [43]

Research has determined that persisters are more capable of forming due to mutation. [40] For example, the *hipA7* mutation increased the formation of persisters in *E. coli* bacteria. Balaban et al. demonstrated that persister formation increased to 20% of the bacterial population, as opposed to the typical value of 0.1%. [40] This mutation was used to test the hypothesis that bacteria and persister bacteria have different phenotypes. [44] The *hipA7* mutation in *E. coli* was used to identify Type I persisters, and experimental results led to the probable conclusion that Type I persisters are different from regular persisters. [44]

In addition to the Type I persisters, Type II persisters were also tested using a mutation in the *hipQ* gene. Persistence was compared to regular *E. coli* persisters. The *hipQ* persisters were more abundant because of this mutation compared to regular *E. coli* persisters. [44]

1.6 Common Cellular Mechanisms and Pathways of Regulation

Guanosine tetraphosphate (PpGpp) Pathway

Mechanistic pathways are responsible for keeping order in bacterial cells. [45] [40] *E. coli* bacteria possess a regulator known as guanosine tetraphosphate (ppGpp) alarmone. This pathway

is responsible for many cellular processes such as DNA transcription and translation (Fig. 5). For instance, the regulator ppGpp controls the expression of about 500 genes, including stress signals known as RpoS and RpoE. These stress signals are activated due to a misfolded protein (Fig. 5). [45] [40] Experimental evidence indicates that the ppGpp inhibits DNA primase and rRNA synthesis in *E. coli* (Fig. 5). [45] [40]

The ppGpp mechanism has been linked to persister formation and dormancy in *E. coli* bacteria.[45] Upon starvation stress in *E. coli, the* ppGpp pathway responds, and a switch causes transcription to decrease and change gene expression, thus leading to dormancy and slow bacterial growth (Fig. 5). [45] [40] It can be shown that ppGpp mechanistically influences the *E. coli* bacterial cell and many mechanistic patterns. Thus, the changes due to starvation and stress affect the ability for persister cell formation (Fig. 5). [40]

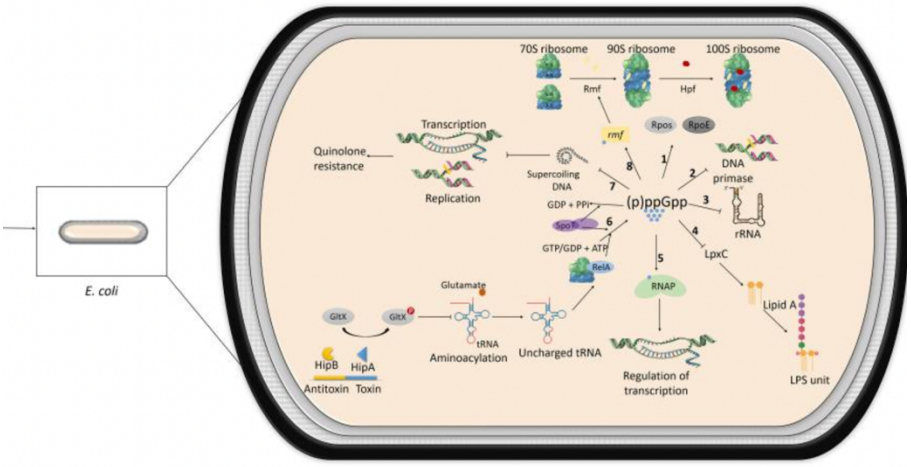

Figure 5: The ppGpp pathway in *E. coli* bacteria. Figure from ref 40.

An increase in ppGpp levels is connected to antibiotic tolerance and persister formation due to issues with DNA replication and transcription (Fig. 5). [45] [40] In a study conducted by Helaine et al. with mouse macrophages known as *Salmonella enterica,* results indicated an increase in ppGpp acidified vacuoles, conditions later linked to persister and bacterial persistence. [45] Another study by Yamaguchi et al. demonstrated that high levels of ppGpp caused the FtsZ protein to be altered, forming a helical structure in DNA instead of a Z-ring structure. [45] This alteration modifies the growth mechanism of the *Salmonella* bacteria and promotes persistence. [45] These findings suggest that ppGpp alters genetic mechanisms and stops the bacteria from growing through the alteration of bacterial division. [45]

Song and Wood's research determined that ppGpp also generates persisters because it causes an inactivation of ribosomes via the proteins ribosome modulation factor (Rmf) and hibernation-promoting factor (Hpf).[45] It is reported that *E. coli persis*ters have 100S inactivated ribosomes, and the 90S ribosomes are converted into 100S ribosomes via Hpf, signaled by ppGpp. The overproduction of Hpf protein leads to a change in the ribosome value and thus the formation of persisters. PpGpp also increases persister formation due to inhibition of ribosome-associated enzyme GTPase Era in *Staphylococcus aureus* bacteria, resulting in alteration of 30S ribosomal subunits.[45]

Toxin-Antitoxin (TA) System

The persister formation mechanistic pathway known as the Toxin-Antitoxin (TA) System causes a bacterium's metabolism to shut down, resulting in a dormant state. [38] A TA system consists of two components, a toxin, and an antitoxin. The stable toxin disrupts either

transcription or translation, while the labile antitoxin prevents the toxic effects of the toxin within the cell. [38]

Antitoxins are classified based on how they prevent the toxicity. An RNA antitoxin that prevents the toxin from translating in the cell and instead changes the translation as an "antisense RNA" is a Type I antitoxin. [6] Type III antitoxins are also composed of RNA, and they prevent toxic damage by binding a toxin protein. Type II antitoxins are composed of proteins and inhibit toxin activity by participating in protein-protein binding. The Type IV antitoxin is a protein that prevents the toxin's ability by binding to a target protein instead of targeting the toxin directly. Lastly, Type V antitoxins are proteins that function by cutting the mRNA toxin.[6]

The link between the TA system and persister formation was first discovered in the 1980s, [39] indicating the TA systems of *E. coli* bacteria have been elucidated to persister formation, such as the *hipBA* system. The toxin, *hipA,* prevents transcription by blocking the EF-Tu transcription factor via phosphorylation. While *HipB* is the antitoxin in this model, [38] other TA system studies have caused persisters, including the TisB/IstR-1 system. The toxin TisB causes a decrease in ATP, which leads to growth inhibition, dormancy, and resistance to ampicillin and ciprofloxacin. [38] In the MqsR/MqsA TA system, the toxin MqsR prevents translation by destroying RNA transcription codons. These cells do not transcribe the correct DNA message and become dormant. [38]

A TA system must respond to a stimulus or stress, such as starvation conditions, to be activated. [6] PpGpp, a stress signal stimulated by outside pressures, activates a TA system. RelA and SpoT stimulate ppGpp to change transcription upon starvation by activating RNA polymerase and stress response sigma factors RpoS and RpoE. The ppGpp molecule acts as a response to a stress signal and causes persistence by altering DNA replication and reducing the

production of proteins. In addition to the TA system, other cellular mechanisms have been shown to promote persistence in bacteria. [6]

Reactivated Oxygen Species

In addition to the ppGpp and TA pathway showing induced the persister phenotype in bacteria, reactive oxygen species also seem to promote persister formation. [45] A reactive oxygen species response (ROS) is produced because of a change in the environment, specifically a change in oxygen levels. [45] ROS response increases when outside environmental stress occurs, such as UV damage, antibiotics, or a bacterium's stress. [45] The increased reactive oxygen species (ROS) response causes damage to the DNA, lipids, and proteins and results in apoptosis, a programmed cell death.[45]

Scientists, Molina-Quiroz et al, have studied the correlation between persister bacteria and reactive oxygen species (ROS). [45] It was observed that persisters do not die in the presence of antibiotics. Instead, the stress caused by oxygen depletion in bacteria signals the ROS response. [45] ROS signaling causes damage to the DNA, which in turn causes cAMP regulators to be turned off. As a result of the halt in transcription, persister formation in *E. coli* bacteria increased.[45]

1.7 Biofilm Formation Mechanism of Staphylococcus epidermidis and Escherichia coli

Biofilms are "dome-like" structures of bacterial cells that contain free-floating bacterial cells that exist in a static structure (Fig. 6). [29] Biofilm formation and structure are unique as they provide bacterial protection for not only free-floating bacterial cells but also persister cells as well. In a biofilm, the percentage of persister cells is significantly higher than that of free-

floating cells. This higher percentage of persister cells has been attributed to the fact that biofilms internally have low nutrients, low oxygen, and free radicals, which is the perfect environment for persister cells to thrive and be easily protected.[29] The increase in the persister cell population that resides in a bacterial biofilm causes issues and concern, as both persister cells and biofilms are harder to treat, thus making them more dangerous and posing more of a threat to the human host.

Penetration of antibiotics through a biofilm from the outside can be an issue, depending on what type of antibiotic is used. However, the primary contributor to this scare is that the human immune system will not be able to respond to a larger mass of cells, as it makes it more difficult for our body's immune system to attack these bacterial cells when they are in this mass-like structure.[46] One possible method of biofilm disruption that has been seen is trying to disperse the biofilm so it's easier for the immune system to detect.[46]

The process of biofilm formation occurs in several stages (Fig. 6)[2 9]. Two polymers involved in biofilm formation occur by a homopolymer known as Polysaccharide Intercellular Adhesin (PIA) and Poly-N-acetylglucosamine homopolymer (PNAG) (Fig. 6)[29]. Biofilm formation in *S. epidermidis* bacteria first requires attachment to a hydrophobic polymer surface, such as an internal medical device. Attachment is aided by surface proteins AtlE, Aae, and Teichoic acids (Fig. 6). The host matrix proteins SdrF, SdrG, SdrH, Ebp, AtlE and Aae attach a matrix to the polymer surface of the bacteria (Fig. 6). After the bacteria are attached via host matrix proteins, exopolysaccharide-specific proteins and other accessory macromolecules are used to provide intercellular aggregation. Finally, bacterial cells express detergent-like peptides and proteolytic activity to help expose the bacteria as a biofilm [29].

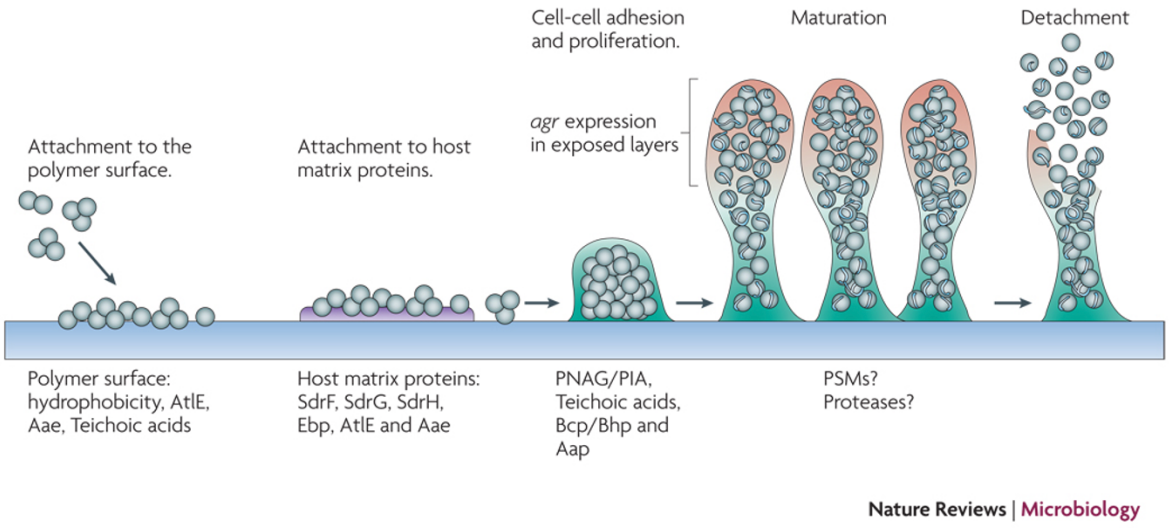

Figure 6: Mechanistic formation of *S. epidermidis* persister biofilms. Figure from ref 29.

