Drew University

College of Liberal Arts

Neroli Essential Oil as a Broad-Spectrum Killing Agent Against Bacterial Persister Cells

A Thesis in Chemistry

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Abstract

Persister cells are a small subpopulation of dormant bacteria that can survive antibiotic treatment while maintaining their genetic makeup. Once antibiotics are removed from a system, persisters repopulate, contributing to both antibiotic tolerance and antibiotic resistance. Essential oils contain antimicrobial properties that may disrupt a cell membrane and directly kill dormant persister cells. Essential oils commonly used in skincare, such as Neroli Essential Oil (NEO), can eliminate Gram-positive and Gram-negative bacteria, including *Staphylococcus epidermidis* and *Escherichia coli*, respectively, through this quick mechanism that can potentially kill infections. This study examines the effects of NEO on persister cells using a biphasic kill curve, anti-biofilm testing, and colorimetric assays. At concentrations of 0.5%, 1%, and 2.5%, NEO eliminated over 99% of persister cells within 1 h and fully eradicated them by 24 h in both *S. epidermidis* and *E. coli*. NEO also effectively killed some persister cells in biofilms, highlighting its potential as an anti-biofilm agent. Upon colorimetric analysis with Erythrosin B, increasing absorbance of *E. coli* demonstrated that the killing mechanism disrupts the cell membrane, though results were inconclusive for *S. epidermidis*. Spectrophotometric assays further confirmed leakage of DNA and proteins from NEO-treated cells, reinforcing a direct, membrane-disrupting killing mechanism that leads to cell lysis. Future work should explore NEO's activity within biofilm environments, isolate the oil's major active chemical components, and investigate drug delivery strategies to translate these findings into practical medical treatments. Overall, NEO demonstrates strong potential as a broad-spectrum natural therapeutic capable of targeting both persister and biofilm-associated infections.

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1. Introduction

1.1 History of Antibiotic Resistance

Antibiotic resistance has emerged as a critical medical challenge, with few significant advancements since the 1980s. According to a 2019 CDC report, over 2.8 million antimicrobial-resistant infections occur annually in the United States, resulting in approximately 35,000 deaths.[1] These fatalities underscore the urgent need to better understand and address this growing medical challenge. In addition, a 2022 CDC report concluded that antimicrobial resistance is the leading cause of death in the world, with a particularly severe impact in low-resource countries.[2] This resistance develops when bacteria evolve to survive antibiotic exposure, often through genetic modifications that allow them to thrive despite treatment.[3] Antibiotics are substances, either synthetically produced or naturally derived, that function as bactericidal agents, killing bacteria, or as bacteriostatic agents, inhibiting their growth and multiplication. Often used synonymously, antimicrobials are agents designed to reduce the risk of infection by inhibiting the growth of microbes, including bacteria, viruses, fungi, and parasites.[4,5] Aside from mutating, bacteria can also acquire resistance through horizontal gene transfer of resistance-encoding genes, which grant the resistant phenotype to the bacterial population.[6] The use of antibiotics is essential for treating infectious diseases and ensuring the success of surgical procedures, such as prosthetic transplants and placing pacemakers. However, growing reliance on antibiotics and increasingly sophisticated resistance mechanisms have

placed a significant financial burden on healthcare systems due to the need for constant adaptation. There remains a critical need to discover new antibiotics and explore uninvestigated drug targets through structural-level insights that can drive research and therapeutic development forward.[7]

Some of the earliest microbially derived antibiotic treatments were found in ancient Serbia, China, Greece, and Egypt. The use of moldy bread for healing wounds or medicinal soil for various ailments have been documented in the Eber's papyrus from 1550 BC.[8] In the 1900s, Paul Ehrlich gained popularity for his "magic bullet" theory in which a drug could directly target diseased cells without killing healthy cells. His synthetic arsenic-based drug was distributed as Salvarsan®, "the arsenic that saves", to treat the causative agent of syphilis (Figure 1).[8,9] Activated by oxidation, the compound targets syphilis-causing bacteria and the arsenic disrupts the bacteria's vital processes.

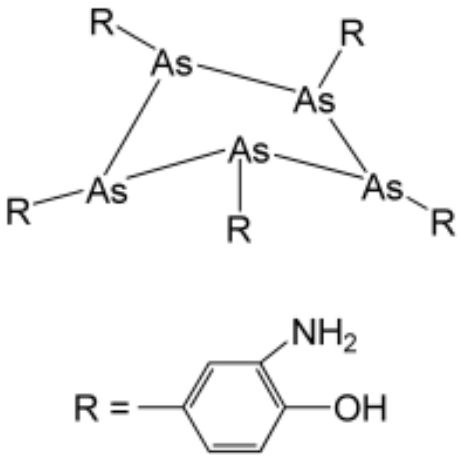


Figure 1: *Structure of the pharmaceutical compound Salvarsan shown by ESI spectrometry to be cyclopolyarsines. Figure created by Lloyd et. al.* [10]

Ehrlich researched dyes that specifically stained bacterial cells or specific cellular structures to understand biological processes. Salvarsan was superseded by Gerhard Domagk's sulfonamide prodrug Prontosil in 1932, a compound also inspired by bacterial cell dyes.[8] He used Prontosil, a bright red azo dye, to treat the streptococcal infection in his daughter's arm that would have otherwise needed to be amputated (Figure 2). Though resistance was reported in the late 1930s, sulfonamides were the first effective broad-spectrum antimicrobial.

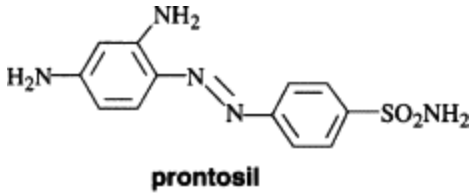


Figure 2: *Structure of Prontosil. Figure created by Silverman.*[11]

Serendipitously, Alexander Fleming accidentally discovered penicillin (Strain: *Penicillium notatum*) on a contaminated petri dish in 1928. On the plate with bacteria, there was a distinct ring around the penicillin mold, revealing its antibacterial property. Penicillin was purified by several scientists at Oxford from an extract of the mold and tested for clinical use on a series of mice. It was first used for treatment in February 1941 and has saved millions of lives since.[12–14] By 1942, however, penicillin resistance was first identified. Scientific debate between Robert Robinson and Dorothy Hodgkin about the structure of penicillin led to the elucidation of the beta-lactam structure in 1945, enabling the development of semi-synthetic derivatives to avoid future penicillin resistance (Figure 3).[8] It shares a common beta-lactam ring with several antibiotics that prevent bacteria from forming strong cell walls.

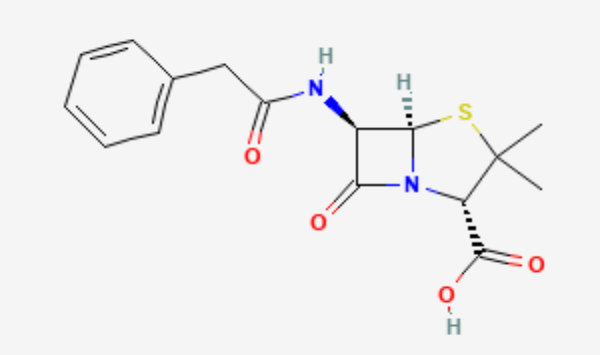


Figure 3: *Benzylpenicillin, the common structure of today's penicillin. Figure created by PubChem.*[15]

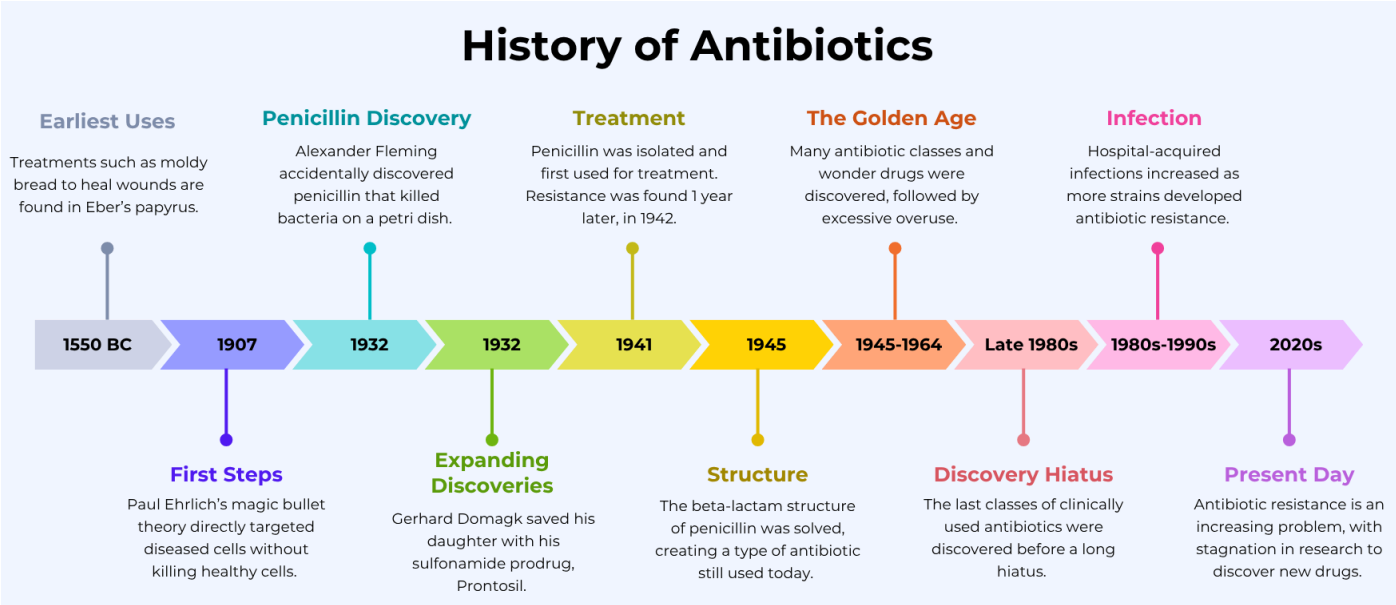


Figure 4: *An outline of the History of Antibiotics.*

The Golden Age of antibiotic discovery was from 1945-1965 where a rapid increase of antibiotic classes and "wonder drugs" were introduced, and were quickly followed by an excessive overuse of these drugs (Figure 4). Antibiotics can be classified in several ways, but they are most commonly grouped by either their chemical structure or their mechanism of action.

Major classes include beta-lactams (such as penicillins and cephalosporins), macrolides, tetracyclines, quinolones, aminoglycosides, lincosamides, and nitroimidazoles.[16] Most newer antimicrobial agents are chemical modifications of these existing classes, and as a result, very few truly new antibiotic classes have been discovered since the 1980s. In addition to a lack of new drug approvals, many bacterial pathogens have evolved to become multi-drug resistant (MDR), such as tuberculosis (Strain: *Mycobacterium tuberculosis*) which caused many hospital-acquired infections in the 1980s-1990s. This strain is known as a superbug, a microbe with "enhanced morbidity and mortality due to multiple mutations endowing high levels of resistance to the antibiotic classes specifically recommended for their treatment".[17] Despite advances in medicine, MDR pathogens continue to pose a major challenge in hospitals today, where they remain a serious risk to patient safety. Although resistance mechanisms to antibiotics are different for every microbe, most involve gene transfer and are evolutionary. For natural resistance, bacteria exhibit reduced permeability of the outer membrane and the activity of efflux pumps. Acquired resistance can occur through transformation, transposition, or conjugation, or through mutations in the bacterial DNA.[18]

Many researchers believe that there are still endless possibilities in the search for new antimicrobial agents. Others, however, remark that inhibitor-target and inhibitor-resistance interactions are not well understood and more structural insight is needed for further developments.[17] Due to the growing financial and emotional burden caused by antibiotic resistance, there is an urgent need for novel therapeutic strategies to effectively treat infectious diseases. One such promising avenue of study being pursued is anti-persister therapy.

1.2 Persister Cells and Their Relation to Antibiotic Resistance

Surviving antibiotic exposure is not solely attributed to resistance; it can also be facilitated by the presence of persister cells, dormant bacterial cells which tolerate antibiotic treatment without making genetic changes. Instead, persister cells are metabolically inactive and remain unaffected by antibiotic effects, allowing them to regrow the parental population as healthy cells that may indirectly mutate or acquire resistance genes. Persisters give rise to a bacterial population that is as susceptible to the antibiotic as the parental population, thus differentiating from the genetic evolution of resistance.[6] Together, resistant and persistent cells contribute to the difficulty and expense of bacterial treatment.

Persister cells are a small subset of dormant bacteria that tolerate antibiotics by surviving environmental stress or population-wide killing, later regrowing to restore the parental population. These cells grow slowly or remain metabolically inactive, allowing them to survive antibiotic treatment. During the exponential growth phase of bacteria, bacterial colonies proliferate rapidly under ideal environmental and nutritional conditions. However, as nutrient sources become scarce and waste products accumulate, bacteria transition into the stationary phase of growth, during which persister cells form. When environmental conditions normalize, persisters can “wake up” and repopulate, leading to a potential relapse of infection. In simpler terms, one can view persister cells like this: in a small town, a few people take a nap and wake up to find that devastation (an antibiotic) wiped out their entire town. In order for their town to flourish once more, these survivors get to work and start to repopulate until the town is alive and thriving once more, just as it was previously. Triggered persistence is a result of stress conditions, such as nutrient limitation, high cell density, acid stress, immune factors, and interactions with other bacterial cells.[6] In this study, antibiotic exposure serves as the stress

signal, mimicking resource depletion and environmental pressure and promoting the formation of persister cells.