S. epidermis bacteria possess a wide range of surface proteins, known as Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs). [29] These proteins interact with matrix proteins and covalently bond to the surface of the *S. epidermidis* bacteria via an enzyme known as sortase A. Other molecules involved in this matrix protein interaction are teichoic acids, surface polymers whose role in biofilm formation is unclear. [29] Fibrin and collagen covalently anchor proteins SdrG, SdrF and SdrH, promoting *S. epidermidis* adhesions. [29] The "dock, lock, and latch" mechanism leads to stability of MSCRAMM-ligand interaction and bacteria biofilm production. [29] Once the bacteria are locked onto the surface, the beginning of a biofilm must meet an internal medical device; full biofilm development occurs through intercellular aggregation via exopolysaccharides and other proteins that help to promote the formation of the extracellular biofilm matrix. This aggregation event is due to teichoic acids and extracellular DNA originating from lysed cells. *S. epidermidis* cells connect in the biofilm via an

exopolysaccharide chain PNAG/PIA (PNAG) as PIA (polysaccharide intercellular adhesion), which allows the connection of *S. epidermidis* cells in the biofilm. This production of PNAG/PIA is significant for forming *S. epidermidis* biofilm. The PNAG/PIA synthesis results from the gene *ica* locus, which involves IcaA and IcaD to produce a chain of activated N-acetylglucosamine monomers. This monomer depends on another IcaC protein. From the N-acetylglucosamine monomers, a partial deacetylation process occurs because N-acetylglucosamine residues are removed by an enzyme known as IcaB, which is a cell surface-located enzyme, and this reaction occurs after export. The deacetylation reaction also induces positive charges into a neutral polymer, which is required for surface binding of PNAG/PIA and helps with biofilm formation. The PNAG/PIA is also under regulatory control, as there are regulatory virulence regulators, excluding, however, *agr.* [29]

Exopolysaccharide PNAG/PIA forms due to a deacetylated b1-6-linked N-acetylglucosamine homopolymer. It is synthesized by N-acetylglucosamine transferase IcaA, which is needed to help IcaD membrane protein to be active. The growing PNAG/PIA chain will then export IcaC membrane proteins. It will help the surface of IcaB de-acetylase remove the N-acetyl groups and give the polymer the ability to attach to surfaces. Ica proteins are encoded in the *ica* gene locus, the *icaADBC* operon and the *icaR* gene encoding protein. The expression of the *icaADBC* operon is regulated directly at the *icaA* promoter of via expression of IcaR by a series of global regulatory proteins. Then, the insertion or excision of the IS256 element may turn on or off PNAG/PIA expression. The organism highly manages PNAG/PIS exopolysaccharide synthesis through virulence regulators, since its production is needed for effective biofilm formation. [29]

Escherichia coli Biofilms

Similar to *S. epidermidis, E. coli* bacteria produce biofilm structures as well. [47] These biofilm formations are more dangerous and pose more of a threat than persister cells in regular planktonic culture. [47] Low nutrients, low oxygen, and free radicals characterize conditions within the interior of a biofilm. As a result of the stressful environment, biofilms have a higher percentage of persisters than the planktonic persister population.[38]

After adhering to a static surface, *E. coli* produce biofilms through several stages (Figure 7). Several stages include adhesion to a surface, quorum sensing or bacterial communication, and biofilm biomass formation.[47] Once the *E. coli* bacteria have adhered to a static surface, the bacteria will then start "talking" via quorum sensing method. These genes include *luxS, fimH, and csgD,* which unlock this biofilm pathogenic ability in the *E. coli* bacteria. The *luxS* gene is required to produce AI-2, allowing biofilm formation and architecture to be maintained. The other biofilm gene *fimH* stimulates fimbriae, which will help biofilm adhesion and formation of the biofilms. [47] *E .coli* bacteria require three stages to produce a biofilm. [48] This first stage is attachment, and after attachment is micro colonization, which is then transformed into a mature biofilm (Figure 7). After the mature biofilm has begun to develop it then begins to build adhesion and formation through hydrodynamic forces and IMFs (Fig 7). [48] There are specific proteins known as OmpA, fibronectin binding proteins, protein A, SasG, and Biofilm associated proteins that help promote the biofilm formation during its initial stages of attachment on surfaces (Fig. 7). [48] In these biofilm structures, bacteria are enclosed in extracellular matrices which are composed of highly polar biomolecules, including proteins, polysaccharides, nucleic acids, and lipids (Fig. 7). [48] This ECM provides a cover from environmental stresses and external environmental factors such as antibiotic exposure. Once the bacteria are done secreting the

extracellular polysaccharide substance, the second stage of biofilm development is irreversible (Fig. 7). This process includes continuing to secrete extracellular polysaccharide substances until the face attachment of bacteria to a surface that is inside a thick complex biomolecular layer (Fig. 7).

A fully mature biofilm structure has a three-dimensional tower-like structure (Figure 4). These formations are little mounds that provide shelter from bacteria and contain small channels for nutrients, water, and waste secretion. [48] What makes Biofilms distinct is the extracellular matrix that is secreted upon biofilm formation. For *E. coli* biofilms, specifically, AidA and TibA proteins are secreted (Fig. 7). [49] In contrast, *S. epidermidis* biofilms are formed via proteins such as Bhp, Aap, and Embp (Fig. 6). [50] These biofilms are cavities that contain bacteria that are shielded from the outside environment, away from outside environmental stresses. Since biofilms shield bacteria from the outside ecological stresses, antibiotic treatment may be ineffective because of cells trapped inside the mounded biofilm structure.

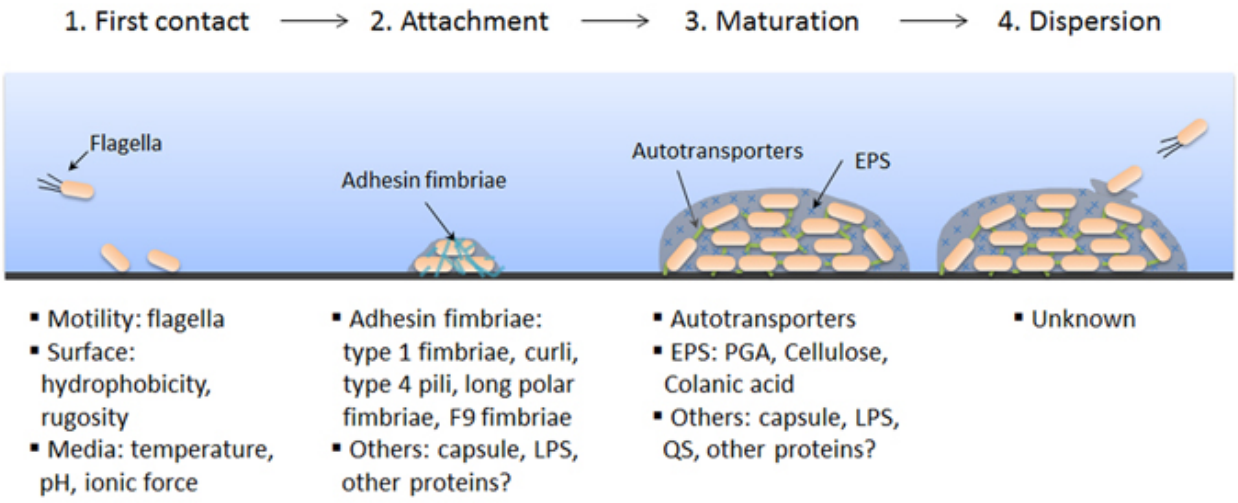

Figure 7: Mechanistic formation of persister biofilms of *Escherichia coli*. Figure from ref 51.

Both *E. coli* and *S. epidermidis* bacteria and their mechanisms of biofilm formation may impact how biofilms are eradicated via dispersal. [52] *S. epidermidis* biofilms seem to be directly related to the iron in the human body and blood. [52] It was found that *S. epidermidis* biofilms can upregulate the genes needed for biofilm production using iron in the human blood. Without the presence of iron, biofilm production stalled due to the lack of PIA/PNAG gene production.[52] So, because *S. epidermis* biofilms have been seen to be impacted by a very common metal in the human body, this prompts *S. epidermis* biofilms to be at the forefront in biofilm research as this issue pertains to human health.[52] For *E. coli* biofilms, the biggest threat to the human body is that *E. coli* is not only a motile organism but can also perform conditioned attachment. [53] This ability for *E. coli* to be motile allows the bacteria to spread more rapidly and easily about a surface before actual biofilm attachment.[53] To add, *E. coli* bacteria will be influenced by environmental stressors and surface structures to attach more or less easily. [53] This means that with the hydrophilic forces and surface adhesion of different surface materials, *E. coli* will attach to surfaces like glass and metal more easily than *S. epidermidis,* thus conditioning film attachment. [53] This ability for *E. coli* to attach to multiple different surfaces because of intermolecular forces will provide *E. coli* biofilms more of an advantage, thus posing more of a threat to health when attached to different medical devices. [53]

1.8 Treatment Methods for Persister Cell Eradication

Studies have been conducted to examine how persister cells can be eliminated.[54] Antiviral compounds have been evaluated as a persister cell treatment strategy. Pan et al determined that the antiviral compound BF8 effectively killed persister bacteria in *E. coli.* [55] BF8 is a molecule involved in QS signaling that helps make the antibiotic ofloxacin more effective in

killing all persister and bacterial cells.[55] Antiviral compound ADEP4, evaluated by Conlon et al, causes ClpP protease to inactivate 400 proteins in the persister cells, causing their death. [55] Starkey et el demonstrated that a signal molecule known as M64 blocks persister formation by blocking the signaling MvfR needed to produce persisters in *P. aeruginosa*.[55]

In addition, new antiviral compounds CD437 and CD1530 showed that *S. aureus* persisters were not formed because these molecules disrupt the lipid bilayer of the persister cell membrane. [55] In addition, persister cells have been treated with antimicrobial peptides, specifically when these peptides are synthesized with neutrophils, which are part of the human immune system. [55] These antimicrobial peptides are more effective and require less time to be active when reacted with the immune system. [56] A study by Yang et al demonstrated that *S. aureus* persisters were killed using P2, P5, and P9 antimicrobial peptides against two strains of *S. aureus*. [55]

Phage Therapy is another strategy that has been explored to treat persister cells. Phage Therapy is when a virus is used to kill bacteria by out-competing them.[55] In a recent study from 2017, Schooley et al. used a bacteriophage consisting of nine lytic phages in conjunction with minocycline, genus here to treat a bacteria known as *A. baumannii.* [55]Another example of Phage Therapy is from Khawaldeh et al. 's 2011 study involving *S. aureus* treatment with a six-lytic phage virus in tandem with the antibiotics meropenem and colistin. [55]

Anti-helminthic drugs have also been shown to destroy the persister membrane of different strains of *S. aureus.* In 2016, Gooyit and Janda reported that the salicylamide drugs, such as rafoxanide and closantel, presented evidence against bacteria *C. difficile,* with no bacteria remaining in the stationary phase. [55] In addition, the drug Nicodamid, also a part of the

salicylamide group, prevented QS signaling molecules from turning on specific genes needed to generate persisters in *P. aeruginosa*. [55]

Persister cells can be eradicated by entirely inhibiting persister formation (Fig. 7). Persister cells can be terminated by either direct killing or sensitization methods (Figure 7). The direct killing of persisters can be accomplished using natural processes such as treatment with essential oils. [12] Due to its microbial properties, tea tree oil was found to eradicate persisters directly. [12] Another technique involves waking the persister cell, followed by drug therapies. [54] To efficiently study persister death, clearly it is essential to understand how persister cells form. The central question for research studies examines how a regular cell becomes a persister cell, as details of the initial transformation mechanism are relatively unknown.

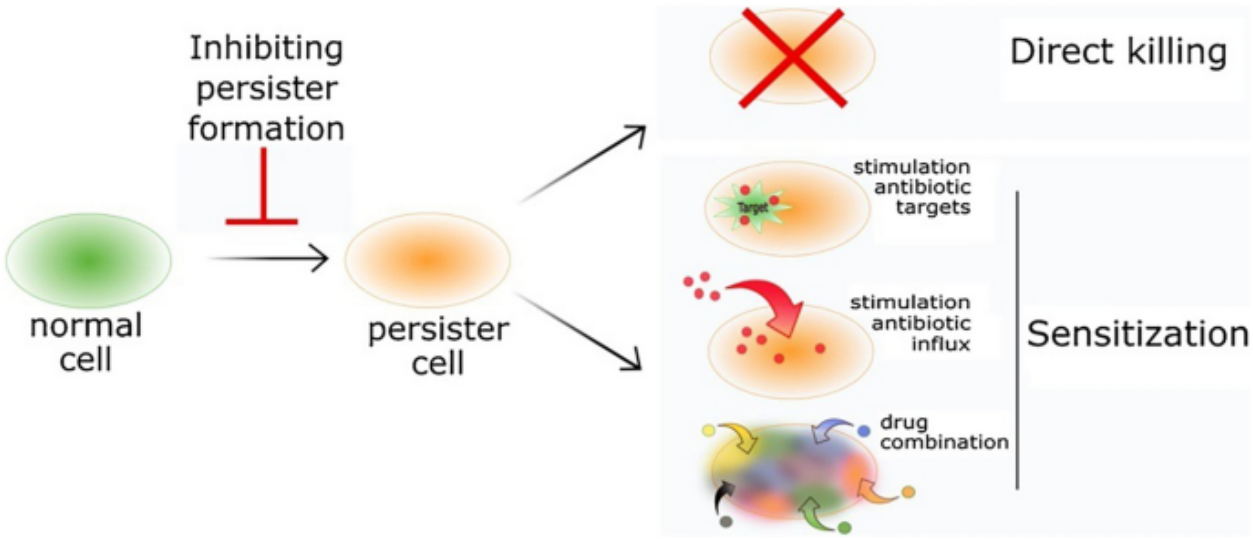

Figure 8: Persister cell eradication methods. Figure from ref 45.

Several treatment methods exist for killing persister cells. Essential oil, specifically as a method of persister eradication, is much more advantageous. Persister cells are hard to treat with

antibiotics because of the persister phenotype. However, essential oils are used to target bacterial membranes as a form of bacterial destruction, thus contributing to their antimicrobial properties and to persister cell death. Essential oils are also commercial products, thus more accessible for large-scale use. Essential oils have been seen to treat planktonic cultures of cells. Studies have evaluated the utility of essential oils in treating persisters in planktonic culture and bacterial biofilms.

1.9 Essential Oils and their Mechanisms against Bacterial Pathogens

In discovering persister eradication methods, essential oils show promise. This is because even though persister cells are dormant, one strategy used even in the destruction of dormant cells is to attack the cellular membrane. Even in a cell that is a dormant cell to be classified and function at the cell level, it needs to contain an enclosed membrane. Essential oils will attack the membrane, thus destroying that cell. Essential oils studies have been done with planktonic, persister, and biofilm bacteria. To fully understand how and why essential oils are effective against these bacteria, an explanation of essential oils and the specifics of these oils is necessary.

Essential oils are derived from plants and have been shown to possess antimicrobial properties. The molecular mechanism of action is not clearly defined at present. [57] The essential oils seem to cause a destabilization in the bacterial cell wall, leading to cell membrane destabilization, enhancing permeability, and disruption of other metabolic functions within the cell. Essential oils easily penetrate the bacterial cell membrane because of the oil's lipophilicity. The destruction of the cell membrane permits the essential oil to destroy the cell envelope and the cytoplasm. [57] Other effects include the leakage of cellular components and ions out of the cell and the disruption of ATP synthesis. [57] Other studies involving tea tree oil suggest that

disruptions in cellular respiration and distortion of the phospholipid bilayer and polysaccharide molecules are involved in the mechanism of action. [57]

In addition, Carvacrol, a component of many essential oils, will cause a change in the fatty acids that are in the phospholipid bilayer and deplete ATP from the bacterial cell. Methyl carvacrol, menthol, citronella, and thymol expand the membrane's phospholipid through passive diffusion or cellular transport. The carvacrol molecule is also found to influence flagellin and disrupt the protein required for moving the bacteria, causing its death.[57] Multiple bacterial enzymes, such as ATPase class enzymes, amylase, histidine carboxylase, and proteases, can be destroyed by the essential oil components known as phenylpropene, cinnamaldehyde, and eugenol. Vanilla was seen to obstruct the K+ ion pump and pH gradient in bacterial cells. [57] In addition, some essential oils will disrupt and cause quorum sensing mechanism to decrease and or stop completely.[57]

While essential oils are derived from different sources, most essential oils are isolated from medicinal and aromatic plants or (MAPs) by secondary metabolism. [57] Essential oils can be used against bacteria, fungi, and viruses due to their wide range of molecules, such as aldehydes, phenolics, terpenes, and other compounds.[57] Essential oils are an important component of medicine. There have been 9,000 plants identified and known to have curing properties, and 250 types of essential oils have been identified for use in pharmaceuticals, treating conditions such as cardiovascular disease, diabetes, Alzheimer's, and cancer.[57]

Essential oils have antimicrobial properties that manifest alone as well as in combination with other synthetic compounds. Essential oils are produced in MAP's in the plant's secretory canals, the glandular trichomes, and the epidermal cells. Most essential oils contain the components of terpenes, terpenoids, aromatic, and aliphatic compounds. [57] Terpenes or

terpenoids can be synthesized in the cell's cytoplasm through the mevalonic acid pathway. Terpenes structures are varied and can be acyclic, monocyclic, bicyclic, or tricyclic. They can further be classified into monoterpenes, sesquiterpenes, diterpenes, and triterpenes. Most essential oils that possess biological activity contain monoterpenes (Fig. 9).

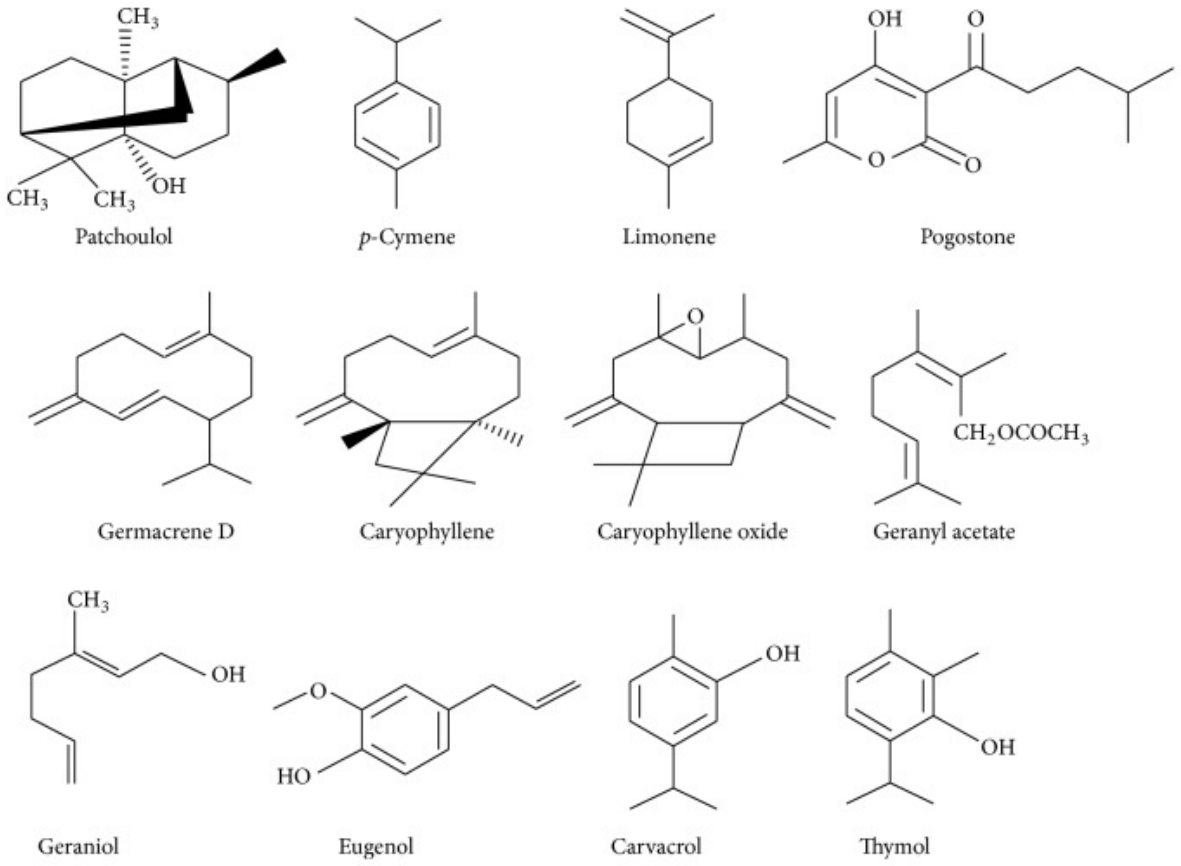

Figure 9: Chemical structures of terpene derivatives. Figure from ref 57**.**

The two ways that essential oils exhibit antibacterial activity are through either cell destruction or inhibition of growth. [57] Sandalwood and Vetiver oil are bacteriostatic with Gram-positive bacteria because they inhibit growth. In contrast, cinnamon, clove, pimento, thyme, oregano, and rosemary prevent antimicrobial activity in *Salmonella typhimurium, S. aureus,* and

P. aeruginosa. [57] Clove oil, which contains carvacrol, thymol, cinnamic aldehyde, eugenol, and *p*-cymene, is bactericidal to the organism. Compounds such as carvacrol, eugenol, and thymol have been shown to prevent *E. coli, Salmonella typhimurium, Listeria monocytogenes,* and *Vibrio vulnificus.* [57] While garlic, ginger, clove, black pepper, and green chili are effective against *Bacillus sphaericus, Enterobacter aerogenes, E. coli, Pseudomonas aeruginosa, S. aureus, S. epidermidis, S. typhi, and Shigella flexneri.* [57] These oils prevented 93% of *S. epidermidis* and *S. typhi* bacteria in a 3-hour incubation time. In addition, Lavender, Egyptian Geranium, Vetiver, Myrrh, Peppermint, and Frankincense are effective against planktonic cells of *S. epidermis* bacteria and bacteria on biofilms. [57]

The essential oil tea tree was found to eradicate persister cells specifically in *S. epidermidis* and *E. coli* bacteria during a 24-hour and 48-hour [12]. The essential oil tea tree was seen to eradicate these persister cells because of the element terpinen-4-ol at the concentrations of 0.5% and 1.0% [12]. The essential oils act as a target for bacterial eradication because they are hydrophobic, which results in the ability of essential oils to be more permeable to the cell membrane, thus causing it to be easily permeable.[58] This permeability will result in cellular contents being able to leak out of the cell, thus causing cells to lose their cell components. [58] This cellular leakage of contents also results in the loss of ATP, which is needed for cellular processes. Essential oils have also been seen to disrupt the cellular membrane and ATP in bacterial cells, disrupt protein synthesis, disturb pH, and alter the DNA, which is essential for control in the bacterial cell. [58] Because of all this, it was seen as the most probable distraction target as persister cells because they are metabolically inactive, thus requiring less ATP than a regular bacterial cell.[58] It is seen that tea tree oil disrupts the membrane of persister cells in both *S. epidermidis* and *E. coli* bacteria. [12]

1.10 Lavender, Egyptian Geranium, and Ylang-ylang

Essential oils have been seen to exhibit antimicrobial properties. This mainly involves the disruption of the cell wall in prokaryotic organisms. Essential oils have also been seen to disrupt the pH needed for the cell to function appropriately. The disruption of prokaryotes with essential oils seems promising, as essential oils are commercial products that are easily accessible. Persister cells in both *S. epidermidis* and *E. coli* bacteria have been destroyed using tea tree oil, as its components specifically destroy the cell wall, which is essential for cellular processes, thus eliminating persister cells [12]. The elements in essential oils seem to help eradicate persister cells, which are a contributing factor in the antibiotic-resistant crisis.