Differences exist between resistant and persistent bacterial cells (Figure 5). When an antibiotic is added to a system, resistant bacteria tolerate the addition and genetically adapt to survive and populate at a greater rate. A genotype is an organism's hereditary genetic information, and thus any changes to this would alter genetic composition. A phenotype is an organism's observed properties and phenotypic changes would appear in development or behavioral in nature.[19] Resistance is a genotypic response due to mutation that changes the genetic makeup of bacteria and ultimately influences how that population functions. In contrast, a susceptible bacterial colony, consisting of typical bacteria that are neither resistant or persistent, is quickly eradicated by antibiotics and tolerant bacteria are still killed, only at a slower rate. In the colonies with persister cells, the population is not entirely eliminated. Persisters embody a bacterial phenotype that relies on metabolism to maintain a dormant state during antibiotic stress and exits this state upon the removal of antibiotics.[6] Unlike resistant bacteria, persister cells show a phenotypic response where they maintain their genetic makeup and go to “sleep” as a stress response and “awaken” after stress condition removal to ultimately present the same as the parental bacteria.

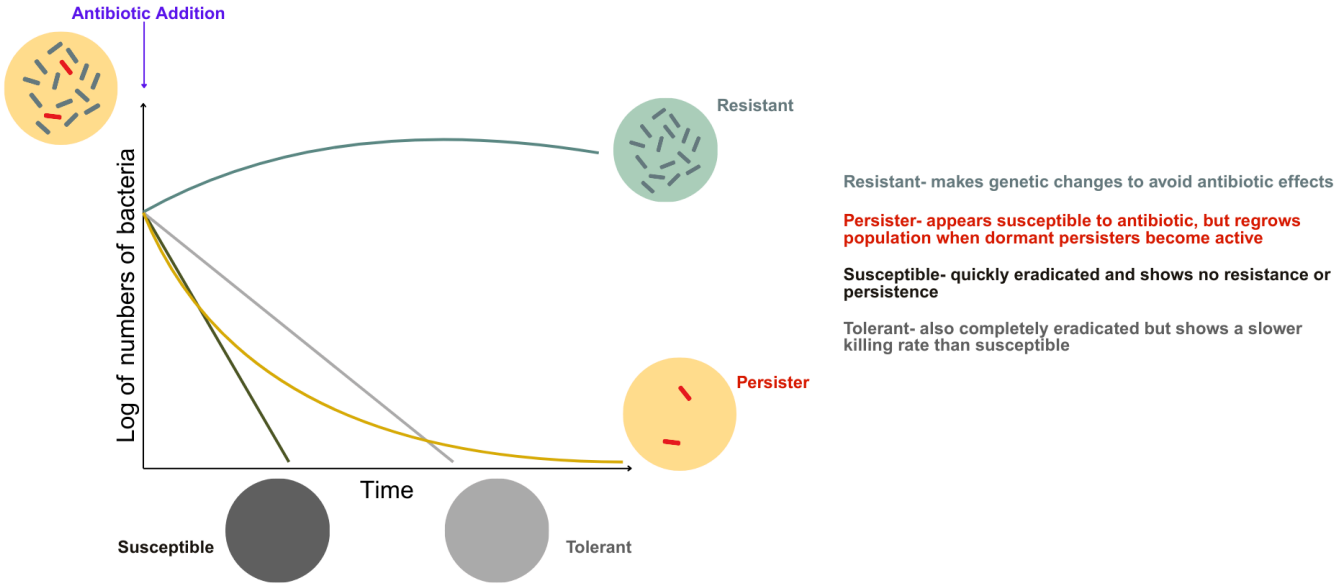


Figure 5: *Killing kinetics of resistant, tolerant, and persister cells. Following the addition of antibiotics, the different bacterial populations are killed at different rates, or grow if they are resistant. Figure adapted from Fisher et al.*[20]

A typical bacterial growth curve helps to illustrate where persister cells originate (Figure 6). Like all bacteria, only a few cells are present during the lag phase, when conditions are not yet ideal for growth. Once conditions become favorable, bacteria grow rapidly during the log phase. Persister formation occurs when resources become competitive in the stationary phase and bacterial killing occurs after antibiotic addition. While resistant bacteria undergo genetic changes that allow them to survive this stress, susceptible bacteria with persister cells appear to die off at a normal rate. As a consequence, persisters are often overlooked, as it seems the bacterial population has been eliminated. Once antibiotic treatment ends, the small number of dormant

surviving persister cells can "wake up" and regenerate the original population.

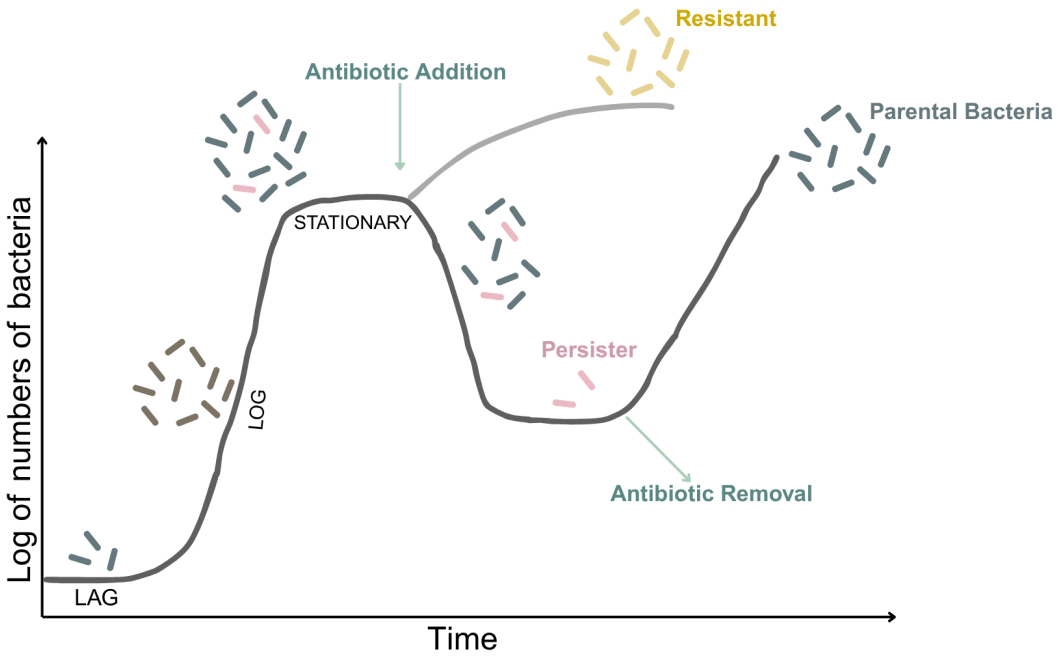


Figure 6: *Bacterial growth curve specific to persister cell formation.*

Antibiotic-tolerant persister cells can both survive antibiotic treatment and re-establish growth afterwards, contributing to many health risks and difficulty with eradication of bacterial infections.[21] Unlike resistant bacteria, persister cells cannot replicate in the presence of antibiotics, but are killed at a much lower rate than the susceptible population.[6] Persister cells show multi-drug tolerance, different from antibiotic-resistant bacteria, that do not result in the mutation of resistance genes.[22] Tolerant bacteria are very similar and are slow or non-growing, whereas persister bacteria are simply non-growing, although this perceived difference is subject to debate.[22] The persister cell's ability to survive antibiotic treatment without being genetically resistant plays a large role in resistant or recurring bacterial infections. The proposed reason is that the immune system cannot detect the pathogen, causing a "failure of the host immune

system to clear the causative organism".[3] Resistant bacteria prevent effective drug binding to their targets, disrupting the drug's killing mechanism. In persistent bacteria, these processes are slowed, and target activity is diminished, resulting in increased bacterial survival.[6] High counts of persister cells have been observed in patients requiring repeated antibiotic doses for infections, highlighting the role of persister cells in contributing to recurrent infections and the importance of investigating their mechanism.[6]

1.3 History of the Discovery of Persister Cells

During the antibiotic discovery boom of the 1940s, researchers observed a strange phenomenon where some bacterial cells, despite being genetically identical to the rest, were able to survive treatment with antibiotics that should have killed them. Gladys Hobby first described bacterial persistence in 1942 when she experimented with penicillin and found that 1% of the bacteria had not been killed by the drug, an example of phenotypic tolerance.[23] In 1944, Joseph Bigger studied the addition of penicillin to staphylococci and found that one out of a million cells survived prolonged antibiotic treatment. He coined the term "persisters", and offered that treatment might be done through alternately administering penicillin and removing treatment to accommodate those bacteria in the persister phase.[24] Ultimately, neither of these researchers received much attention for their work on persistence, as their findings were not well understood at the time.

The phenomenon of persistence was overlooked for another 30 years until Alexander Tomasz discovered genotypic tolerance *in vitro* in 1970. He found a pneumococcal mutant that does not lyse during penicillin exposure, despite being as sensitive to penicillin growth inhibition as the wild-type parent strain. He concluded that this could be a result of bacterial persistence.[25]

Four years later, Gary Best identified the first genotypically tolerant *S. aureus* (strain Evans) and subsequent findings on its reduced autolysing activity under antibiotic stress. This study provided early mechanistic insight into how some bacteria, without inherent genetic resistance, survive lethal antibiotic exposure.[26] This discovery marked the beginning of recognizing the importance of persisters and paved the way for subsequent findings. In 1976, a high prevalence of persisters was found by Mayhall et al. among several clinical isolates of staphylococci.[27] The next year, Diane Horne and Alexander Tomasz observed a tolerant response in *Streptococcus sanguis*.[28] Then, in 1983, Harris Moyed carried out research in the lab of Alexander Tomasz and identified the first persister gene *hipA*. He found three highly persistent (*hip*) mutants with 10-10,000-fold increased persister cell fractions upon incubating with penicillin.[29,30] These findings became frequently used as a research tool for persistence. This same year, Brennan and Durack identified a connection between the degree of *S. sanguis* persistence and efficacy of treatment, where tolerant bacteria survived better than non-tolerant bacteria. The researchers concluded that combination therapy could help with knowledge of tolerance mechanisms.[31] These foundational studies opened the door for a growing body of research that would continue to uncover the biological basis, clinical relevance, and broader implications of bacterial persistence in the decades that followed.

Persister research regained traction again in the 1990s-2000s when biofilm-associated infections increased with the usage of indwelling medical devices, such as stents and catheters.[32] Biofilms are dense bacterial communities that adhere to surfaces and are notoriously difficult to treat, often leading to persistent infections. This increase of infection also went alongside a rise in immunocompromised patients, such as those undergoing cancer treatment or those with HIV. Kim Lewis discovered the link between bacterial persistence and biofilm infections in 2001, and

it was found soon after that biofilms contain persisters which is what makes biofilm associated-infections so difficult to treat.[33] With the usage of mathematical models and computer simulations, it was shown persistence could extend the duration of antibiotic treatment which may lead to treatment failure. This phenomenon is called "non-inherited antibiotic resistance", or simply put, a type of resistance that bacteria genetically develop after treatment failure.[34] A later study on tuberculosis highlighted the clinical significance of bacterial persisters and the importance of understanding the extent of their formation. It showed that pyrazinamide, by targeting persisters, could shorten the course of tuberculosis treatment and help reduce relapse rates, ultimately highlighting the value of anti-persister strategies in improving therapeutic outcomes.[35] So, decades after the discovery of persister cells, antibiotic resistance has caused persister research to be much more medically relevant, highlighting the importance of understanding persister formation and killing mechanisms at a deeper level.

1.4 Persister Cell Formation

Persisters exhibit phenotypic heterogeneity that can be affected by colony size and species.[22] Many bacteria display persister cell formation, though the fractions of persister cells in a population varies by species and environmental factors.[21] A majority of persisters are formed in the stationary phase of bacterial growth. Studies have shown ATP levels are significantly reduced in the stationary phase of *S. aureus,* while exponential growth requires higher ATP concentrations.[36,37] As a result, the proportion of persister cells is higher in the stationary phase than in the logarithmic phase.[22] A continuation of this study found that stationary-phase-specific promoters were activated in persisters with reduced ATP, suggesting that metabolic differences between growth phases contribute to antibiotic evasion.[22,37] These findings support the

conclusion that ATP levels play a universal role in persister cell induction, although most known mechanisms involve toxin-antitoxin systems.[22]

It is generally understood that persisters remain in a distinct phenotypic state with the expression of toxin-antitoxin (TA) systems that are formed in stationary phase bacteria.[21,36] TA systems refer to linked genes responsible for encoding a toxic protein and its corresponding antitoxin to form a neutral complex easily degradable by ATP dependent proteases.[36] These toxins consist of a stable toxin, which disrupts essential cellular processes, and a labile antitoxin that prevents toxicity.[38] The toxins may be translation inhibitors which downregulate protein synthesis and induce dormancy, allowing the cells to evade antibiotic effects.[36,37] When antibiotics are added to bacteria, they experience stress conditions, such as nutrient limitation or pH changes. During this stress, the cells try to activate genes in attempt to resist and convert part of the population to a dormant state to survive through inactivity.[39] It is believed that the most optimal scenario for a cell is to remain a persister until the stress condition is no longer present. This survival strategy permits the cell to continue to grow once the stressor is removed.[21] Further, the cell response to stress activates TA systems through alarmone guanosine tetraphosphate, or ppGpp, that changes transcription and reduces DNA replication and protein synthesis.[38]

Persister formation greatly varies from species to species, and the mechanism of formation has not yet been completely elucidated. A 2010 study found that ciprofloxacin induces several TA genes in *E. coli* and causes persister formation by activating the TisB toxin, a membrane peptide that decreases proton motive force and ATP levels to induce dormancy.[39] While many researchers have supported the idea that TA systems contribute to persister formation, some have questioned whether stress-induced activation of certain toxins is the

primary mechanism, suggesting instead that ATP depletion alone may drive dormancy.[40] A 2017 study found that the persister marker rrnB P1 in *E. coli* persisters is activated by a drop in ATP levels, concluding that stochastic variation in ATP is the main mechanism of persister formation.[40] This explanation aligns with antibiotic mechanisms that rely on corrupting energy in a cell, and a decrease in ATP production could explain drug tolerance.