Lavender essential oil is commonly used for its healing properties since it helps to rejuvenate skin. During Ancient Rome, it was known for its antiseptic properties, and it was used on cuts and wounded skin. [59] Lavender is found in Mediterranean countries where the weather and soil conditions are ideal for the *Lavandula* plant. The species within the *Labiatae* family have three different plants that produce the lavender oil used in the cosmetic industry: *Lavandula officinalis, Lavandula latifolia, and Lavandula angustifolia.* [60] The antimicrobial properties of lavender are associated with compounds such as linalool, linalyl acetate, landulo, geraniol, bornyl acetate, borneol, terpineol, eucalyptol, and levandule acetate. [60] Several compounds, specifically eucalyptol, linalool, terpineol-4ol, and alpha-terpineol, possess a wide range of antimicrobial activity. [60] Eucalyptol is also an antifungal, while linalool and linalyl acetate can be used for antiseptic purposes. [60] Compounds such as alpha terpineol, terpinene-4-ol, eucalyptol, and linalool exhibit antiviral activity against the influenza strain. Finally, eucalyptol, terpinene-4-ol, thymol, and carvacrol exhibit anti-inflammatory effects on the skin. [60]

Lavender as an essential oil has been tested against pathogenic bacteria, specifically against methicillin-resistant *S. aureus* strain (MRSA). [60] The lavender essential oil, in combination with the antiseptic octenidine dihydrochloride (OCT), [59] increased the effectiveness of the antiseptic OCT against the MRSA strain. [59] The topical antiseptic octenidine dihydrochloride functions in tandem with the lavender by destroying the bacteria's cell wall. [59] Even though this antiseptic ointment shows promise, it still experiences antibiotic resistance similar to a standard antibiotic treatment.[59] It was found that the mutations in the *norA* and *norB* genes that encode for the efflux pump of proteins would be responsible for causing the resistance towards this antiseptic. Because just by itself, octenidine dihydrochloride seems less effective at killing the MRSA bacterial strain due to genetic mutations, it was examined what essential oils, in addition to the antibiotic, would do to its effectiveness in treatment. [59] The lavender essential oil that was tested was from the plant *Lavandula angustifolia* and what was hypothesized that because of its antibacterial properties that this essential oil when added would help increase the antibacterial activity of the OCT against MRSA by destroying the bacterial wall making the bacterial more susceptible to treatment yet the molecular mechanism by which Lavender helps to impact the cell wall integrity remains unknown. [59]

From the study, it was found that lavender essential oil, in addition to the OCT antiseptic ointment, increased in efficiency when both were applied to the MRSA bacterial strands. [59] The Lavender essential oil is shown to have most of its antimicrobial properties because of linalool and linalyl acetate, which are molecules in the lavender essential oil. [59] It was revealed that the lavender contains 34.1% of linalool and 33.3% linalyl acetate, which was shown to be most effective when combating MRSA in addition to OCT. [59] In addition to lavender's antimicrobial properties, another oil that shows promise is geranium.

The Geranium essential oil is obtained from the stems, flowers, and leaves *of P. graveolens, P. odoratissimum, P. zonale,* and *P. roseum*. [61] It can benefit patients suffering from gastric ulcers, diabetes, and certain skin conditions. The most effective geranium essential oil comes from the plant *P. graveolens*, which exhibits high antimicrobial activity against *S. aureus, Bacillus cereus,* and *B. subtilis*. [61] Geranium oil from Pelargonium graveolens has antimicrobial effects on S. aureus (ATCC 43300) and *S. aureus* bacteria sourced from a medical clinical setting at 0.25 μg/mL concentration. Additionally, geranium essential oils have shown therapeutic effects against the bacteria *S. epididymitis*. [61] With the Geranium oil, the S. aureus strain (ATCC 433000) was inhibited using 0.25 μg/mL of the Geranium oil. It was found that 47 out of 70 tested bacterial strains were sensitive to geranium oil at an oil concentration of 1.00 μg/mL or lower. [61] All *S. aureus* strains were isolated from the nasal cavity, skin lesions, postoperative wounds, intubation tubes, or patients' throats. [61]

There has been a growing concern about *S. aureus's* resistance to antibiotics. The bacterial strain has resisted the beta-lactam and glycopeptide antibiotic classes. [61] Because of this concern, using essential oils as a new way to help treat bacteria is becoming increasingly popular. [61] It was found that *S. aureus* strains are susceptible to the antibiotics tigecycline, rifampicin, trimethoprim-sulfamethoxazole, linezolid, fusidic acid, quinupristin-dalfopristin, vancomycin, and daptomycin.[61] The geranium oil was found to have antimicrobial activity against the clinically isolated strains of *S. aureus* and *S. aureus* (ATCC 433000). This is due to Geranium oil having citronellol and geraniol, which take up around 40% of all the major components of geranium oil.[61]

The geranium essential oil is seen to be most effective against the *S. aureus* strain ATCC 700699, and this is because a method known as disk-diffusion was used to test its antimicrobial

activity on the *S. aureus* ATCC 7000699 strain. [61] It was found that using these methods, the geranium oil with a concentration of 12.8 mg/mL was able to inhibit the growth of *S. aureus* strain ATCC 25923. However, the strain *S. aureus* ATCC 433000 found that at a low concentration of 0.25 uL/mL, the growth was inhibited using the geranium oil at this low concentration. [61] Lavender and geranium both, because of their promising antimicrobial properties, may provide some help in eradicating antibiotic-resistant bacteria. Treatment with essential oils is more favored as they show killing of antibiotic-resistant bacteria that cause life-threatening infections. In addition to lavender and geranium, the essential oil ylang-ylang against bacteria has also been studied, however, not as extensively.

The essential oil ylang-ylang, extracted initially from the *Chromolaena odorata* tree, [62] is native to Asia.[63] A study examined the essential oil ylang-ylang and its antimicrobial effects when tested against Gram-negative and Gram-positive multidrug-resistant bacteria, including *S. aureus, S. epidermidis,* and *E. coli.* [63] It was found that when ylang-ylang essential oil was used against these bacteria using the agar-well diffusion method, killing of all strains ensued. [63] An aromatic profile was also performed, and it was found that when ylang-ylang oil was used, the linalool compound was found. [63] This linalool compound was the most abundant compound present, as it had the highest percent composition of the oil [63]. This same component is found in lavender essential oil, which contributes, to its antimicrobial effects. Thus, a link has been established between linalool and antimicrobial properties. [63] This is due to linalool disrupting key cellular enzymes and denaturing key cellular proteins at high concentrations. [63] Another component of ylang-ylang oil, second to linalool, was a compound known as a-gurjunene. [63] This compound was shown to also have antimicrobial properties against *E. coli* bacterial strain because of its destruction of the cell wall and disruption of internal cellular mechanisms . [63,64]

In addition, a minimum inhibitory concentration (MIC) assay against ylang-ylang was performed against the two most susceptible bacterial strains, *S. aureus* and *E. coli.* [63] It was found that the most growth inhibition was for ylang-ylang concentration up to 500 μg/mL.[63] This all shows rising research using these oils and means of antibacterial and antimicrobial agents to help combat drug-resistant bacteria. However, these are all regular bacterial strains; studies involving the treatment of persister cells using essential oils are limited.

From these studies, it is important to note that essential oils have been examined to have killing effects against regular Gram-positive and Gram-negative bacteria. However, studies with persister cells have been limited, except for using tea tree oil as a method of persister cell eradication. Tea tree oil, however, has been shown to eradicate persister cells in both *E. coli* and *S. epidermidis* bacteria. [12] It was found that when treated at different concentrations starting from concentrations as low as 0.5% up to 2.5% tea tree oil at different time points, persistent cell eradication. [12] At the different time points, 1hr, 2hr, and 24 hr persister cells were eradicated in *E. coli* bacteria when treated with all concentrations of tea tree oil, this started as early as the 1hr time point. [12] However, *S. epidermidis* bacteria showed little growth at both the 1-hr and 2-hr time points; however, it was completely eradicated at the 24-hour time point. [12] So, in planktonic persister cell culture, tea tree oil was seen to cause persister cell death in Gram-negative and Gram-positive bacteria. [12]

Another aspect of this research was examining the specific components of tea tree oil. [12] It was seen that the primary killing agent of tea tree oil was Terpinen-4-ol. [12] This component showed that even at the 1-hr time point, all persister cells were eradicated, even at that early time, and even at the lowest concentration in both *S. epidermidis* and *E. coli* bacteria. [12] This

could be because this component has the biggest antimicrobial effect, even against *S. epidermidis* and *E. coli* strains. [12] Tea tree oil effectively eradicates persister cells, and even the individual components of the oil seem to exhibit anti-persister properties against both *S. epidermidis* and *E. coli.* This brings some light as persister cells are a real concern regarding patient infections. This tea tree oil research led to questions about other essential oils and their potential properties that could cause possible persister eradication. These questions sparked the inspiration for this research.

1.11 Our Contribution

Due to their antimicrobial properties and affordability, essential oils can be vital tools in combating antibiotic-resistant bacteria. In commercial products, essential oils are widely used in various skin and personal care products. This project focuses on addressing the major concerns of the antibiotic crisis and treating antibiotic-resistant bacterial strains with essential oils. One major issue contributing to antibiotic resistance is the formation of persister cells. These persister cells are dormant, indicating metabolic inactivity in response to environmental stress. Many of these persister cells are found in biofilm structures, contributing to the difficulty of treating antibiotic-resistant bacteria and many septic infections. Biofilm structures are responsible for post-operative infections due to the formation of biofilms on internal medical devices. In response to stress, these bacteria enclose persister cells in a hard lipopolysaccharide coating, thereby protecting the persister cells and preventing the penetration of antibiotic treatments. This research addresses whether lavender, geranium, and ylang-ylang essential oils can effectively eradicate persister cells in a planktonic persister culture and in biofilms. Both Gram-positive and Gram-negative bacterial strains, *S. epidermidis* and *E. coli*, will be used to examine each oil's effects on persister cells and biofilm structures. Fighting antibiotic-resistant bacteria with

essential oils could lead to the development of topical ointments that incorporate these oils, providing a more time- and cost-effective method for antibiotic-resistant bacterial eradication.

Chapter 2: Materials and Methods

2.1 Materials

The two bacterial strains used in this study include *Escherichia coli* MG1655 and *Staphylococcus epidermidis* RP62A, which were acquired from the American Type Culture Collection (ATCC; Manassas, VA, USA). Ylang-ylang, lavender, and geranium essential oils were purchased from Plant Therapy (Twin Falls, ID, USA). A 70% *v/v* essential oil solution was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and filter sterilized after solutions were made using a 0.22 μm filter.

The liquid media used to promote growth of *S. epidermidis* is known as tryptic soy broth (TSB). This media was made using 15 g of TSB premix purchased from Thermo Fisher Scientific (Waltham, MA, USA). For *E. coli*, lysogeny broth (LB) was used. LB media contained 5 g of NaCl, 5 g tryptone, and 2.5 g yeast extract and was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Both media were used to promote growth in both planktonic and biofilm experiments of *S. epidermidis* and *E. coli* bacteria. Agar plates were used to promote growth for both bacteria. For the bacteria *S. epidermidis,* tryptic soy broth (TSB) premix was combined with 15 g/L agar purchased from Thermo Fisher Scientific (Waltham, MA, USA). For *E. coli,* LB broth (5 g of NaCl, 5 g tryptone, and 2.5 g yeast extract) was mixed with 15 g/L agar, all purchased from Thermo Fisher Scientific (Waltham, MA, USA), to help count colony-forming units (CFUs). Colony-forming units (CFUs) were measured by manually counting colonies from 10 to 100. In the planktonic experiments, ciprofloxacin and ofloxacin were used

for *S. epidermidis* and *E. coli* and purchased from Sigma-Aldrich (St. Louis, MO, USA). Ofloxacin and ciprofloxacin were prepared in 0.1 M NaOH and 0.1 M HCl, respectively, and then filter sterilized using a 0.22 μm syringe filter.

In both experiments, colony-forming units (CFUs) are analyzed. The plating procedure to produce CFUs for each bacterium is standard. Contents are placed into a microcentrifuge tube. Each microcentrifuge tube is centrifuged at 10000g for one minute. Once finished spinning, a series of washes are performed by removing 900 uL of the supernatant and adding back 900 uL of PBS. Pelleted cells are resuspended in 900 μL of PBS before placing the microcentrifuge tube back into the centrifuge. These series of washes are performed a total of three times. After the third wash, 900 uL of supernatant is removed from each microcentrifuge tube, and 100 uL of pelleted cells are resuspended and placed into well one of a 96-well round-bottom plate. In wells 2-6 of the 96-well plate 90 uL of PBS is placed into each well. 10 uL of the solution is taken from well one and a 10 uL dot is placed onto the agar plate. 10 uL from well one is then add to well two thus, performing serial dilutions. After mixing 10 uL of the solution from well two is placed onto the agar plate. Continue this procedure for all samples and until all samples are plated. Plates were placed in the incubator at 37 °C overnight. The following day, colonies were counted, and colony-forming units (CFUs) were calculated from dilutions. When counting CFUs, plates with a range of 10–100 colonies were used to calculate the percent of survival and determine the efficacy of the treatments.