1.5 Strategies for Eliminating Persister Cells

Most strategies for eliminating persister cells rely on a process called sensitization, where dormant cells are reactivated metabolically, making them susceptible to antibiotics. Examples of strategies to sensitize persister cells include promoting resuscitation within the dormant population to prompt active target production, stimulating persister metabolism to trigger a phenotypic switch from dormant to antibiotic-sensitive, and manipulating drug influx rates.[36] This method typically involves targeting ATP-dependent processes, as many antibiotics require activated cells to function. Other mechanisms include the destruction of DNA cross-linking, inhibition of important enzymes, and the generation of reactive oxygen species, which are highly unstable.[36] Ultimately, persister cells have significantly lower ATP levels compared to metabolically active bacteria, rendering these treatments less effective.[40] Therefore, an alternative approach is to focus on non-ATP-dependent methods, such as directly disrupting the cell membrane.

By targeting the cell membrane, cell lysis can occur in its dormant state without requiring metabolic activation. This method has the advantage of bypassing the need for ATP and avoiding triggering unpredictable and complex intracellular pathways that might form during antibiotic treatment. Such metabolic pathways could allow the bacterial population to survive and adapt,

potentially leading to antibiotic resistance or bacterial persistence and the continued spread of infection. Direct killing by membrane disruption eliminates this risk and is a promising avenue for treating persistent bacterial infections. The precise molecular mechanism of action of direct killing, however, still remains unclear.

1.6 Persisters Associated with Biofilms and Biofilm Formation

Persister cells can be found in the planktonic state, or free-floating in solution, in the body. When these cells adhere to a surface, many others join and begin to form a sticky, sugary, polymer around themselves. This structure is known as a biofilm, a resilient community of microorganisms attached to a surface that plays a significant role in persistent bacterial infections. Biofilms are primarily water but also contain a complex matrix of polysaccharides, proteins, extracellular DNA, lipids, membrane vesicles, nutrients, and metabolites that together embed the cells and support the structure of the mature biofilm.[38,41,]

Biofilm-associated infections are linked with in-dwelling medical devices such as pacemakers, prosthetic joints, or catheters.[41] When there is a foreign object in the body, bacteria gravitate towards and stick to it, leading to a layer of well-protected bacteria. Within a biofilm, bacteria exhibit significantly greater antibiotic tolerance compared to their planktonic form. This increased resistance is attributed to the protective physical barrier of the matrix surrounding the biofilm, which shields bacterial cells from external threats and provides nutrients, creating an environment where persister cells can thrive in much higher concentrations. As a result, biofilms are of importance when exploring persister cells. The outer membrane of bacteria are often negatively charged, which explains their tendency to colonize surfaces with opposite charges in the body.[41] Biofilms still occur on living tissue where bacterial adhesion is facilitated by surface

adhesins.[41] These biofilms can be found in moist, nutrient dense environments, such as on teeth or on respiratory and gastrointestinal surfaces.

Bacteria can switch between planktonic and biofilm form and do so as a survival mechanism to avoid being washed away by water or blood.[7] The biofilm's strong surface increases cell density which can allow for plasmid exchanges through conjugation that encodes for antibiotic resistance.[7] Additionally, persisters are dormant due to their low metabolic activity, leading to a significantly reduced growth rate that is characteristic of persisters in biofilms, offering the foremost reason for reduced susceptibility of biofilms to antibiotics.[42] Biofilm formation is influenced by several factors, beginning with the initial deposition of bacteria. This initial step can occur through weak interactions such as Van der Waals forces, sedimentation, and Brownian motion, which bring bacteria into close proximity and facilitate the early stages of biofilm formation. Since the surface of bacteria typically carry a negative charge, they are naturally repelled from environmental surfaces at about 10–20 nm. As a means of overcoming this barrier, bacteria rely on attractive forces like Van der Waals interactions, as well as structures such as fimbriae and flagella, to stick to these surfaces.[7] For some species that can only attach to themselves rather than another surface, these cells may utilize cell-cell communication, otherwise known as quorum sensing, to colonize.[43] Once the bacterial cells have congregated, they begin producing a polar extracellular matrix that surrounds and protects them from environmental stressors and potential threats, marking the transition into the maturation phase. During this irreversible binding, and until the biofilm is complete, extracellular polysaccharide substances are secreted. At this point, a mature biofilm is three dimensional and consists of many "towers" of the matrix. The small channels between these towers are for transportation of water, nutrients, and waste, and they offer a safe space for planktonic bacteria to inhabit.[43] This

structure protects bacteria from any immune system response through failed activation of phagocytes.[43] After the biofilm reaches maturity, environmental stress or other unfavorable conditions can trigger the release of planktonic cells from the biofilm towers. This leads to the final stage, dispersion, where bacteria can spread and colonize on a new surface.[7]

Despite ongoing research, clinical drugs specifically targeting bacterial biofilms remain in the development phase.[44] The biofilm matrix surrounding bacteria enhances their tolerance and resistance to antibiotics, contributing to many chronic diseases that are challenging to treat effectively.[43] This unmet medical need underscores the importance of investigating potential anti-biofilm agents to help prevent or treat biofilm-associated infections.

1.7 Gram-positive and Gram-negative Bacteria

The assessment of persister cell killing approaches across both Gram-positive and Gram-negative bacteria is important for developing broad-spectrum therapeutics, as treatments are more valuable when they are effective against multiple bacterial types. Gram-positive and Gram-negative refers to the structural differences in the bacteria's cell membrane, and killing mechanisms may vary between bacteria. Gram-positive bacteria, such as *S. epidermidis*, have a thick peptidoglycan layer in their cell wall and lack a distinct outer membrane. In contrast, Gram-negative bacteria, like *E. coli*, possess a lipid-rich outer membrane and a thinner peptidoglycan layer. However, both types have a plasma membrane composed of a phospholipid bilayer.[45]

Gram-positive bacteria are largely contained by the peptidoglycan cell wall, which serves as a "scaffold for the attaching proteins, polysaccharides, and ribitol-phosphate polymers".[46] While the core chemical structure of peptidoglycan is similar across many bacterial species, there

are variations in bridge structure, chain length, and the overall physical organization within the cell wall.[46] Generally, peptidoglycan is composed of repeating disaccharide N-acetyl glucosamine-N-acetyl muramic acid units that are cross-linked, or bridged, by pentapeptide side chains.[47] The variation in peptidoglycan structure can inhibit the function of some antibiotics, further complicating the ongoing issue of antibiotic resistance. One study found that *S. aureus* has a tightly packed peptidoglycan structure that, when interacting with vancomycin antibiotics, causes the peptidoglycan pores to clog and prevent drug penetration. The study proposes that the reduced antimicrobial activity may be due to bulky covalently linked glycopeptide dimers.[46] In other words, the dense peptidoglycan layer poses a challenge to penetration and may reduce the overall effectiveness of treatment.

Gram-positive bacteria lack an outer membrane which serves to protect the cell from environmental stress, provide structural support, and prevent the entry of harmful molecules. In order to compensate for this shortcoming, these bacteria have a layer of peptidoglycan which is much thicker than that of Gram-negative bacteria. Both Gram-positive and Gram-negative bacteria contain peptidoglycan, though the thickness of the layer and composition of the cell wall vary. Gram-positive bacteria have a thick peptidoglycan membrane of 30-100 nm that contains many layers, whereas Gram-negative bacteria have a cell wall thickness around 10 nm, with peptidoglycan thickness of 2 nm to 3 nm.[47,48] Anionic teichoic and lipoteichoic acids are interwoven in the cell wall and anchored to the head groups of the membrane lipids, and these components account for over 60% of the cell wall mass.[47,48] Surface proteins are also present and associate closely with, or are anchored to, the lipid membrane, as Gram-positive bacteria lack the containment of the outer membrane.[47] These structural differences effect a molecules ability to penetrate the membrane and cause direct killing of a bacterial cell.

Gram-negative bacteria have a much more complex membrane with different components such as the outer membrane, the peptidoglycan wall, and the inner membrane. The outer membrane is a lipid bilayer composed of lipopolysaccharide and phospholipids with proteins attached to it. Lipopolysaccharide is a glucosamine disaccharide with acyl chains and O-antigen chains connected to a polysaccharide core. This nonfluid molecular matrix forms a barrier to hydrophobic molecules, and the efflux pumps and porins of the membrane limit molecules of around 700 Daltons or larger.[47] The thin peptidoglycan layer can be found directly below the outer membrane, and this combination makes the cell wall selectively permeable. The inner membrane is a phospholipid bilayer where many proteins for cellular function are located. Of all these layers, the outer membrane makes the most substantial contribution to the lack of permeability of the Gram-negative bacteria. Permeability is a factor that complicates the treatment of Gram-negative bacteria with antibiotics more than Gram-positive bacteria.

This project utilizes two bacterial species to model treatments for both Gram-positive and Gram-negative organisms. *Staphylococcus epidermidis* (*S. epidermidis*) is a common Gram-positive bacterium of the human skin microflora that typically has a benign relationship with its host.[44] While generally harmless on the skin surface, this bacterial species can become opportunistic when burns, scratches, wounds, or other breaks compromise the skin barrier, allowing it to enter the bloodstream and form biofilm-associated infections, particularly on implanted medical devices such as cardiac devices, prosthetic joints, and catheters.[49] Bloodstream infections occur in 4-5 out of every 1,000 central venous access catheter insertions in the United States, with 22% of these caused by *S. epidermidis*.[44] *S. epidermidis* serves as a low biohazardous Gram-positive bacterial model that can be compared to the functions and properties of many other Gram-positive bacteria.

Escherichia coli (*E. coli*) is a common Gram-negative bacterium found in the intestinal flora where it can cause intestinal and extraintestinal illnesses. Although typically harmless as part of a healthy intestinal microbiota, *E. coli* can also be found outside of the intestinal tract and can cause urinary tract infections, pneumonia, bacteremia, and more.[50] This bacterium serves as an excellent Gram-negative model for studying both planktonic and biofilm persisters. By experimenting with both Gram-positive and Gram-negative bacteria, conclusions can be drawn that may suggest a broad-spectrum approach.

1.8 Obtaining Persister Cells

Persister cell formation occurs after antibiotic stress conditions, thus obtaining persisters for investigation begins by treating bacteria with antibiotics. In this study, *E. coli* is treated with ciprofloxacin and *S. epidermidis* is treated with ofloxacin. Ciprofloxacin is a broad-spectrum bactericidal antibiotic belonging to the fluoroquinolone drug class that is most potent against Gram-negative bacteria.[49] This antibiotic prevents DNA replication through inhibition of bacterial DNA topoisomerase and DNA-gyrase, with the GyrA subunit of gyrase being the primary resistance mechanism for *E. coli*.[49] Very similarly, ofloxacin is a broad-spectrum antimicrobial fluoroquinolone used to treat bacterial infections, notably having little therapeutic advantage over ciprofloxacin.[51] Ofloxacin binds to and inhibits DNA-gyrase and topoisomerase IV which interrupts DNA replication, transcription, and repair.[51] In general, fluoroquinolones are synthetic therapeutics that are concentrated intracellularly which result in rapid intracellular killing.[51,52]

After treating and incubating bacteria with these antibiotics, the removal of the antibiotic creates stress conditions that lead to persister formation, an important assumption for the methods used in this investigation. In order to isolate persisters for experimentation, it is assumed that

centrifugation will isolate the persister cells and any remaining dead cell content from treated bacteria. This step is essential, as persisters must be obtained from the population of previously sensitive bacteria exposed to the antibiotic. Now that a method for obtaining persister cells has been established, and it is understood that traditional bactericidal antibiotics are ineffective against them, an alternative approach must be considered. This study investigates the use of a familiar substance that has a long history of health benefits: essential oils.

1.9 Essential Oil Properties and Neroli Essential Oil

Recent studies highlight the promising antimicrobial activity of essential oils from medicinal plants, primarily through membrane disruption.[53] Applied in healing practices by ancient civilizations such as Egypt, China, and India, essential oils have long been used for their antioxidant and anti-inflammatory properties in familiar oils such as tea tree, lavender, and eucalyptus oil. Known for their aromatherapy benefits, the concentrated components of essential oils have historically been incorporated into cosmetic formulations and medicinal ointments, serving as therapeutic agents for a wide range of internal and external ailments. Research suggests that essential oils can even destabilize cell membranes, causing leakage of organelles and ultimately leading to cell death.[54] They can have toxic effects on cell membrane integrity, fluidity, rigidity, and function due to their hydrophobic nature. Research suggests that this mechanism is a series of events that involve the cell wall and the interior of the cell, such as cytoplasm or DNA content. Additionally, essential oils have been shown to have potent effects on both Gram-positive and Gram-negative bacteria.[45] This direct killing mechanism effectively eliminates persister cells without triggering intracellular pathways. Quantitative measures of membrane permeability include protein leakage, membrane potential, and leakage of genetic

materials such as DNA and RNA.[45] Due to their therapeutic potential, essential oils are a promising anti-persister agent with a potential pharmacological future, offering a natural alternative to conventional treatments.