2.2 Testing Essential Oils on Planktonic Persister Cells

In a baffled Erlenmeyer flask, 25 mL of media (TSB or LB) was inoculated with *S. epidermidis* or *E. coli* from a -80°C stock. The cultures were placed in a shaking incubator for 24

hours at 37°C and 250 rpm. The next day, the cultured flasks were treated with the respective antibiotics for both *S. epidermidis* and *E. coli* at their respective concentration, 10 μg/mL on *S. epidermidis* and 5 μg/mL on *E. coli.* The flasks were then placed into the shaking incubator for either 24 or 48 hr, depending on the cultured bacteria. For the *E. coli* flask, a 24-hour incubation after antibiotic treatment, and for *S. epidermidis,* 48 hours, respectively, at 37 °C and 250 rpm. To prepare the persister cell culture 25 mL of culture was removed from the flask and added to a 50 mL conical tube. Once a blank was prepared, the culture was centrifuged for 10 minutes at 4000 rpm (BECKMAN GS-6KR Centrifuge, 3750 RMP MAX). Once centrifugation has finished, the supernatant is removed, leaving a pellet at the bottom, and then resuspended in 12 mL of PBS.

Three treatment times were tested (1 h, 2 h, and 24 h) for each concentration (0.5%, 1% and 2.5%) of 70/30% essential oil along with a PBS/DMSO (70/30%) control. The 70/30% solutions were made by adding 700uL of either essential oil or PBS to 300uL to the DMSO solvent. An 800 uL amount of resuspended culture was added to each tube containing 200 uL of essential oil solution thus, making each tube contain 1 mL of solution. Once solutions were prepared, an untreated sample was plated, and the remaining tubes were placed into the shaking incubator at 37°C 250 rpm. At every time point the cultures were plated using that standard plating procedure and incubated at 37°C for approximately 24 hours. After 24 hrs, colonies were counted, and colonies ranging from 10 to 100 were used in the data analysis calculations. Then, after CFUs, the percent survival was calculated and graphed. Error bars and statistical analysis was performed using the standard deviation of the means.

2.3 Testing of Essential Oils on Established Biofilms

A bacterial culture was made by inoculating 5 mL of media in a test tube with a stock of pre-aliquoted culture. The culture was placed into the shaking incubator for 24 hours at 37 °C and 250 rpm. After the incubation, an OD600 was measured, and the culture was diluted (OD600=0.01). A 200 µL amount of bacteria was added to a flat-bottom 96-well plate. The plate was incubated for 24 h at 37 °C to establish biofilms. After 24 h, the plate was washed with PBS twice to ensure that any planktonic or loosely attached cells are removed. Once washed 200 uL of 70/30% essential oil solution and 200 uL of 70/30% DMSO/PBS solution were placed onto the biofilms at varying concentrations (0.25%, 0.5%, 1%, 2%, 4%, and 8%).

After the biofilm plate was treated, it was placed back into the incubator for 24/48 hours at 37 °C. After the desired incubation, the media/treatments were removed, and the biofilms were washed twice with 200 uL of PBS. 200 µL of PBS was added to the wells, and the biofilms were removed from the bottom of the plate wells by scraping the bottom and the walls of the flat-bottom plate with a pipette tip. The biofilms extracted from the bottom of the plate in the 200 µL of PBS were placed into a microcentrifuge tube filled with 800 µL of PBS. The samples will then be plated on agar plates using the standing procedure and incubated at 37°C for approximately 24 hours. After 24 hrs, colonies were counted, and colonies ranging from 10 to 100 were used in the data analysis calculations. Then after CFUs and percent survival were calculated and graphed. Error bars and statistical analysis were performed using the standard deviation of the means.

In addition to the different experimental trials being tested on both *S. epidermidis* and *E. coli* bacteria, each experiment was performed in triplicate as it is the standard protocol to ensure accurate results.

Chapter 3: Results

3.1 Persister Cell Isolation using Antibiotics

All antibiotics are used and designed to specifically target one or multiple cellular mechanistic processes. For example, Penicillin and its derivatives are used to target and destroy the bacteria's cell wall. This cell wall targeting is a smart feature for penicillin, as prokaryotic cell walls are structurally different compared to an eukaryotic cell wall. The quinoline antibiotics such as ciprofloxacin and ofloxacin, target the bacterial DNA. Ofloxacin is used as a method of persister isolation for *E. coli* bacteria; this antibiotic treatment will kill all bacteria except for the persisters. [11,14,46] Similarly, ciprofloxacin is used to isolate *S. epidermidis* persister cells. Both ciprofloxacin and ofloxacin have antibacterial effects on *S. epidermidis* and *E. coli* without killing any persister cells.[12] In previous work, persister cell isolation was graphed in both *S. epidermidis* and *E. coli* bacteria. It was found that as the ofloxacin concentration increased to values greater than 5ug/mL and ciprofloxacin concentrations increased to concentrations greater than, 10ug/mL isolated the same percentage of *E. coli* and *S. epidermidis* persisters (Fig. 10 a and Fig. 10c) .[12] As shown with graphing percentage survival against concentrations of 5ug/mL and above for *E. coli,* and 10ug/mL for *S. epidermidis* the percentage of persister cells despite increases the concentrations of antibiotic remained the same thus giving a plateau (Fig 10a and Fig 10c).

In addition, both ofloxacin and ciprofloxacin successfully isolated persister cells at various time points using the concentrations of 5ug/mL and 10ug/mL respectively, (Fig. 10b and

Fig. 10d). [12] After 5ug/mL of ofloxacin and 10 ug/mL of ciprofloxacin were added to bacteria in stationary phase at different time intervals the data shows a plateau on the percent survival vs time graph (Fig 10b and Fig 10d). The plateau line represents just the persisters cells, that is why this plateau is at the same percentage of survival, suggesting that all other cells besides persisters have died (Fig. 10b and Fig. 10d).[12] So, despite increasing both drug concentrations and exposure time, roughly the same fraction of persisters were isolated as represented by all of the plateaus in the percent survival plots (Fig. 10).[12] It also can be found that despite an increase in antibiotic concentration against both bacteria the quantity of persister cells were not impacted (Fig 10a and Fig 10c). [12]

These prior results are significant because they suggest that persisters form at the same percentage each time. If persister formation was due to random chance, a different number of persisters should have formed in the bacterial population: the amount of less than <1% is verified by these results (Fig. 10). [12] With this, we now have confirmed the successful isolation of persister cells and plan to evaluate the effects essential oils.

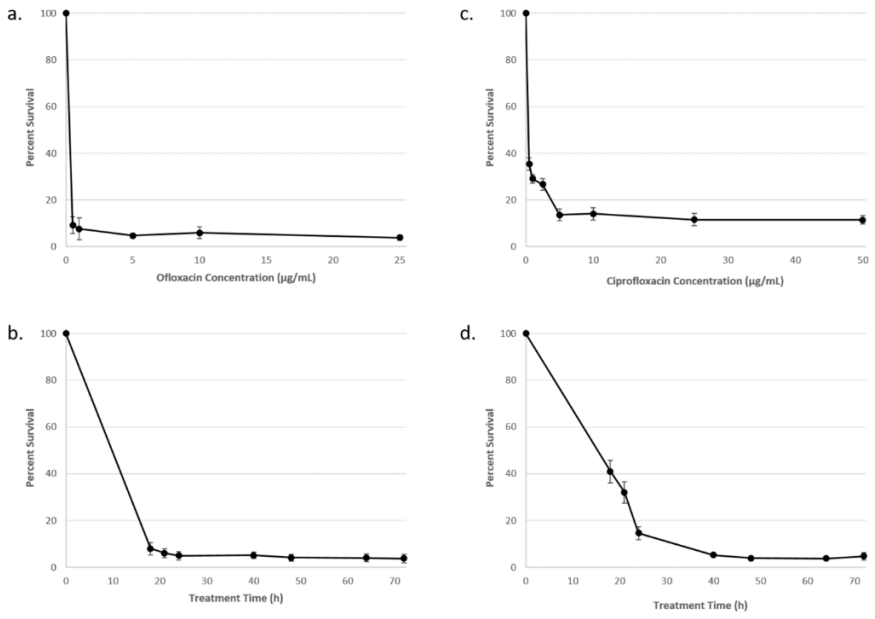

Figure 10: Graphical representation of **(a)** *E. coli* and **(c)** *S. epidermidis* persister cell isolation with increasing of both ofloxacin and ciprofloxacin concentration. In addition, to antibiotic concentrations increasing **(b)** treatment time of 5ug/mL of ofloxacin and **(d)** increasing treatment time of 10 ug/mL of ciprofloxacin to respective bacteria to isolate persisters. Figure from ref 12.

3.2 Quantification of Data

Standard plating and colony-forming units (CFUs) were counted to quantify the results of all experiments performed. The percent survival was calculated, and the average of all three trials was taken. After calculating the average percent survival, it was graphed to produce bar graphs. When graphing data if an average percent survival for any given essential oil concentration acceded 100% survival the value was just labeled as 100%. Anything over 100% survival continued to grow despite treatment with either the essential oil or in the presence of the DMSO control. In addition, the standard error of the mean was used to produce error bars and provide a statistical data analysis.

When performing experiments, treatment of the three essential oils chosen, lavender, ylang-ylang, and geranium, was performed on both a gram-negative and a gram-positive bacterial strain. The gram-positive bacteria *S. epidermidis* were one of the organisms tested in the study. An essential feature of *S. epidermidis* is that this microorganism is viable on both the external and internal surfaces of the human body. This makes *S. epidermidis* an opportunistic pathogen because it is viable in different environments and quickly adapts to a given condition. This knowledge led to the overall inspiration for testing essential oils against these specific bacteria. Many essential oils are found in skin and personal care products, so the hope is that these oils, lavender, ylang-ylang, and geranium, against bacterial strains will provide insight into whether essential oils have any multipurpose use.

3.3 Treatment of Planktonic S. epidermidis persisters with Essential Oils

In planktonic culture of *S. epidermidis* bacteria, it was seen that when treated with lavender essential oil at 1% concentration and a 1-hour treatment time point, there was a significant decrease in persister cell survival (38.945%) in comparison to the DMSO control (100%) (Fig. 11a). It should be noted that all time points DMSO grew to 100% survival (Fig 11 a). At a 2.5% lavender concentration at the 1-hr time, further decrease was seen in survival (11.81%). At the 2-hr time point and a 1% lavender concentration, the percent survival continued to decrease (1.036%), and at the 2.5% lavender concentration, only (0.385%) of persister cells remained. Finally, at 24 hours, all persister cells were eradicated (0%), given both the 1% and 2.5% essential oil concentrations (Fig. 11a).

When testing the geranium essential oil, the data showed that starting at 1 hour at a 0.5% essential oil concentration, a significant decrease in persister cells survival occurred (9.101%) compared to the DMSO control (100%). The DMSO control, which grew at all three time points, had 100% survival. The 1% and 2.5% geranium concentration at the 1-hr time point also decreased persister cell survival (3.127% and 3.528%), respectively (Fig. 11b). At the 2-hour time point the concentrations of 0.5% and 1% oil showed almost complete persister cell death (0.419% and 0.58%). However, the 2% oil concentration effectively eradicated all persister cells (Fig. 11b). At the 24-hour time point the 0.5% essential oil and 2.5% geranium eradicated all persisters (Fig. 11b), however, interestingly the 1% geranium after 24 hours still had a remaining persister survival of (0.673%) (Fig. 11b).

The ylang-ylang essential oil against the *S. epidermidis* bacteria showed complete eradication of persister cells at all essential oil concentrations (0.5%, 1%, and 2.5 %) at all three time points

(1hr, 2hr, and 24hr) (Fig. 11c). The DMSO control showed (100%) survival at all three time points (Fig. 11c).