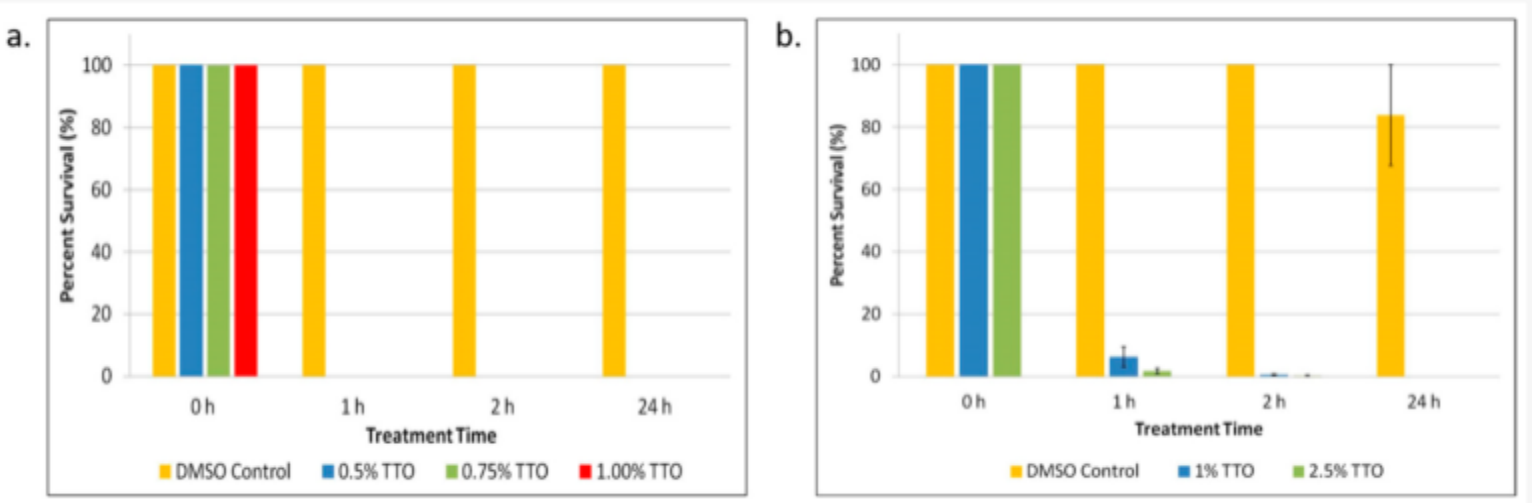


Figure 7 (a) *E. coli persister cells and* ***(b)*** *S. epidermidis treated with varying concentrations of Tea Tree Oil and a DMSO control show complete killing of persister cells treated with Tea Tree Oil.*[55]

Previous research focused on the effects of tea tree essential oil (TTEO) on killing Gram-positive and Gram-negative persister cells.55 Within 1 h of TTEO treatment, 99% of *E. coli* persister cells were killed, indicating a rapid rate of cell lysis (Figure 7a). These findings suggests that TTEO directly killed the bacteria through cell membrane disruption, rather than by triggering a metabolic pathway responsible for cell death. By 24 h, 100% of the persister cells were completely eradicated. Similar results were obtained in experiments with *S. epidermidis* persisters (Figure 7b). Most persisters were eliminated within the first hour, with 100% killed after 24 h, demonstrating the effectiveness of TTEO as an anti-persister agent.[55]

Based on the anti-persister success with TTEO, the investigation to evaluate the potential use of neroli essential oil (NEO; *Citrus aurantium*) for treating Gram-positive and Gram-negative bacteria began. This Egyptian oil is obtained through the hydrodistillation or steam distillation of the flowers from the blossom of the bitter orange tree, with more than 90% of the worldwide production sourced in Tunisia and Morocco.[56] NEO is commonly used in skincare products to address acne, aging, dry complexions, and various skin traumas, such as cuts, eczema, psoriasis, and scars.[57] It also possesses analgesic and anti-inflammatory properties, making it a promising candidate for antibacterial activity.[58] Previous studies found NEO to effectively kill stationary phase *S. aureus* after 3 day exposure at low concentrations of 0.5%, 0.25%, and 0.125%.[59]

Given the demonstrated ability of TTEO to eliminate persister cells in both Gram-positive bacteria (*S. epidermidis*) and Gram-negative (*E. coli*), it is hypothesized that NEO, with its similar antimicrobial properties, will exhibit comparable effects in targeting persister cells. This research study aims to determine whether NEO can effectively kill *S. epidermidis* and *E. coli* persister cells by disrupting the bacterial membrane in both planktonic and biofilm forms, providing a novel approach to combating persistent infections. The molecular mechanism behind this killing effect, however, remains unclear and is also further explored.

Ultimately, the objective of this project is to eradicate persister cells in *S. epidermidis* and *E. coli* to reduce the risk of pathogenic infections and gain a deeper understanding of persister cell elimination. Given the role of persister cells in biofilm-associated infections, this investigation seeks to identify effective anti-persister and anti-biofilm agents. Unlike conventional antibiotics, which rely on bacterial metabolic activity, essential oils are explored as an alternative antibacterial approach. As natural products, they do not require synthetic

processes, making them an appealing option for alternative therapies. By examining the membrane-disrupting properties of essential oils, this study aims to uncover new methods for targeting persister cells, thereby reducing infection risk and limiting bacterial survival through non-traditional pathways during treatment. The determination of a broad spectrum therapeutic is ideal for further studies in medicinal applications.

2. Materials and Methods

2.1 Bacterial Strain and Other Materials

Staphylococcus epidermidis RP62A and *Escherichia coli* MG1655 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and neroli essential oil (NEO) was purchased from Plant Therapy (Twin Falls, ID, USA). A 70% v/v NEO solution was prepared using dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as the solvent.

Media components, including tryptic soy broth (TSB), agar, NaCl, KCl, Na_2HPO_4, and KH_2PO_4, were sourced from Thermo Fisher Scientific (Waltham, MA, USA). TSB was used for planktonic growth with *S. epidermidis* and TSB-agar (TSB + 15 g/L agar) was prepared to measure CFUs. For *E. coli*, lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used for planktonic growth, and LB-agar (LB + 15 g/L agar) was prepared to measure CFUs.

HCl, ciprofloxacin, and ofloxacin were also purchased from Sigma-Aldrich. Stock solutions of ciprofloxacin in 0.1 M HCl and ofloxacin in 0.1 M NaOH were prepared and diluted with ultrapure water.[55]

2.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) for NEO against *S. epidermidis* and *E. coli* were determined by growing each stock solution of bacteria in its respective media (TSB or LB). These were incubated in a conical tube for 24 h at 37 °C with shaking at 250 rpm. Bacteria were diluted to a starting OD_{600} of 0.005. Test tubes were inoculated with 8 CFU/mL test tubes of

E. coli or *S. epidermidis* and treated with 70% NEO at the following concentrations: 0.0625%, 0.125%, 0.25%, 0.5%, 1%, 2%, 4%, and 8%. These were compared to two controls, one of just bacteria and one of just media. Visual inspection of solution turbidity was used to determine bacterial growth. The MIC for each bacterial species was determined to be in between the concentrations showing visible bacterial growth and no visible growth. One trial was performed.

2.3 Obtaining Persister Cells

An aliquot of *S. epidermidis* stock (stored at –80 °C) was added to 25 mL of TSB media and incubated in a 250 mL baffled flask for 24 h at 37 °C, with shaking at 250 rpm. Similar methods were used for *E. coli* and the stock was added to 25 mL of LB. The following day, a 250 µL of 25 µg/mL solution of ciprofloxacin (or 5 µg/mL of ofloxacin for *E. coli*) was added to the *S. epidermidis* flask and incubated for 48 h (24 h for *E. coli)*. After the treatment period, the culture was centrifuged at 4000 × *g* for 10 min, the supernatant was removed, and the pellet was resuspended in 15 mL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 at pH 7.4).[55]

2.4 Planktonic Killing Assay

Twelve aliquots of persister cells suspended in PBS were prepared, as explained in Section 2.3. The cells were combined with NEO at concentrations of 0.5%, 1%, and 2.5% and with a 2.5% DMSO control for treatment at three separate times, resulting in three sets of the four concentrations of test tubes. These samples were made from a 70% NEO solution diluted in DMSO, and a 70% DMSO solution diluted in PBS. Later methods use this same preparation up to this point. The test tubes were collected for sampling timed at 1, 2, and 24 h and incubated at

37 °C with shaking at 250 rpm. After incubation, the samples were centrifuged at 10,000 × g for 1 min, the supernatant was removed entirely, and bacteria were resuspended in 1 mL of PBS. The samples were then serially diluted in PBS in a round-bottom well plate and plated on TSB-agar (for *S. epidermidis*) or LB-agar (for *E. coli*) in 10 µL spots. Plates were incubated for 24 h at 37 °C, and CFUs were counted. Colony counts between 10 and 100 were used to calculate CFU/mL values. Trials were performed in biological triplicate, and data is displayed with standard error of the mean bars.

For the lower concentration assay, a similar method was used but NEO concentrations were diluted. The standard 0.5%, 1%, and 2.5% NEO were diluted ten fold to result in 0.05%, 0.01%, and 0.25% NEO and 0.25% DMSO. The rest of the methods in Section 2.3 to 2.4 were then followed. One trial was completed for this assay.

2.5 Killing Persister Cells in Biofilm

Early Trial Method:

An overnight culture of *S. epidermidis* was analyzed with UV-Vis spectroscopy (Cary 60 UV-Vis, Agilent Technologies, recorded at 600 nm) and diluted to prepare a solution with an OD_{600} of 0.01. Varying concentrations of NEO and DMSO (0.25%, 0.5%, 1%, 2%, 4%, and 8%) were prepared. In a flat-bottom well plate, the perimeter wells were filled with PBS to regulate evaporation, and *S. epidermidis* was added to the interior wells. The bacteria were grown for 24 h and treated with varying concentrations of NEO, and then the agar plate was incubated at 37 °C for 24 and 48 h. The following day, the media was removed, and the cells were washed twice with PBS, resuspended by scraping the wells with a micropipette tip, and further diluted in PBS. The samples were then serially diluted in PBS in a round-bottom well plate and plated on

TSB-agar in 10 μL spots. After incubation, CFU counts were taken. One trial with *S. epidermidis* was completed.

New Trial Method:

Overnight cultures of *S. epidermidis* and *E. coli* were prepared and diluted to an OD_{600} of 0.1. In a flat-bottom well plate, PBS was added to the perimeter wells to minimize evaporation, and 100 μL of each bacterial suspension was added to 30 wells for overnight incubation. The following day 100 μL of each bacteria was added again to prevent drying. After two days of incubation, media were removed from the bacterial wells, and NEO solutions were prepared from the DMSO-solubilized stock. Corresponding DMSO control solutions were also prepared in PBS at the same concentrations. Appropriate volumes of 70% stock solution of NEO and 70% stock solution of DMSO were diluted to obtain final concentrations of 8%, 4%, 2%, 1%, 0.5%, and 0.25%.

To each well, 100 μL of either NEO or DMSO solution was added to the generated biofilms and incubated for 24 or 48 hours at 37 °C. Following incubation, NEO and DMSO solutions were carefully removed, and wells were gently washed once with 200 μL PBS. Fresh PBS (200 μL) was then added, and the biofilm at the bottom of each well was disrupted using a micropipette tip. The resulting biofilm suspension was transferred to a round-bottom well plate containing 5 rows of 90 μL PBS for serial dilution. From each dilution, 10 μL were plated on the appropriate agar media following standard serial dilution procedures. Plates were incubated overnight, and resulting colonies were counted and converted to percent survival.

2.6 Assessing Membrane Integrity through a Colorimetric Assay

After repeating the culture and standard concentration essential oil preparation from Section 2.3 and 2.4, the samples were treated with erythrosin B (EB) to perform a colorimetric assay that determines cell membrane integrity. Two sets of samples were prepared to normalize the data: control samples containing 500 µL of PBS and 500 µL of treated culture, and samples containing 500 µL of PBS and 500 µL of the cultures dyed with EB. These samples were incubated at room temperature for 15 min. Following incubation, samples were centrifuged (10,000 × g for 1 min), the supernatant was removed, and the cell pellet was resuspended in 1 mL of PBS. This process was repeated 2 times for the dyed samples to wash out the EB stain. The absorbance of the samples was measured with a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, recorded at 530 nm), and data were analyzed and compared to the untreated control. One trial was performed for *S. epidermidis*, and two trials were performed for *E. coli*.

2.7 Assessing DNA Content Spillage with UV-Vis Spectrophotometry

After preparing the culture and standard essential oil solutions from Section 2.3 and 2.4, the samples were centrifuged (10,000 × g for 1 min). Following centrifugation, 200 µL of the supernatant was removed, and 800 µL of PBS was added to a microcentrifuge tube. Using a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, recorded at 260 nm), absorbance values were recorded to measure DNA concentration. The cuvette was washed with PBS between each measurement. For *S. epidermidis,* three trials were performed and the results were analyzed with standard error of the mean bars. Two trials were completed for *E. coli*.

2.8 Assessing Protein Content Spillage with a Bradford Assay

After preparing the culture and essential oil preparation from Section 2.3, the samples were centrifuged (10,000 × g for 1 min). Following centrifugation, 500 µL of the supernatant was placed in a cuvette along with 1.5 mL of Bradford Reagent for *S. epidermidis* (Thermo Fisher Pierce™ Bradford Plus Protein Assay Kit). For *E. coli*, 50 µL of supernatant was used instead as this was the first iteration of the method. The samples were incubated for 10 min at room temperature. Absorbance values were recorded (Cary 60 UV-Vis, Agilent Technologies, recorded at 595 nm) and analyzed. One trial was performed.