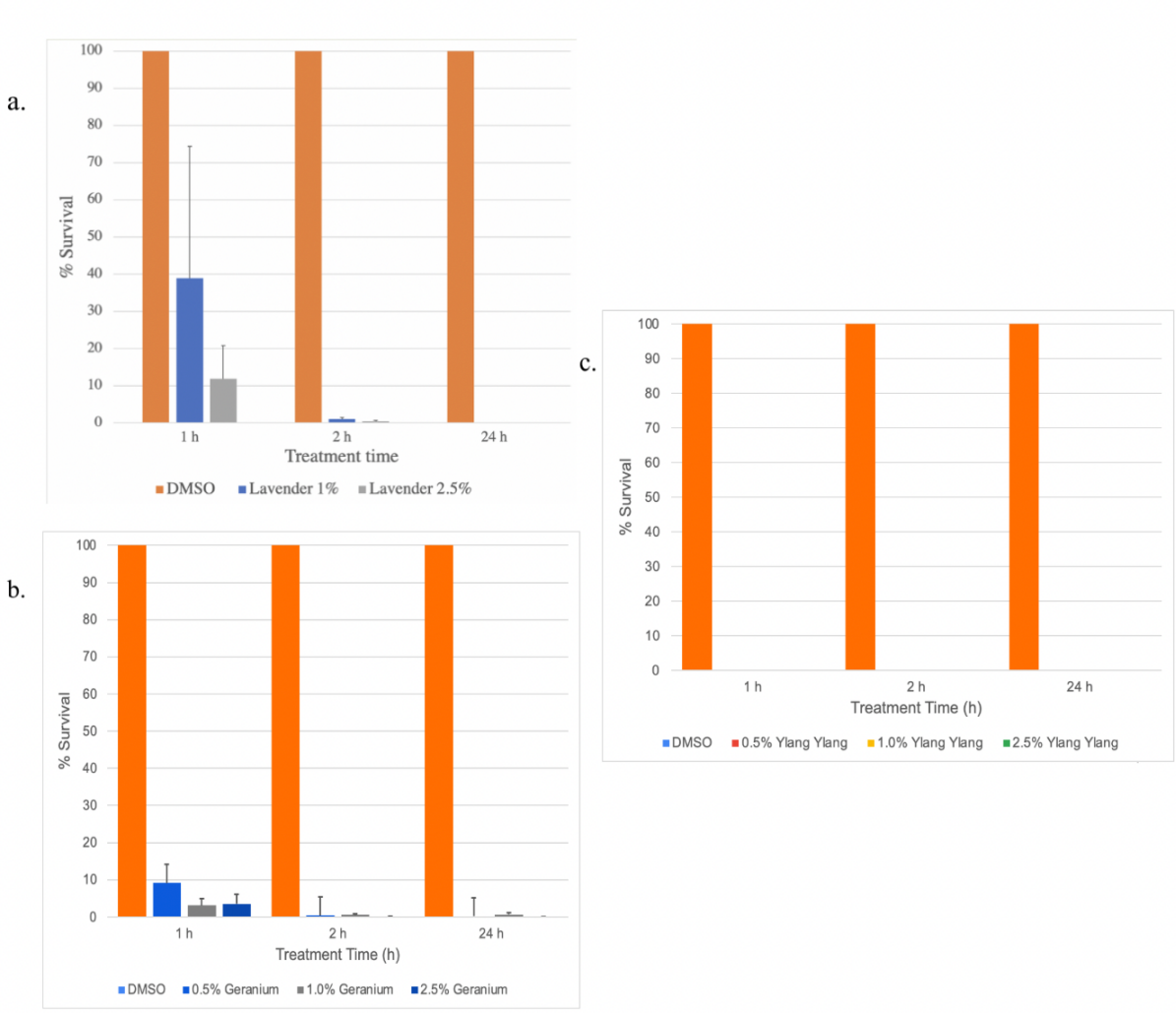

Figure 11: Treatment of essential oils against planktonic persister cells of **(a-c)** *S. epidermidis* bacteria. *S. epidermidis* bacteria were treated for 1 hr, 2 hr, and 24 hr with different concentrations of the following essential oils: **(a)** lavender, **(b)** geranium, **(c)** ylang-ylang. After treatment, persister cells were determined, and the percent survival was calculated. The percent survival of the untreated DMSO control was also plotted. The data represents three replicate trials and error bars represent the standard error of the mean.

Given the results from the *S. epidermidis* bacteria, which is a Gram-positive strain, a further investigation of a Gram-negative bacterial strain was conducted; in this case, *E. coli* bacteria were used.

3.4 Treatment of Planktonic E. coli persisters with Essential Oils

When treated with both geranium and lavender essential oil, it was seen that even at the lowest concentration of 0.5%, all planktonic persister cells were eradicated at the 1-hr, 2-hr, and 24-hr time points (Fig. 12a and Fig. 12b). The DMSO control for the geranium essential oil showed full growth at all time points (100%) survival (Fig. 12b). For the lavender DMSO control specifically at the 2hr time point it was seen that persister cells did not have complete survival (100%) instead the DMSO at 2hrs showed (62.42%) survival (Fig. 12a). However, at the 24-hour time point the DMSO grew to the max percent survival (100%) similarly to the DMSO control at the 1-hr time point.

The ylang-ylang essential oil showed at the 1-hour treatment time, specifically at the low concentrations of 0.5%, some slight persister survival was observed (0.5493%) (Fig. 12c). However, not only is this percent survival valued at less than 1% but, in addition to the comparison to the DMSO control this concentration of 0.5% ylang-ylang showed a significant decrease in persister cell survival (Fig.12c). At both the 2-hr and 24-hr time points for all concentrations of ylang-ylang oil, it was observed that all persister cells were eliminated, thus (0%) survival (Fig. 12c).

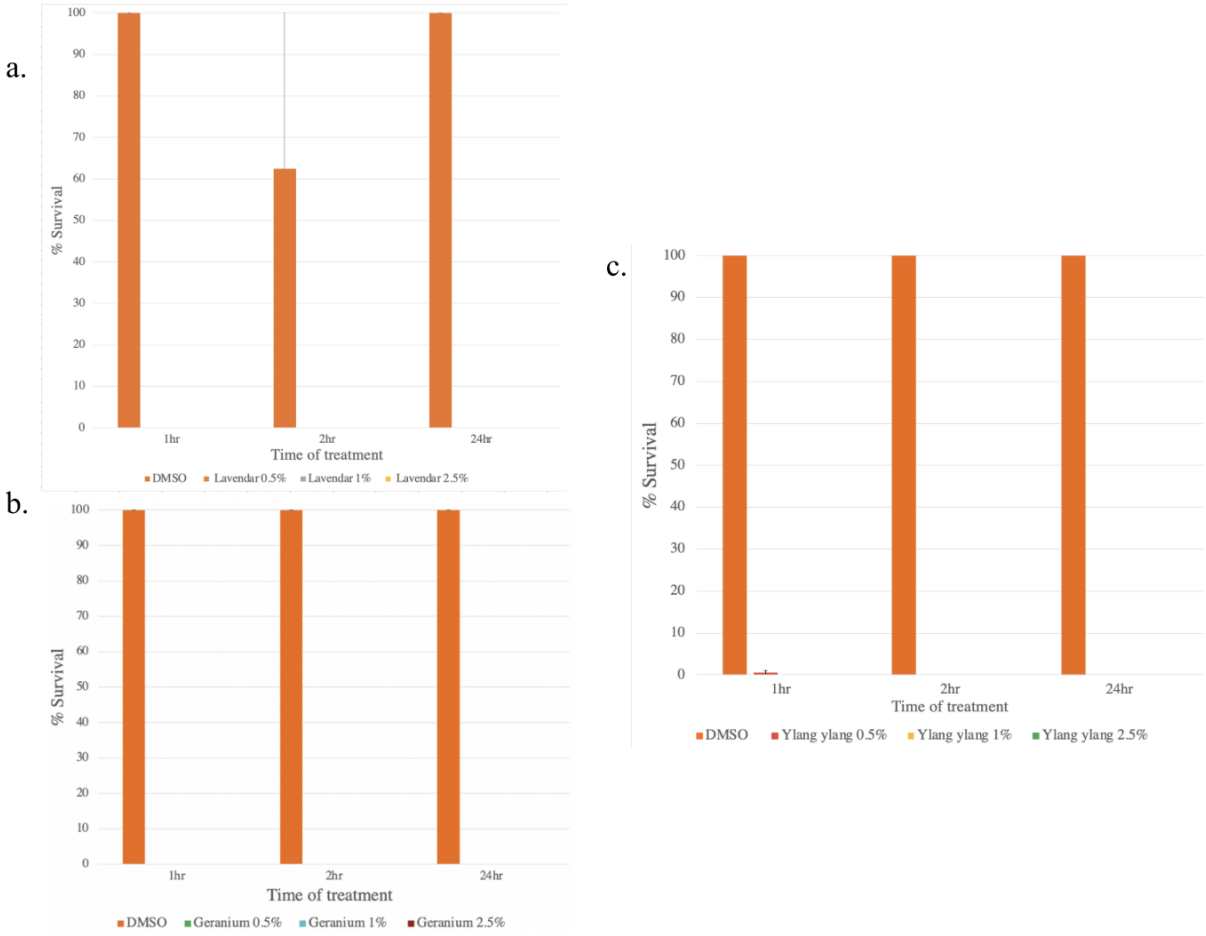

Figure 12: Treatment of essential oils against planktonic persister cells of (**a-c**) *E. coli* bacteria. *E. coli* bacteria were treated for 1 hr, 2 hr, and 24 hr with different concentrations of the following essential oils: **(a)** lavender, **(b)** geranium, **(c)** ylang-ylang. After treatment, persister cells were determined, and the percent survival was calculated. The percent survival of the untreated DMSO control was also plotted. The data represents three replicate trials and error bars represent the standard error of the mean.

The three essential oils effectively eradicated planktonic persister cells in both *S. epidermidis* and *E. coli* bacteria. This indicates promising results that could promise a solution to combating persister cells within both these bacteria in the medical field. Now that planktonic cultures were examined, how essential oils would affect biofilm structures created by both these bacteria was studied.

3.5 Treatment of S. epidermidis Biofilms with Essential Oils

Given our success with treating both *S. epidermidis* and *E. coli* persisters with essential oils, the effect essential oils have on the biofilm structures of both bacteria was then examined. Not only do persister cells present a concern regarding the antibiotic crisis, but biofilms are arguably more of a concern. Biofilm structures contain more persister cells encased with a polysaccharide coating, making them hard to penetrate with antibiotics. Biofilm structures are a perfect environment for an abundance of persisters to thrive. Biofilms pose a threat specifically in hospitals as these structures are prevalent on pacemakers, catheters, and internal medical devices; thus, when integrated into a patient's bloodstream, infection occurs. Interesting results were found when examining the effect of the three different essential oils against the two bacterial biofilms.

When *S. epidermis* biofilms were treated with lavender essential oil, it showed some intriguing results in comparison to the geranium and ylang-ylang essential oils (Fig. 13a). The lavender essential oil is most effective at eradicating biofilms at lower concentrations, specifically the 0.25% and 0.50% at the 24-hour time point. Showing 0.25% essential oil had a percent survival of (5.149%). For the 0.5% essential oil concentration, specifically at 24 hours, it was seen that (3.17%) biofilms survived. These values are significantly lower in comparison to the biofilm survival present at the 1% essential oil concentration, which was (95.09%) (Fig. 13a). To continue at 24-hrs, the 2%, 4%, and 8% essential oils had percent survivals of (71.106%, 77.33%, and 29.54%) respectively (Fig. 13a). So, at 24 hours the most effective concentration that promoted the most biofilm eradication was 0.5% lavender. (Fig.13a). At the 48-hour time point, specifically for 0.5% and 0.25% concentration, the percent survival was (19.88% and 10.76%). As the concentration increased to 1%, 2%, 4%, and 8% concentrations, the biofilm

killing continued to decrease with the acceptance of 2% (Fig. 13a). The percent survival of biofilms at these concentrations are (3.45%, 5.65%, 0.75% and 0.68%) respectfully (Fig. 13a). This result shows that the most killing occurred at the 48-hr time point for the highest essential oil concentration (Fig 13 a). For the DMSO control at the 24-hr and 48-hr time points, the percent survival was valued at (46.899% and 15.5%) (Fig. 13a).

For the bacteria *S. epidermidis,* when treated with geranium at various concentrations for 24 and 48 hours, it was shown that at both time points and 0.25% concentration of essential oil, biofilms were not eradicated and had (100%) survival (Fig. 13 b). At the 0.5% concentration, at 24 hr had a percent survival of (61.1%), and at 48 hr time had a percent survival of (65.5%) (Fig. 13 b). At the 1% oil concentration at the 24-hour and 48-hour time, a percent survival of (20.27% and 33.7%) was seen (Fig. 13 b). For 2%, 4%, and 8% concentration of lavender at both 24 and 48 hours, fully eradicated the bacterial biofilms (Fig. 13 b). The 24 and 48 hours DMSO control had a percent survival of (100%) (Fig. 13 b).

When using the ylang-ylang essential oil to treat *S. epidermidis* biofilms, it was seen at 24hrs 0.25%, 0.50%, 1%, and 4% essential oil concentration, the percent survival was (100%) (Fig. 13c). For the 2% and 8% concentrations the percent survival was (88.5% and 0.003%) respectively (Fig 13 c). For the 48-hour time point, the 0.25% oil concentration had a percent survival of (100%), 0.5%, 1%, and 2% concentrations had percent survival values of (61.3%), (71.2%), and (89.9%) (Fig. 13c). At the 4% and 8% essential oil concentration for 48hrs the percent survival was (18.8 and 6.6%) (Fig 13 c). So, it can be observed that the 8% concentration of oil was the most effective at both time points, despite not fully eradicating all biofilms (Fig. 13c). Both DMSO controls at 24 and 48 hrs showed (100%) survival (Fig. 13c).

Once *S. epidermidis* was tested against these essential oils, a Gram-negative bacterial strain, *E. coli,* was tested. As previously mentioned, biofilms are complex to treat due to their outer coating and habitable environment to house a higher number of persister cells, thus causing concern with antibiotic resistance. *E. coli* is a Gram-negative bacterium, which means that the cell wall of *E. coli* is different than the cell wall of *S. epidermidis,* which is a Gram-positive bacterium. Gram-negative bacteria are generally harder to treat as they have a more layered cell wall composed of outer and inner membranes. In addition, Gram-negative bacteria have special lipids that coat their outer cell wall, which are known as Lipopolysaccharides (LPS). These specialized structures add layers of protection against antibiotics for the Gram-negative bacteria. Finding treatments for Gram-negative and Gram-positive bacteria is essential to create a broad-spectrum solution to the antibiotic-resistant crisis. The essential oils were tested against *E. coli* at all the same concentrations; 0.25%, 0.5%, 1%, 2%, 4%, and 8% for 24 and 48 hours.

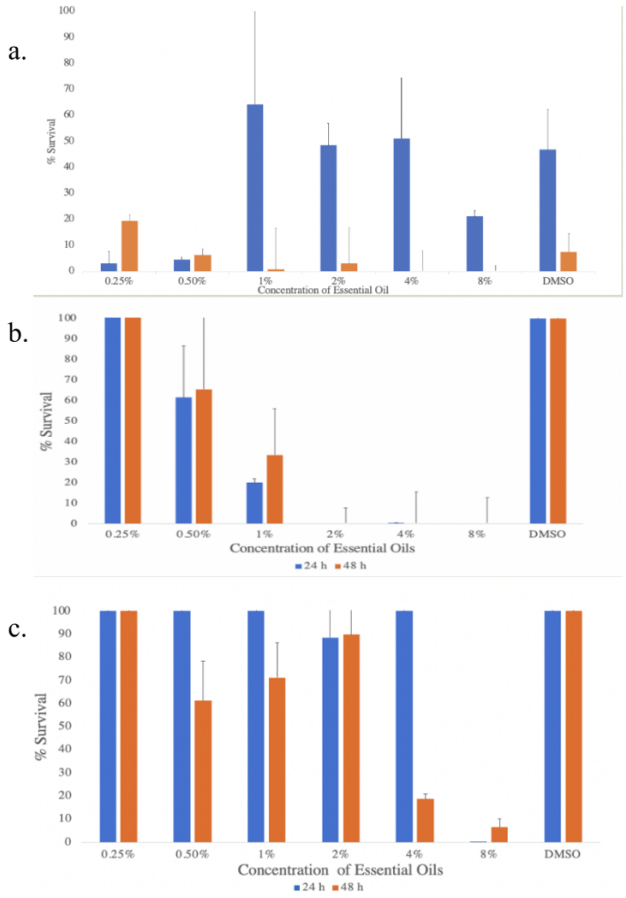

Figure 13: Treatment of essential oils against biofilms of (**a-c)** *S. epidermidis* bacteria. *S. epidermidis* bacteria were treated for 24 and 48 hours with different concentrations of the following essential oils: **(a)** lavender, **(b)** geranium, and **(c)** ylang-ylang. After treatment, biofilms were determined, and the percent survival was calculated. The percent survival of the untreated DMSO control was also plotted. The data represents three replicate trials, and error bars represent the standard error of the mean.

3.6 Treatment of E. coli Biofilms with Essential Oils

The essential oil lavender was then tested against the *E. coli* biofilm structure. It was seen that at the lower concentration of the essential oil, specifically 0.25% and 0.5%, at the 24-hour time point, showed a percent survival of (2.65% and 0.0988%) (Fig. 14a). At concentrations of

1%, 2%, 4%, and 8% at the 24 hr time point the percent survival of biofilms was shown (33.35%, 100%, 100%, and 67.61%) (Fig. 14a). This increase in percent survival show a lack of biofilm killing at these higher concentrations at the 24 hr time point (Fig. 14a). At the 48-hour time point, similarly, there was a decrease in percent survival of biofilm structures at the higher concentrations (Fig. 14a). At 0.25% and 0.5% the percent survival was (25.01% and 11.23%) respectfully (Fig. 14 a). At 1%, 2%, 4%, and 8% essential oil concentration at the 48-hour time, the percent survival was (41.91%, 41.16%, 39.08%, and 27.61%) respectively (Fig. 14a). The DMSO control for both the 24 and 48hr time points showed growth respectfully the percent survival of DMSO for 24hr and 48hr were (58.95% and 44.85%) (Fig. 14a).

It was found that when *E. coli* was tested against geranium at concentrations of 0.25%, 0.5%, 1%, 2%, 4%, and 8% specifically at a 24-hour time point, the percent survival was calculated to be (95.25%, 100%, 13.33%, 2.817%, 0%, and 8%) respectively (Fig 14 b). Thus, at a 24-hour time point, it was found that a complete eradication of persisters is shown with geranium at 4% and 8% essential oil concentrations (Fig. 14 b). At 0.25%, 0.50%, 1%, 2%, 4%, and 8% essential oil concentrations, the percent survival was found to be (100%, 84.4%, 0%, 16%, 0%, and 0%) at 48 hours (Fig. 14 b). It is interesting to note that the 1% and 2% essential oil concentrations led to a significant amount of killing compared to 0.25%. However, the 0.50% essential oil against biofilms (Fig. 14 b) demonstrated that at both 24 and 48-hour time points, the 4% and 8% essential oil concentrations eradicated all biofilms using geranium (Fig. 14 b). The DMSO for both 24 and 48 hours resulted in (100%) survival (Fig. 14 b).

When *E. coli* was tested against different concentrations of ylang-ylang for both 24 and 48 hours, it was found that the 8% essential oil concentration killed all biofilm structures (0.0183%) (Fig. 14c). At the 24-hour time point for 0.25%, 0.5%, 1%, 2%, and 4% essential oil,

the percent survival was found to be (71.97%, 100%, 81.31%, 12.25%, 30.6%) (Fig. 14 c). At the 48-hour time point, specifically 0.25%, 0.5%, 1%, 2%, and 4% essential oil, the percent survival was found to be (100%, 100%, 20.5%, 14.2%, and 10.3%) (Fig. 14c). As previously mentioned, the 8% essential oil for the 48-hour time point showed complete biofilm eradication (0%) (Fig. 14c).

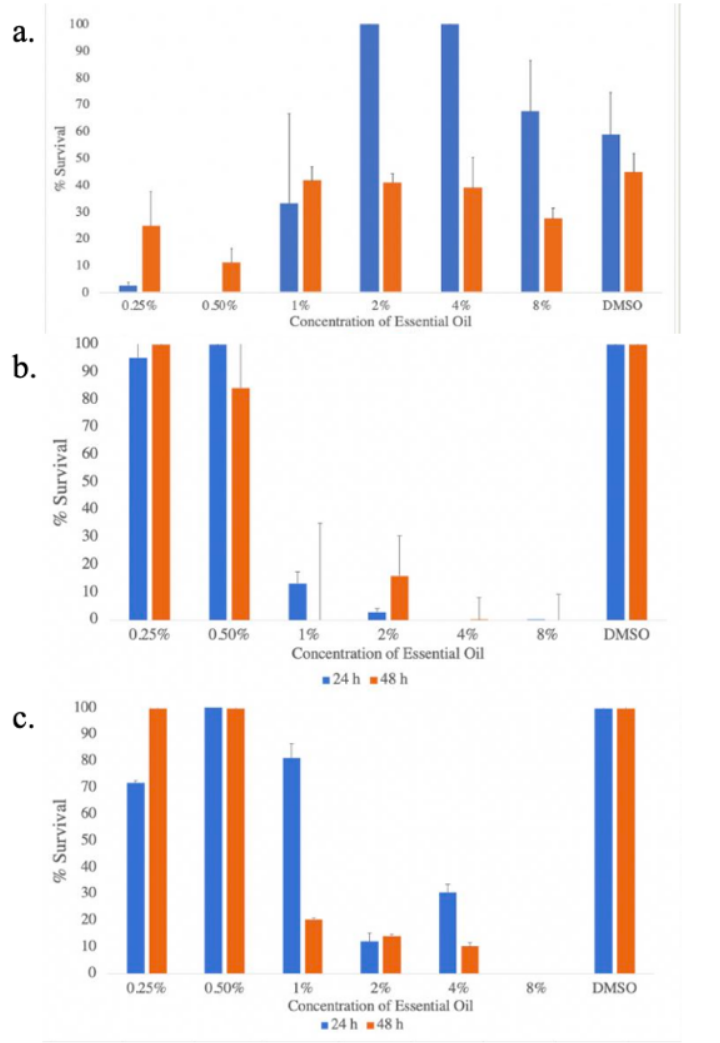

Figure 14: Treatment of essential oils against biofilms of **(a-c)** *E. coli bacteria*. *E. coli* bacteria were treated for 24 and 48 hours with different concentrations of the following essential oils: **(a)** lavender, **(b)** geranium, and **(c)** ylang-ylang. After treatment, biofilms were determined, and the percent survival was calculated. The percent survival of the untreated DMSO control was also

plotted. The data represents three replicate trials, and error bars represent the standard error of the mean.

Analyzing both the planktonic and biofilm experimental data using the two bacteria revealed some interesting results that warrant further examination. It can be determined that a trend has emerged from the analysis of the data. All essential oils, including lavender, geranium, and ylang-ylang, have been shown to cause eradication of persister cells in both *S. epidermidis* and *E. coli* bacteria, primarily at the 24-hour time point and at the highest concentration (2.5%) across all oils (Fig. 11 and Fig. 12). However, in biofilm structures of both *S. epidermidis* and *E. coli,* it was found that lavender was effective at lower concentrations (0.25% and 0.5%) at both the 24 and 48-hour time points against *E. coli* bacteria (Fig. 14a). In comparison, when treated with *S. epidermidis,* the 48-hour time point showed the most biofilm eradication, as lavender at both 4% and 8% achieved complete biofilm eradication (Fig. 13a). Both geranium and ylang-ylang oils also demonstrated effectiveness at higher concentrations (4% and 8%) at both the 24 and 48-hour time points in both *S. epidermidis* and *E. coli* bacteria (Fig. 13 b,c and Fig. 14 b,c).