To achieve greater sensitivity, a BCA protein assay kit (Thermo Fisher Pierce™ BCA Protein Assay Kit) was used according to the manufacturer's instructions. Following preparation from Section 2.3 and the standard centrifugation, 25 µL of supernatant was placed in a flat bottom well plate along with 200 µL of the kit's reagent. An additional lysed bacterial sample was prepared from the same culture by sonicating at 120 W for 20 seconds, repeated three cycles, to ensure complete cell disruption. Samples were incubated at 37 °C for 30 min and then cooled at room temperature for 10 min. Absorbance values were measured using a plate reader (Molecular Device**s**, recorded at 562 nm) and analyzed. One trial was performed.

2.9 Statistical Analysis

All results were analyzed using Microsoft Excel, and error bars were added with the function showing standard error of the mean. Colony-forming units (CFUs) for sections 2.4 and 2.5 were converted to percent survival using the following equations:

$$\frac{\text{\# of colonies (CFU) x dilution factor}}{\text{volume of spot (mL)}} = \text{CFU/mL} \qquad \text{(Equation 1)}$$

$$\% \text{ survival} = 100 \times \frac{\text{sample (CFU/mL)}}{\text{t = 0 (CFU/mL)}} \quad \text{(Equation 2)}$$

For sections 2.6 and 2.7, values were represented in bar charts to display absorbance value trends.

For section 2.8, protein concentration (μg/mL) was calculated based on a standard curve with known BSA protein concentrations provided by each kit. The resulting linear equation for each curve was used to relate the recorded absorbance values to calculated protein concentrations, and results were displayed in bar charts.

3. Results

3.1 Minimum Inhibitory Concentration of S. epidermidis and E. coli

First, we set out to determine if neroli oil was effective against *S. epidermidis* and *E. coli* bacteria by determining how much neroli oil was needed to prevent bacterial growth. The minimum inhibitory concentrations (MIC) that prevent bacterial growth in *S. epidermidis* and *E. coli* were determined (Table 1). For *S. epidermidis*, since bacterial growth was seen in cultures treated with 4% NEO but not in cultures treated with 8% NEO, the MIC is between 4% and 8%. For *E. coli*, bacterial growth was seen at 0.5% NEO but not when treated with 1% NEO, so its MIC is between 0.5% and 1%. Ultimately, any concentrations lower than 4% NEO should not prevent bacterial growth in *S. epidermidis,* and anything lower than 0.5% NEO should not prevent bacterial growth in *E. coli*. The MIC of *S. epidermidis* is between 4% and 8% and between 0.5% and 1% for *E. coli* (Table 1).

***Table 1**: Growth of S. epidermidis and E. coli against various concentrations of NEO*

Concentration (%)	*E. coli Growth*	*S. epidermidis Growth*
8%	No	**No**
4%	No	**Yes**
2%	No	Yes
1%	**No**	Yes
0.50%	**Yes**	Yes
0.25%	Yes	Yes
0.13%	Yes	Yes
0.06%	Yes	Yes
(+ control) should grow	Yes	Yes
(- control) should not grow	No	No

The change between lack of growth and growth determines the MIC range for each bacteria.

3.2 Neroli Essential Oil Kills Planktonic Persister Cells

Once it was established that neroli oil is effective against regular *S. epidermidis* and *E. coli* cells, the effect was studied on persister cells. Persister cells were acquired by treating stationary-phase cultures with antibiotics and methods modeled from the previous TTEO study.[55] To compare results to the TTEO study, NEO concentrations were initially chosen to match that study. After isolation, the bacterial cultures were treated with varying concentrations of essential oil solubilized in DMSO (0.5%, 1%, 2.5%) and a 2.5% DMSO control suspended in PBS. Within

1 h of treatment, NEO killed more than 99% of planktonic persister cells in *S. epidermidis*. By 24 h, the persister cells were completely eradicated. DMSO, the solubilizing vehicle for NEO, was used as a control to rule out that it is not killing the persisters. Ultimately, no significant killing of the samples treated with DMSO was observed (Figure 8). The 2 h sample of DMSO has a lower percent survival on average due to one trial's accidental mixing in a flat bottom as opposed to a round bottom well plate, causing the mixing to be less even and possibly lowering CFU counts.

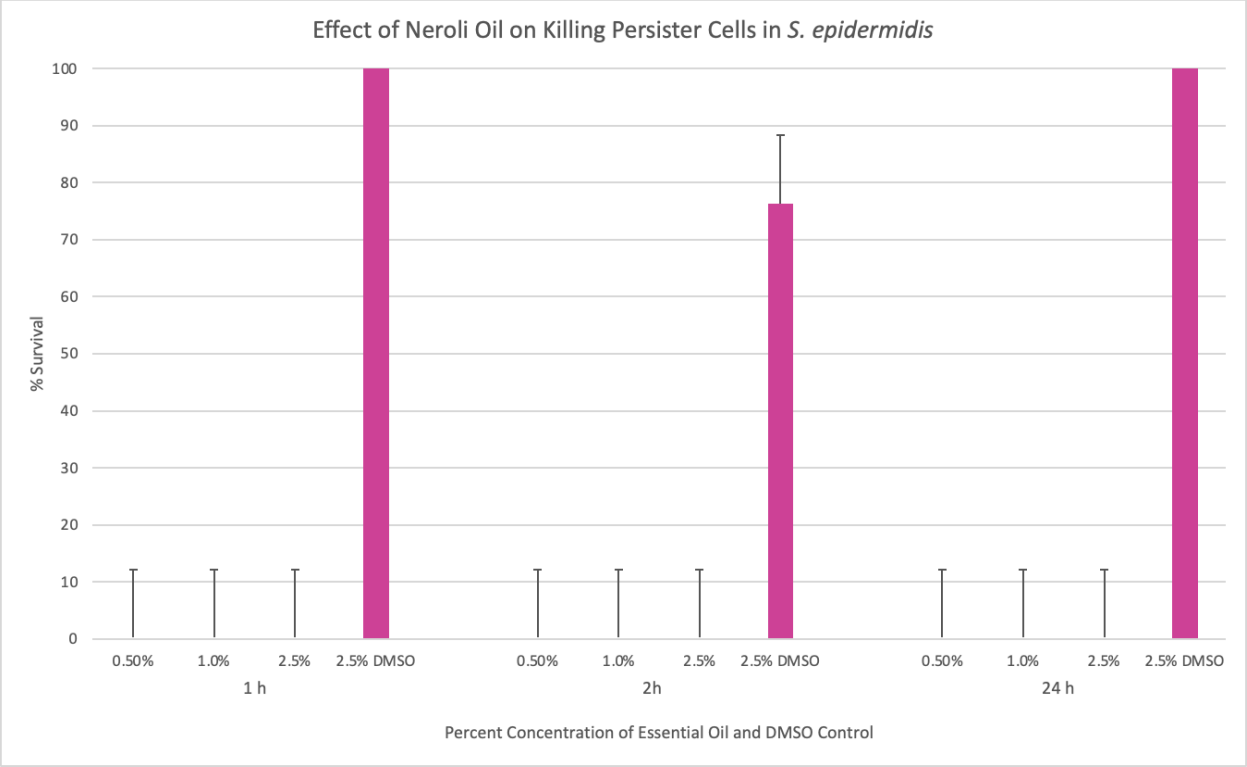


Figure 8: *Neroli Essential Oil treatment of S. epidermidis with varying concentrations of NEO solubilized in PBS over a period of 1 h, 2 h, and 24 h. After treatment, persister cell CFUs were calculated and converted to % survival. The percent survival against the untreated control sample (2.5% DMSO) was plotted. Depicted results represent three trials, including standard error of the mean bars.*

To explore NEO's potential as a broad-spectrum agent, NEO was tested against *E. coli* planktonic persister cells. After 1 h of treatment, over 99% of the persister cells were killed when treated with 0.5% and 1% NEO, and 100% of the bacteria treated with 2.5% NEO were killed. By 2 h and again in 24 h, all of the persister cells were completely eradicated, showing an increased killing rate compared to the Gram-positive samples. DMSO was consistently used as a control, showing no significant killing (Figure 9). The key finding is that the majority of persister cell killing occurs rapidly, with substantial reduction observed within the first hour of treatment and further decrease by the second hour. The rapid rate suggests that the mechanism is unlikely to involve metabolic pathway activation, which typically requires more time. Instead, the data support a model of direct bacterial killing, rather than sensitization or reactivation of dormant cells.

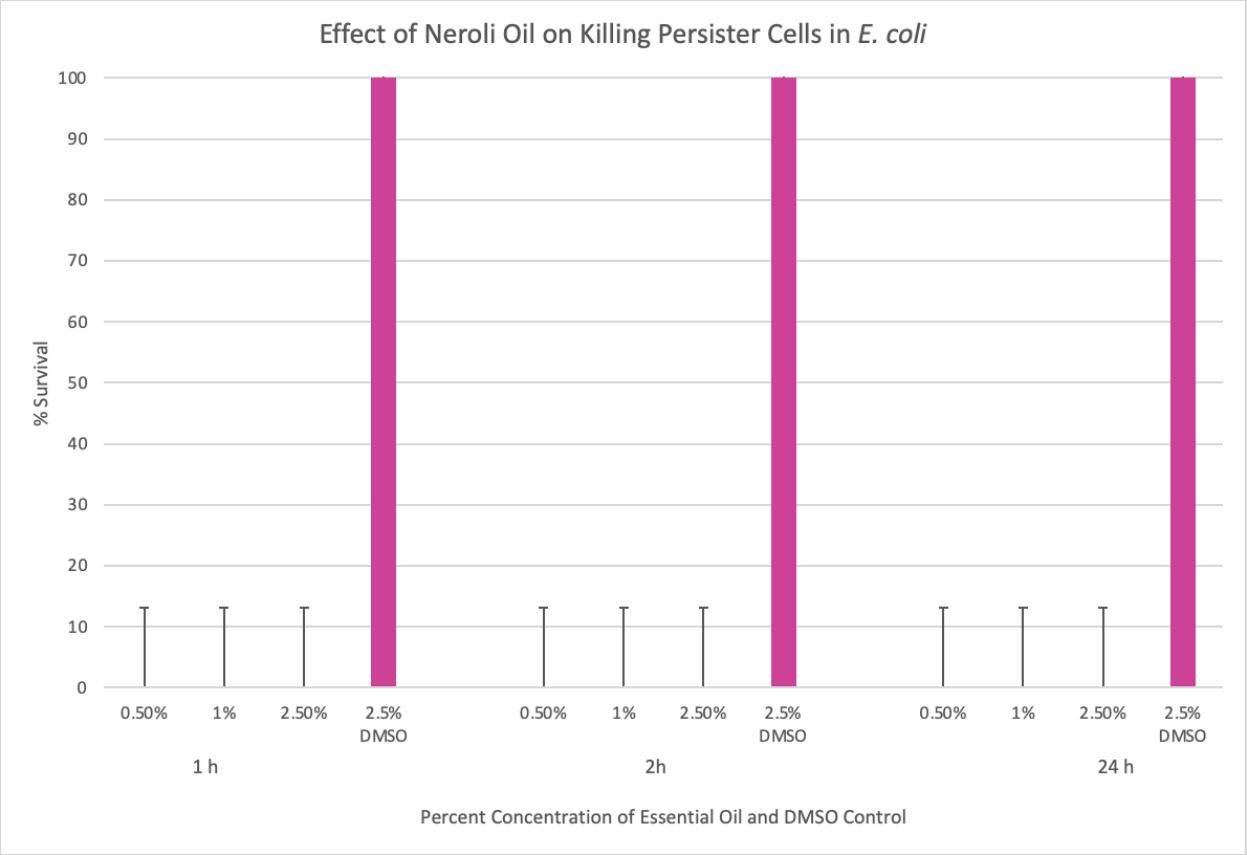


Figure 9: *Neroli Essential Oil treatment of E. coli with varying concentrations of NEO solubilized in PBS over a period of 1 h, 2 h, and 24 h. After treatment, persister cell CFUs were calculated and converted to % survival. The percent survival against the untreated control sample (2.5% DMSO) was plotted. Depicted results represent three trials, including standard error of the mean bars.*

3.3 Neroli Essential Oil as a Possible Anti-Biofilm Agent

NEO-treated biofilms are an important part of this project due to their growing medical relevance, particularly in infections involving *S. epidermidis*. To begin exploring NEO as an anti-biofilm agent, biofilms were generated in a well plate using *S. epidermidis* culture. The

cultures were treated with varying concentrations of NEO and DMSO (0.25%, 0.5%, 1%, 2%, 4%, and 8%) and incubated for 24 h and 48 h. In this early method, only 1 trial was tested, leaving some unanswered questions to address. As shown in Figure 10, within 24 h, a large portion of biofilm cells were killed by both the NEO and DMSO. However, by 48 h, 100% killing occurred only in the samples treated with NEO. CFU counts appeared inconsistent on the TSB plates, possibly due to environmental contamination, such as airborne bacteria. The presence of random error in this trial was significant, suggesting that modifications to the experimental method are necessary.