Chapter 4: Conclusions

4.1 Overview

The properties of essential oils have been extensively studied. Essential oils have been used as natural remedy treatments since 2800 BC.[65] Essential oils exhibit antimicrobial properties, which have been tested against specific antibiotic-resistant bacteria such as *S. epidermidis* and *E. coli.* These studies have involved the use of regular bacterial cells. Treatment of both persister cells and biofilm structures with essential oils has become an active area of research. [66] Both persister cells and structural biofilms contribute to the antibiotic crisis, a public

health problem predicted to become responsible for 40 million deaths by the year 2050.[1] These persister cells are metabolically inactive and remain dormant during antibiotic treatment. [48] Biofilm structures are a breeding ground for these persister cells, as biofilm-forming bacteria develop a hard, protective coating consisting of lipopolysaccharide that encloses persister cells. Biofilm structures are complicated to treat as their outer coat protects them from antibiotics. The internal environment inside biofilm structures is also where nutrients and oxygen are limited, further driving these cells into the persister state. The primary concern regarding biofilms involves their prevalence on medical devices such as pacemakers and catheters. [12] Persister cells released during a biofilm burst pose a threat if they are infiltrated into the human bloodstream. Essential oils may prove helpful in the treatment of both persisters and biofilms. [67]

Essential oils are a favored treatment method as they terminate the persister cells' membrane and cell wall.[38] Essential oils are able to penetrate the bacterial cell wall, causing bacterial cell death. [12] The essential oils that have been studied in this work include lavender, germanium, and ylang-ylang. When tested against both *S. epidermidis* and *E. coli* planktonic persister cells, these essential oils caused termination (Fig. 11 and Fig. 12). Geranium and ylang-ylang were the most effective at eradicating biofilm structures in both bacteria. While lavender gave mixed results (Fig. 13 and Fig. 14).

4.2 Planktonic Persister cells treated with Lavender

Results of this study indicate that against *S. epidermidis* and *E. coli* planktonic persisters, the essential oil, lavender showed effective killing. Previous work involving lavender oil further supports the results obtained in this study. Lavender essential oil is effective when tested against antibiotic-resistant bacterial strains such as *Staphylococcus aureus* (MRSA), resistant to

methicillin, *Enterococcus* resistant to vancomycin, and *E. coli* which is resistant to penicillin. [68] Lavender was able to destroy the bacteria by penetrating its outer cell membrane as determined by bioluminescence methodology. [69] A specific component of the lavender oil, the monoterpene linalool, is responsible for the destructive activity against both biofilm and persister cells in *Candida auris*. [71] Crystal violet assays revealed penetration of biofilm formation in *Candida auris* at low lavender concentrations. [71] Despite favorable experimental results, against both *E. coli* and *S. epidermidis* persisters and biofilms, lavender still receives minimal attention. In addition, these bacteria *S. epidermidis* and *E. coli* are more prevalent in starting infection in humans therefore more relevant for additional studies. [72]

The results presented indicate that lavender worked best against *S. epidermidids* persisters starting at the 2hr time point and continued to the 24hr time both the 1% and 2.5% concentration (Fig. 11a). These results suggests that lavender and its specific component linalyl acetate need more than 1hr of treatment time to fully penetrate and break the cellular membrane in these persister cells. The Log P value measures the hydrophobicity of a compound and suggests the likelihood of the components partitioning between polar and non-polar solutions. [68] Compounds with a larger Log P are more nonpolar. Linalyl acetate's Log P value of 3.09 indicates that this compound is more likely to penetrate a non-polar component. [68] It is less likely to penetrate the thick polar peptidoglycan layer compared to *E. coli's* non-polar lipopolysaccharides. In *E. coli* bacteria, lavender completely eradicated persister cells starting at 1 hour of treatment time (Fig. 12a). It is plausible to think that the Gram-positive *S. epidermidis* bacteria have a thicker peptidoglycan layer, which makes it harder for lavender to integrate into the cell membrane. In contrast, lavender may more easily penetrate a Gram-negative organism, *E. coli,* due to a thinner peptidoglycan layer. Since lavender is a significant ingredient in skin and

other personal care products, it is also plausible that *S. epidermidis* bacteria, common to human skin, may already tolerate this oil and its properties.

4.3 Planktonic Persister cells treated with Geranium

Geranium essential oil has also been found to exhibit antimicrobial properties against antibiotic-resistant bacterial strains, including *Staphylococcus aureus*. [61] [69] Previous research suggests that geranium oil is effective against *S. aureus* bacteria in the stationary phase with concentrations as low as 0.25%.[70] This study indicates that persister cells were most likely present during this assay. The Minimum Inhibitory Concentration (MIC) of geranium essential oil was determined to be 0.5%. [70] Based on the previous MIC results, studies involving both *S. epidermidis* and *E. coli* bacteria were performed with this concentration in mind. A significant decrease in *S. epidermidis* persister cells was observed starting at the low concentration of 0.5% at 1 hr. Geranium oil, like the lavender, needed at least 2 hrs of treatment time before entire killing occurred (Fig. 11b). In *E. coli* bacteria, geranium completely eradicated all persister cells at all concentrations even at the 1hr time point (Fig.12b). One of geranium's components Citronellol, has a Log P value of 2.75. The non-polar nature of this compound will make it easier to penetrate the non-polar lipopolysaccharide layer of the Gram-negative bacteria. In contrast, citronellol will not easily penetrate a polar peptidoglycan layer of a Gram-positive bacteria. [71]

4.4 Planktonic Persister cells treated with Ylang-Ylang

The final oil tested against planktonic persister cells was ylang-ylang oil. Ylang-ylang oil, similar to lavender and germanium, exhibited antimicrobial properties against the opportunist pathogen as *Bartonella henselae*. [72] Previous experimental findings suggest that ylang-ylang oil exhibited antimicrobial properties against both *B. henselae* and *S. epidermidis* persisters. [72]

Ylang-ylang inhibited *S. epidermidis* persister growth starting at the lowest concentration, 0.5%, it continued to exhibit this killing trend with the highest concentration (1%) at both the 1hr and 2hr time point (Fig. 11c). When tested against *E. coli,* ylang-ylang oil worked the best as all persister cells at all time points and at all concentrations were eliminated (Fig. 12c). Similarly, previously reported research on the Gram- negative opportunist pathogen *B. henselae, S. epidermidis* bacteria were killed and eradicated. The properties of Gram-negative bacteria may make them more susceptible to the ylang-ylang oil, including the *E. coli* in our study. Furthermore, ylang-ylang may also have other life applications, such as insect control, because the research suggests ylang-ylang's ability to attack other Gram-negative bacterial species. [72]

Ylang-ylang oil's different main chemical component may be responsible for this increased the effectiveness of eradicating *E. coli* persister cells. Compared with lavender and geranium ylang-ylang oil's major component is benzyl acetate, which has a Log P value of 1.96. [73] [74] Enhanced lipophilicity of benzyl acetate likely results in enhanced eradication and easier penetration in a Gram-positive bacteria compared to *E. coli* bacteria. [75] Additional research is necessary to make definitive correlations between the chemical structures associated with biological activity.

Another component of this research project was the examination of biofilms. Biofilms lead to the production and storage of persister cells, making them essential structures for experimental study. Persister cells and biofilm structures are directly correlated, and it is crucial to test all three essential oils against both persisters and biofilms of each bacterium. In addition to examining persister cell eradication, lavender, geranium and ylang-ylang were tested at various concentrations against biofilms for 24 and 48 hours to determine the impact of those essential oils.

4.5 Biofilms treated with Lavender

When lavender essential oil was treated against *S. epidermidis* biofilms, the 48-hour time point worked the best at eradicating all persister cells, specifically at the 4% and 8% essential oil concentration (Fig. 13a). Those results suggests that lavender needs both a higher concentration and more time to penetrate the biofilm outer coating. Previous published experimental studies indicate that lavender oil requires a 16% v/v concentration to eradicate all biofilms of the food pathogen *Listeria monocytogenes.* [76] In this work, results suggest that after 48 hrs, both 4% and 8% lavender oil were sufficient to eradicate all biofilms of *S. epidermidis* bacteria. However, at the 24hr time point, at lower concentrations, such as the 0.25% and 0.5% lavender, showed the most biofilm killing. Higher concentrations seemed to have had less of an impact on biofilms (Fig.13a). In addition, lower concentrations 0.25% and 0.5% promoted the most *E. coli* biofilm killing (Fig. 14a). This could be the result of the lavender oil causing biofilm premature attachment. The process of attachment is vital as biofilms favor hydrophobic surfaces and electrostatic interactions. [77] In addition, detachment of biofilms is also essential, as demonstrated by studies that suggest that biofilm matrix synthesis either needs to stop completely or get disrupted by covalent bonds present between the actual matrix. [77] It is plausible to hypothesize that at the beginning of treatment time for biofilms, lavender at high concentrations disrupts the biofilm matrix, and promotes early detachment but not biofilm eradication. Potential future experiments will focus on the mechanism of action between oil and biofilm to elucidate a possible explanation for these findings.

4.6 Biofilms treated with Geranium

Geranium oil at the higher concentrations of 2%, 4%, and 8% showed complete biofilm eradication of *S. epidermidis* bacteria at both the 24- and 48-hour time points. (Fig. 13b). This result is not surprising as geranium oil killed planktonic persisters effectively at the 2.5% oil concentration in excess of 2.5% were expected to fully kill the biofilms (Fig. 11b). Complete eradication of *E. coli* biofilms was observed at the 4% and 8% oil concentration at both 24 and 48 hours (Fig. 14b). This data suggests Geranium oil not only breaks the outer coating to attack the biofilm but, also is effective at killing the persisters inside. Previous findings indicate that geranium as an effective treatment against *S. aureus* bacteria due to its alcohol-based components, citronellol, geraniol, and linalool. A working hypothesis is that the compounds will terminate cells by disrupting the cell membrane. [78] The proposed mechanism of action for the geranium oil components can explain the killing of both planktonic and biofilms in both *S. epidermidis* and *E. coli.*

4.7 Biofilms treated with Ylang-ylang

The final oil tested against both *S. epidermidis* and *E. coli* biofilm structures was ylang-ylang. Killing of *S. epidermidis* biofilms was observed starting at the 24 hr time at the 1% concentration (Fig. 13c). Partial eradication of biofilms was observed at the 4% oil concentration (Fig. 13c) while complete eradication of biofilms took place at the 8% oil concentration (Fig. 13c). For the 48-hour time point, killing ensued beginning with the low 0.25% concentration and continues through 4% oil concentration (Fig. 13c). At the 8% concentration, complete killing *S. epidermidis* biofilm structures at both the 24- and 48-hour time was observed (Fig. 13c). Biofilm

killing was observed at the 1% essential oil concentration for *E. coli* bacteria at 48 hours (Fig. 14c). At the 8% essential oil concentration at both time points 24- and 48-hour, complete biofilm killing occurred (Fig. 14c). For comparison, ylang-ylang from the previous persister assay performed in this study exhibiting killing effects at both the 1% and 2.5% concentration on *S. epidermidis* (Fig. 11c). In addition, it also showed complete killing of *E. coli* persisters at all three time points (Fig. 12c). The need for a greater essential oil concentration could be a result of the biofilm structure. Larger doses of essential oil may be needed to fully penetrate the outer biofilm layer. In addition, ylang-ylang essential oil has the same major chemical component, linalool, that is present in both lavender and geranium essential oil. Linalool may be responsible for the high antibiofilm activity. [79]

4.8 Conclusions

Results obtained from this research project indicate that lavender, ylang-ylang, and geranium, as essential oils, are able to eradicate planktonic persister cells in both *S. epidermidis* and *E. coli* bacteria. However, geranium and ylang-ylang oils seem to be the most successful at eradicating biofilm structures for both bacterial organisms, specifically at high concentrations. The essential oil of lavender still needs further research attention, as experimental results obtained to date are not conclusive.

Chapter 5: Future work

5.1 Future Projects

The effect of essential oils on both persisters and biofilm structures from antibiotic-resistant bacteria is a field that needs further research attention. The use of lavender specifically

against the biofilms needs further investigation, because results from this study show non-definitive results. Since lavender, geranium, and ylang-ylang were shown to eliminate persister cells, further attention should be devoted to the development of an antibiotic cream incorporating essential oils to stop external persister infections. In addition, the use of essential oils contained in intravenous lines to stop bloodstream persister infections can be explored. The potential benefit of adding essential oil soaks to implants and medical devices before patient insertion should be explored to combat the risk of biofilm and persister post-operative infection. [78] [79] In the future, it would also be interesting to examine the effect of essential oils on biofilms found on teeth to treat acidogenic acid-tolerant bacteria that result from poor oral hygiene [81]. These studies could provide some information regarding integrating essential oils into toothpaste and mouthwash. The experimental results surrounding lavender, geranium, and ylang-ylang suggest that additional research experiments into further applications of essential oil treatments of persisters and biofilms hold promise.

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