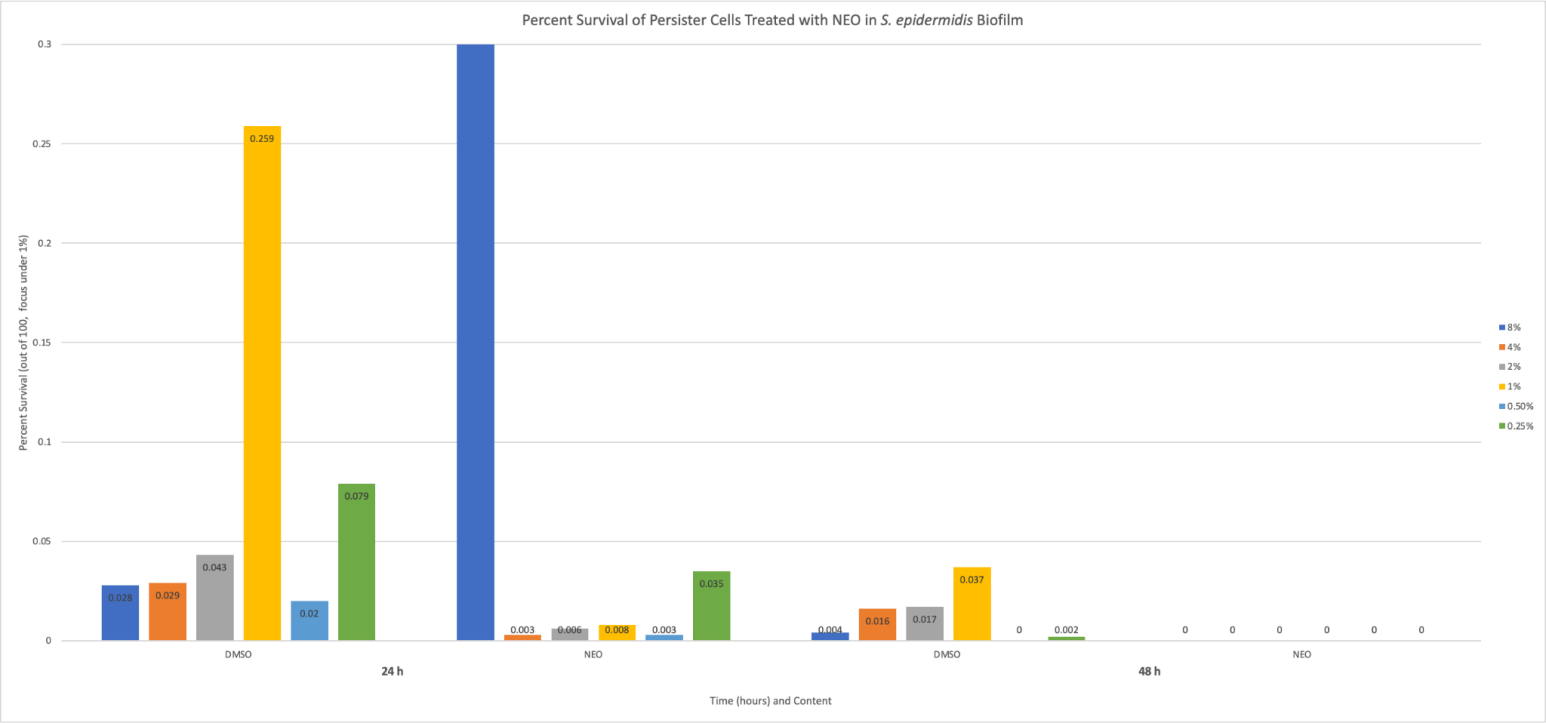


Figure 10: *NEO treatment of biofilms generated with S. epidermidis, including persister and other bacterial cells. DMSO control and NEO concentrations solubilized in PBS are plotted side by side to represent 24 h and 48 h treatment. Depicted results represent a single trial.*

To address these initial experimental issues, a revised method was implemented, resulting in somewhat clearer outcomes. For *S. epidermidis*, significant killing is observed across all samples when comparing 24 h to 48 h treatment, with many values decreasing from around 100% survival to below 70% survival (Figure 11). This reduction of percent survival is ideal for the samples treated with NEO. However, a similar level of biofilm killing was also observed in the DMSO control. It is worth noting that the persister cells in the biofilm are not isolated, and this is simply an investigation of NEO's anti-biofilm killing activity, with the assumption that persister cell concentrations in the biofilm are high. While the 24 h treatment with a wide range

of NEO concentrations shows clear effectiveness in killing *S. epidermidis* biofilm, significant cell death is also observed in both experimental controls, complicating the interpretation of NEO's specific impact.

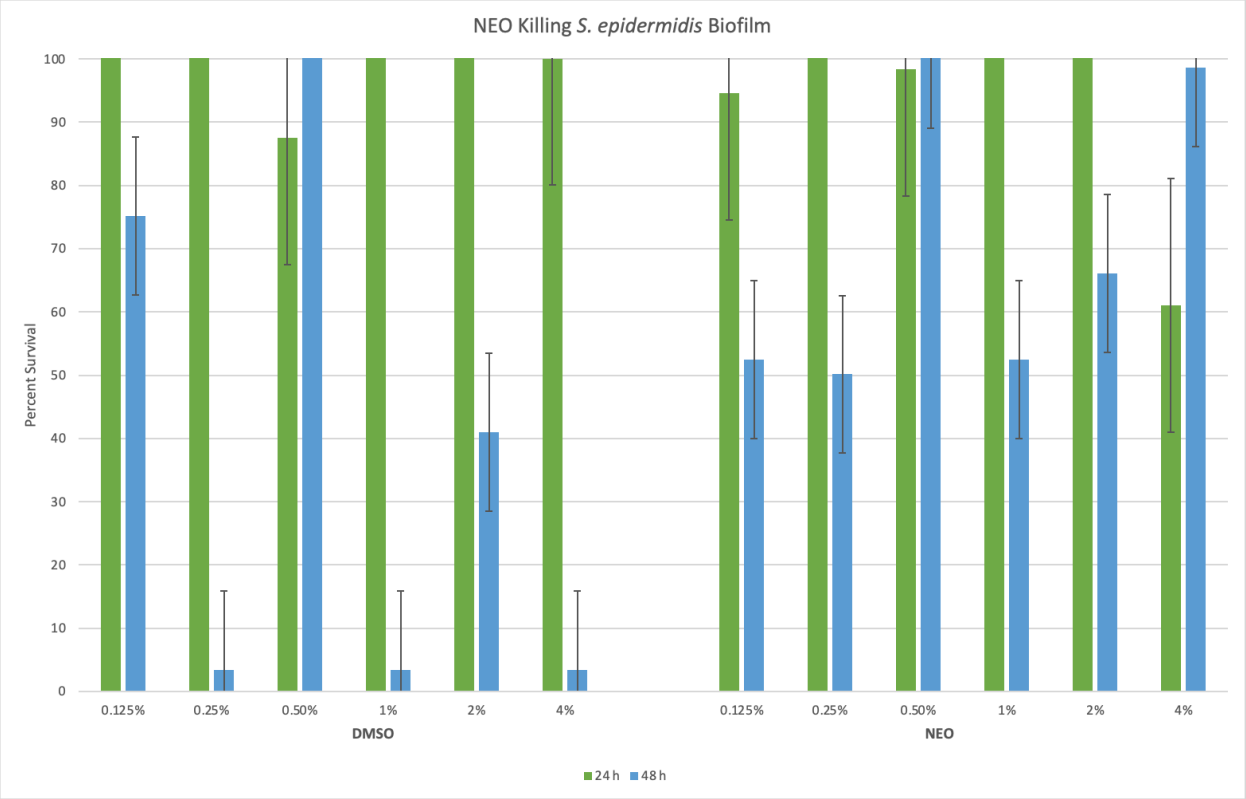


Figure 11: *NEO treatment of biofilms generated with S. epidermidis, including persister and other bacterial cells. DMSO control and NEO concentrations are plotted side by side to represent 24 h and 48 h treatment. Three trials were performed with standard error of the mean bars.*

When compared to *S. epidermidis,* the results for NEO treatment of *E. coli* biofilms exhibits more variability (Figure 12). For the samples treated with NEO, there are results showing some concentrations were able to kill the biofilm more within 24 h, such as 4% NEO and 2% NEO. However, 4% NEO exhibits an increase in percent survival after 48 h of treatment,

and some of the DMSO samples show significant decrease in percent survival, both unanticipated results. This inconsistency reveals an unclear relationship between NEO concentration and its antibacterial effectiveness against *E. coli* biofilms. DMSO controls also exhibited biofilm killing, although the effect of DMSO was less pronounced in *E. coli* than in *S. epidermidis*. Since the percent survival in many of the DMSO-treated samples is significantly higher than in the NEO-treated samples, it appears that DMSO contributes less to bacterial killing than the essential oil itself.

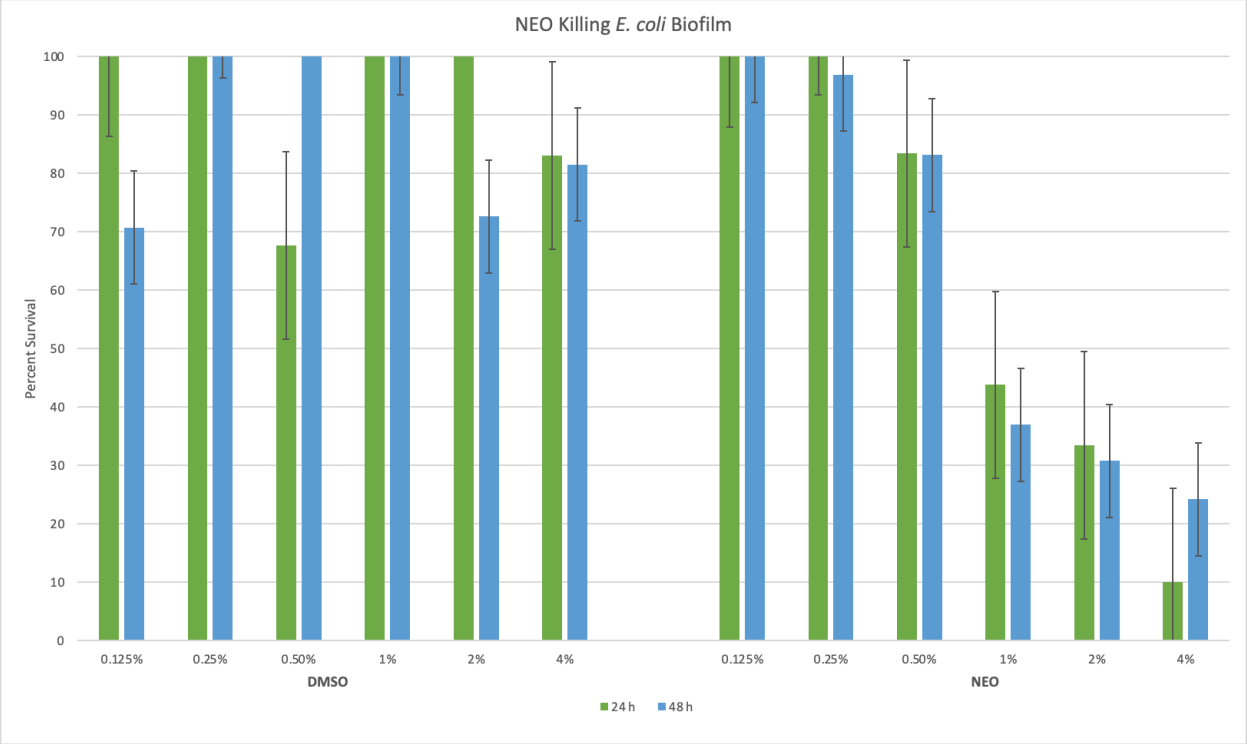


Figure 12: *NEO treatment of biofilms generated with E. coli, including persister and other bacterial cells. DMSO control and NEO concentrations are plotted side by side to represent 24 h and 48 h treatment. Three trials were performed with standard error of the mean bars.*

3.4 Neroli Essential Oil May Disrupt Persister Cell Membranes

Assessing Membrane Permeability

Given the conclusion that NEO can kill planktonic persister cells, the next step of the project was to study the killing mechanism. With the goal of direct killing, rather than sensitization or activating persister cell metabolism, this trial investigated if the cells were being killed by cell membrane disruption. Previous research involving a colorimetric assay concluded that Tea Tree Oil killed persister cells, via this mechanism. This colorimetric assay was performed using erythrosin B (EB), which is a membrane impermeable dye, meaning it will dye cells with a disrupted membrane and not dye those with an intact membrane.[60] In order to determine if cell membrane disruption was the cause of the killing, the persister cells were incubated with EB after culture treatment with NEO. Spectrophotometer readings were recorded at 530 nm due to the maximum wavelength of EB. Treatment of 1 h and 2 h were recorded because the planktonic assays showed rapid persister killing within the first two hours, emphasizing the importance of focusing closely on these time points.

One trial was performed for *S. epidermidis* using varying concentrations of NEO (0.5%, 1%, 2.5%) and compared to a 2.5% DMSO vehicle control (Figure 13). No clear pattern was observed when plotting absorbance results (Figure 6). After 1 h, 0.5% and 1% NEO may have killed persister cells. The positive absorbances recorded suggest that minimal killing may be accomplished through cell membrane disruption due to a near zero absorbance. However, the remaining data include negative absorbance values, which reflect instrument error and are effectively equivalent to zero, indicating a potential issue with the trial or the experimental methodology.

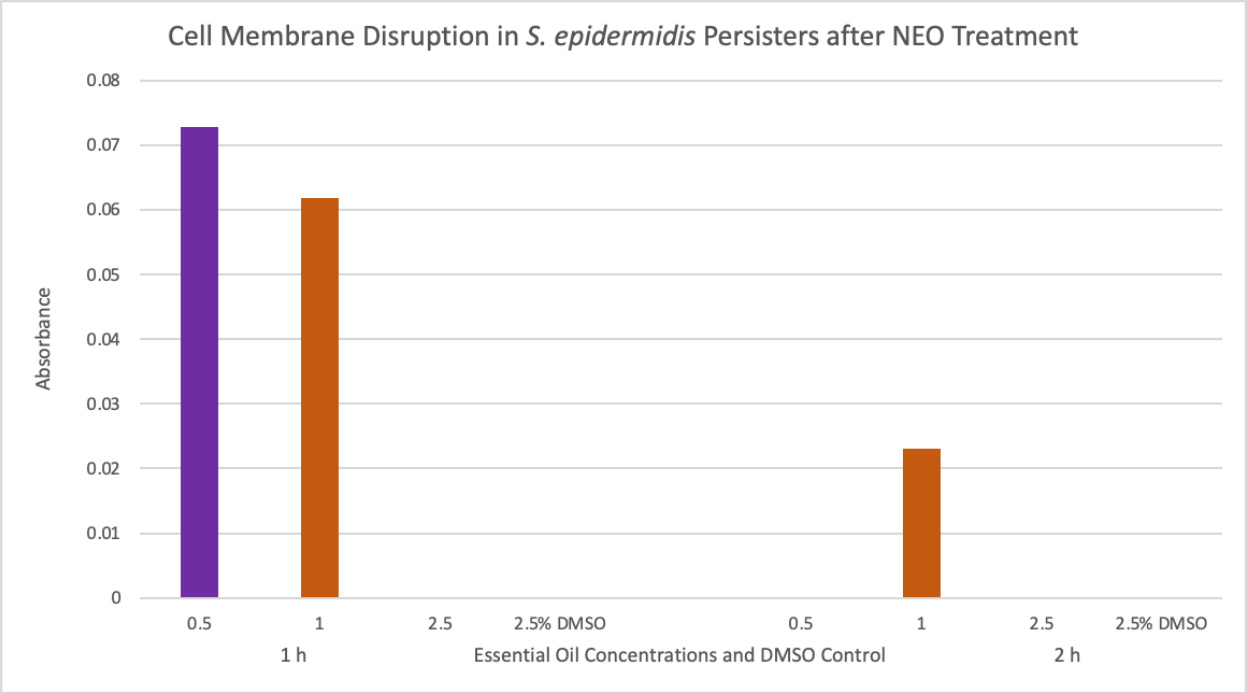


Figure 13: *Determination of membrane integrity of S. epidermidis persister cells after treatment with NEO solubilized in PBS over 1 h, 2 h, 24 h. Sample absorbance was measured at 530 nm and trials were normalized. This represents one trial and is plotted next to a DMSO control of 2.5%.*

The two trials of the colorimetric assay for *E. coli* exhibited more promising results (Figure 14). After 1 hour of treatment, the absorbance of the samples treated with NEO was positive across all concentrations and decreased from 0.5% to 2.5%. Minimal absorbance was recorded for the DMSO control. After 2 hours, the absorbance of the treated samples increased, but did not follow a consistent pattern. *E. coli* treated with 1% NEO had the highest absorbance, followed by 2.5% and then 0.5% NEO. Given these significant absorbance values and noting that EB dye binds to cells with compromised membranes, it is likely that the Gram-negative bacterial lysis was caused by membrane disruption.

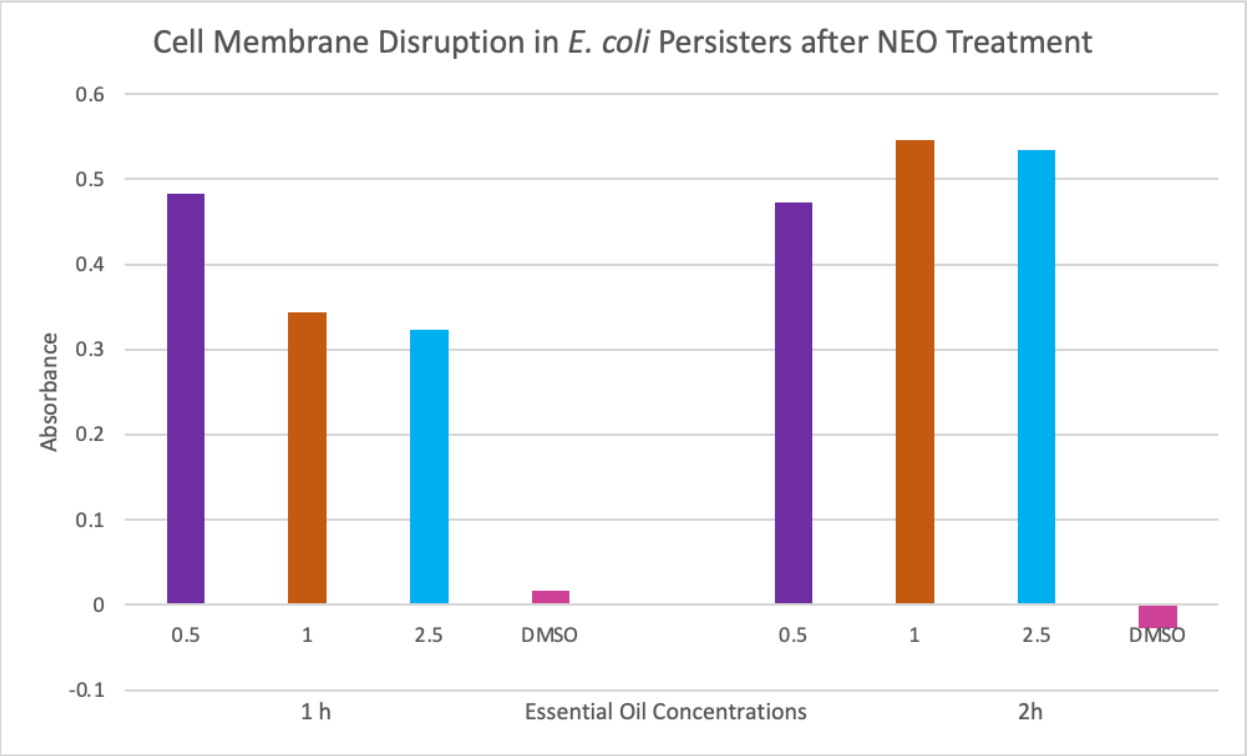


Figure 14: *Determination of membrane integrity of E. coli persister cells after treatment with NEO solubilized in PBS over 1 h, 2 h, 24 h. Sample absorbance was measured at 530 nm and trials were normalized. This represents two trials and is plotted next to a 2.5% DMSO control.*

Assessing DNA Content Spillage

Because of some inconsistent results with the EB dye, an additional assay was done to determine if the membrane was disrupted in the killing. An assay to measure DNA leakage was performed in order to better understand if persister cell death was a result of membrane disruption.. Following persister cell treatment with NEO, the supernatant was collected, and the absorbance at 260 nm was measured to detect DNA. DNA is a large complex biomolecule that is membrane impermeable and would not be expected in the supernatant unless the cell membrane was compromised. For *S. epidermidis*, the three trials performed showed a very consistent

increase in DNA absorbance from treatment with 0.5% to 2.5% NEO (Figure 15).

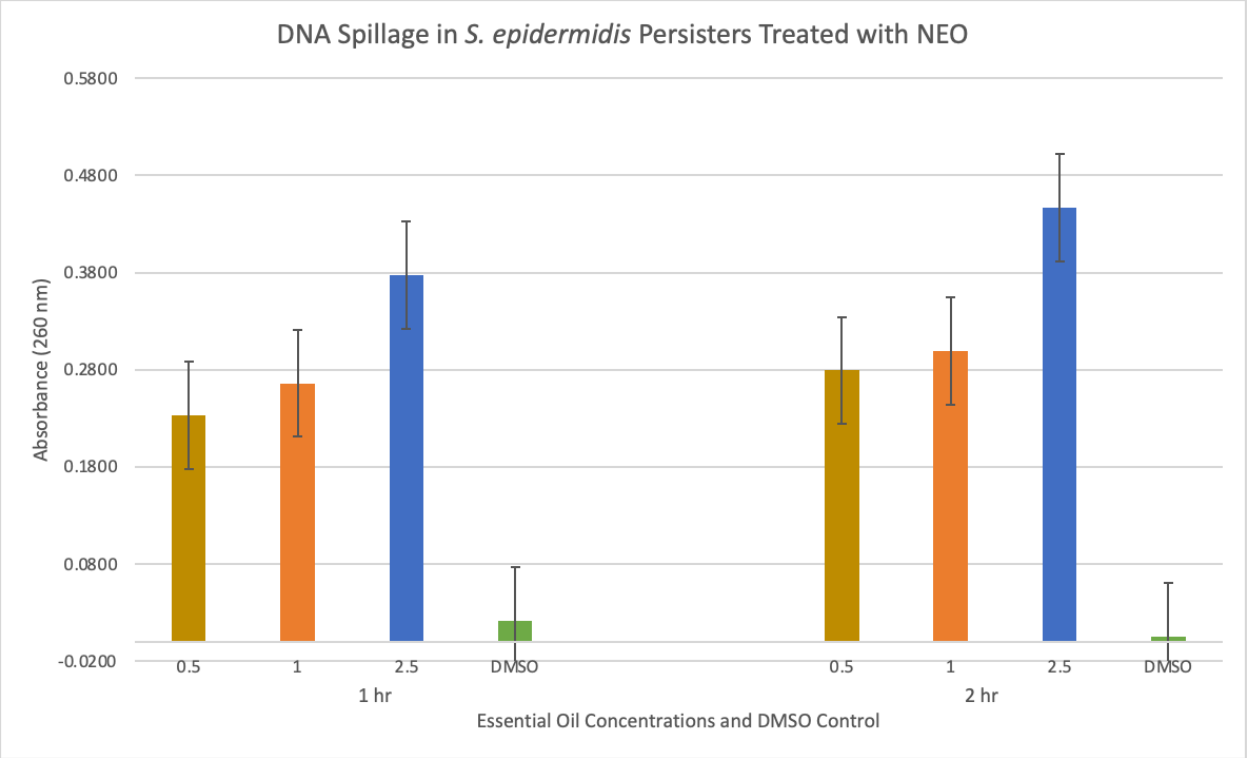


Figure 15: *Comparison of DNA absorbances, which can be loosely interpreted as concentration of DNA spillage, resulting from S. epidermidis treatment with various concentrations of NEO solubilized in PBS. Three trials were measured at 260 nm with standard error of the mean bars are shown.*

The trials for *E. coli* exhibit the same behavior as with *S. epidermidis*, showing an increase in DNA absorbances as the concentration of NEO increases (Figure 16). The highest recorded absorbance here was 0.370, which is lower than that of the Gram-positive bacteria, indicating a possible lower concentration of DNA spillage. For both bacteria, there was a clear increase in absorbance leading to the conclusion that DNA leaked from sufficiently large holes in the cell membrane with a minimal though notable increase from 1 to 2 h of treatment (Figures 15

and 16). This experimental result aligns with the slight increase in planktonic persister killing observed between these time points, corresponding to a small rise in DNA leakage. The absorbance of DMSO is extremely low and near zero, accounting for any killing that the solubilizing agent could have been responsible for.As a loose measure of relative DNA concentration, *S. epidermidis* exhibited a maximum absorbance of 0.477 which is larger than that of *E. coli*.

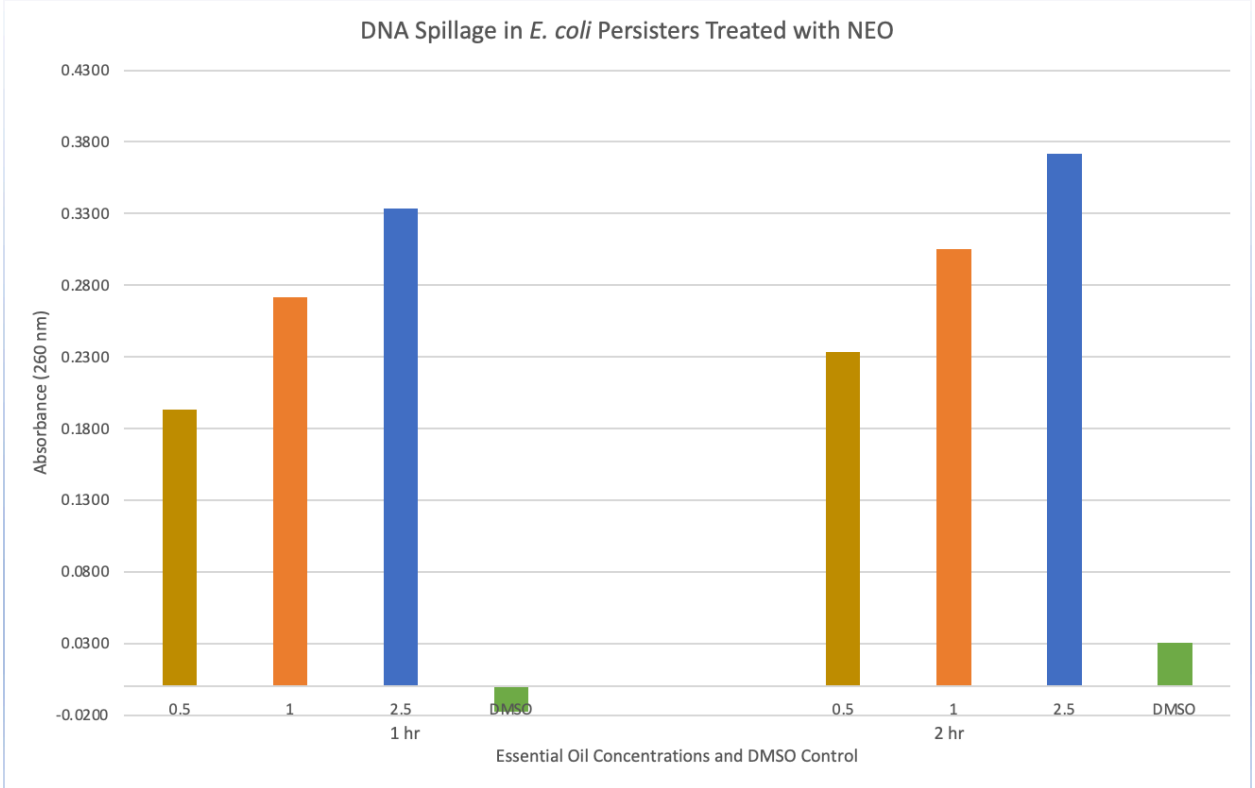


Figure 16: *Comparison of DNA absorbances, which can be loosely interpreted as concentration, resulting from E. coli treatment with various concentrations of NEO solubilized in PBS. Results are compared to a DMSO control and 2 trials are shown (260 nm).*

Assessing Protein Content Spillage

Bradford Assay:

To further investigate the bacterial cell content spillage, a Bradford assay was performed (Figure 17). The Bradford assay is able to account for spillage of nonspecific protein content which is generally smaller in size than DNA. Therefore, the purpose of the Bradford assay in this context is to determine whether smaller protein molecules leak from bacteria following cell membrane disruption.

The Bradford assay trials between the two bacterial species cannot be directly compared due to differences in supernatant volume. In the first trial for *E. coli* the results are unclear due to a low supernatant volume of 50 µL (Figure 17). All treated samples begin with a higher protein concentration at 1 h and then show a decrease by 2 h. This decline is likely due to a methodological error, as protein that has already leaked from the cells cannot simply disappear. All samples show a similar initial concentration of about 1.6 µg/mL and lower to around 1.4 µg/mL within 1 h of treatment. Because the protein concentration values across all samples are so similar, there is insufficient variability to support a clear interpretation. As a result, no definitive conclusion can be drawn regarding the effect of the treatment under these specific conditions.

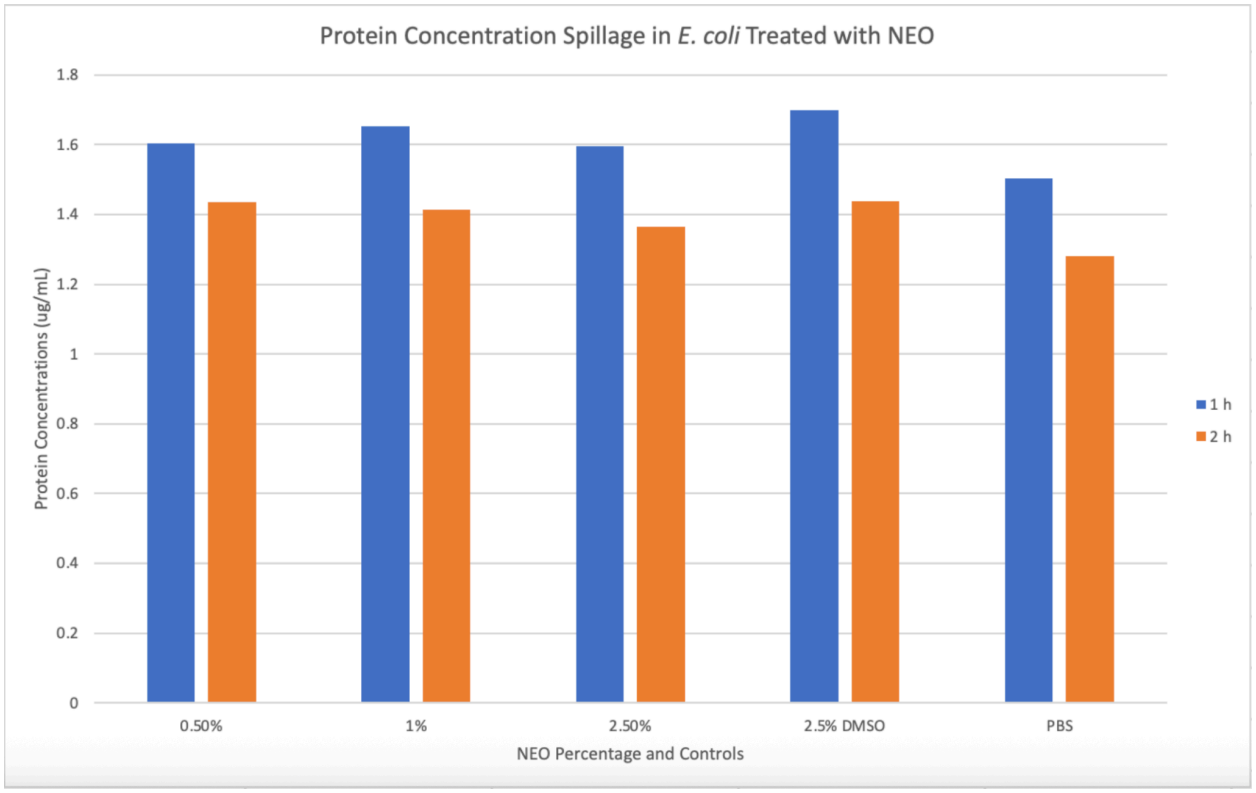


Figure 17: *Bradford Assay Measurement of nonspecific protein spillage from E. coli persisters after NEO treatment, including treatment with 2.5% DMSO and PBS controls (595 nm). Protein calculations are based on known BSA standards and one trial was performed.*

A second trial was performed for this assay, using 500 µL of *S. epidermidis* supernatant as opposed to 50 µL of *E. coli* supernatant in the first trial. The increased amount produced more distinctive results due to the higher amount of cellular content, making it more likely that a meaningful conclusion can be drawn from this figure. For all concentrations of NEO, there was an increase in protein leakage from 1 h of treatment to 2 h (Figure 18). The protein concentration for both controls were lower than that of the NEO treated samples, reinforcing that significant

protein content released following essential oil treatment in *S. epidermidis*, independent of the solubilizing vehicles.

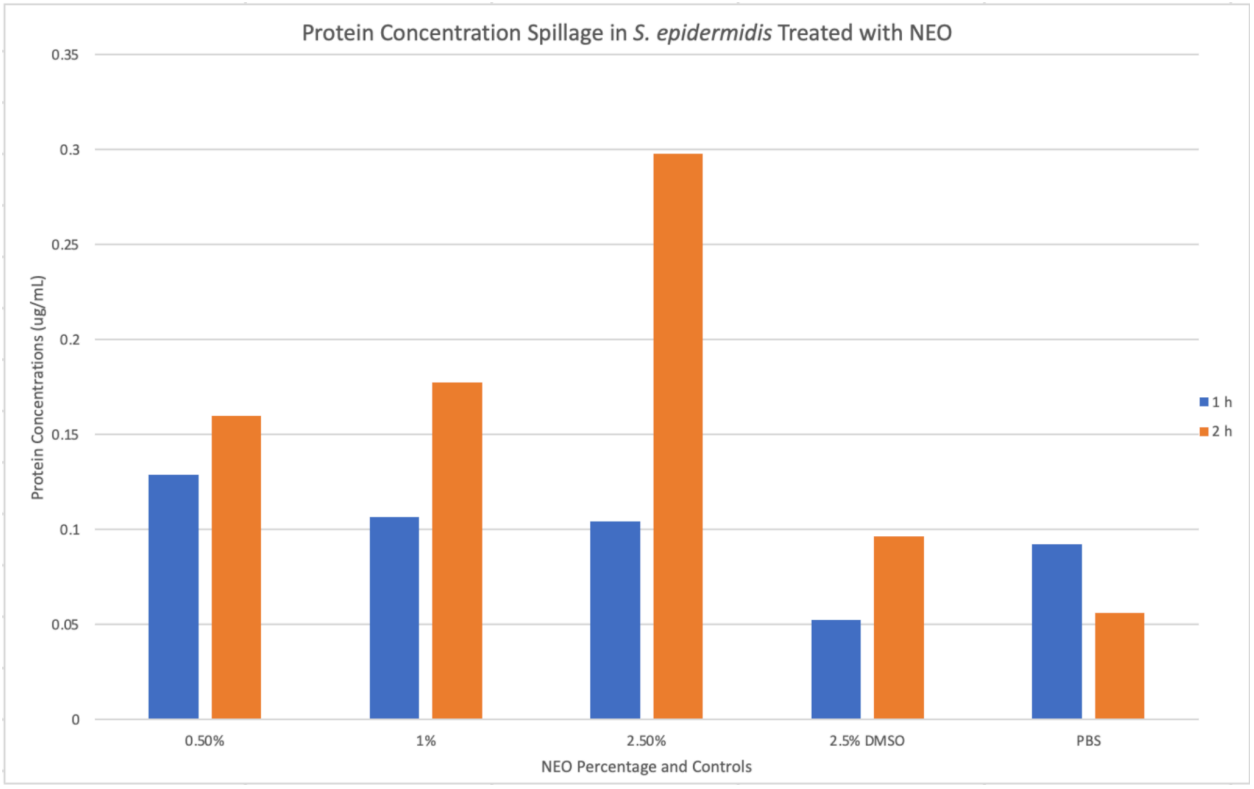


Figure 18: *Bradford Assay Measurement of nonspecific protein spillage from S. epidermidis persisters after NEO treatment, including treatment with 2.5% DMSO and PBS controls (595 nm). Protein calculations are based on known BSA standards and one trial was performed.*

BCA Protein Assay Kit:

After observing an unclear pattern of protein leakage using the Bradford assay, the BCA (Bicinchoninic acid assay) protein kit, with its more sensitive dye, was employed to achieve more precise measurements. For *S. epidermidis*, an additional sample of untreated bacteria lysed through sonication was included for comparison (Figure 19). In all NEO-treated samples except

0.5% NEO, the concentration of leaked protein increased from 1 h to 2 h. The 0.5% NEO sample showed a slight decrease over time, which does not follow the overall trend. This observation may be attributed to variability and can be clarified with additional trials. Most of the NEO treated samples leaked over 80 µg/mL of protein within 1 h. The DMSO and PBS controls did, however, leak similar protein contents, with the most notable value from 2 h of the DMSO treated sample. The remaining values were below 60 µg/mL of proteins for all of the other samples tested.

These results suggest that NEO treatment may lead to greater protein leakage than the controls, though the difference is not yet strong enough to draw a confident conclusion. Interestingly, the sonicated sample which is expected to show the highest protein concentration due to deliberate lysis actually yielded a calculated protein value below zero. This indicates that an error likely occurred during the sonication process, or that the bacterial lysis was incomplete.

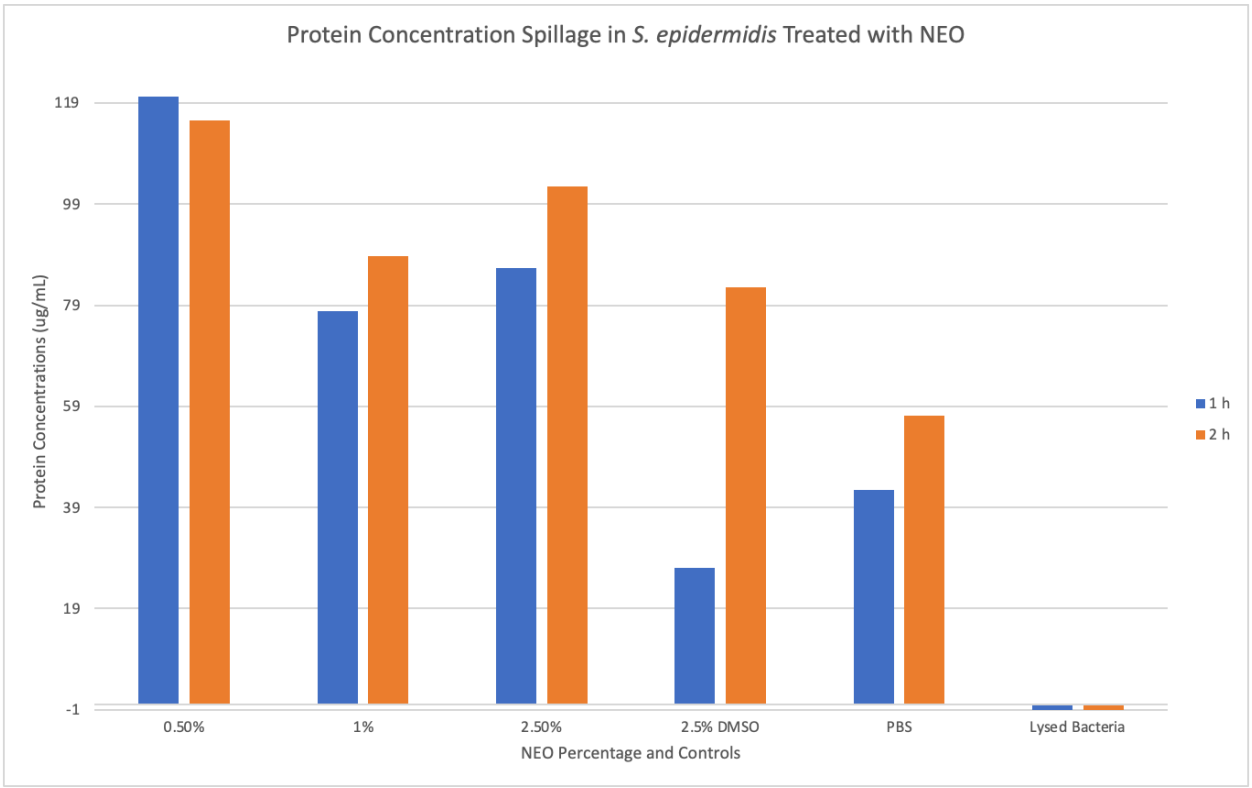


Figure 19: *BCA Protein Assay Kit measurement of nonspecific protein spillage from S. epidermidis persisters after NEO treatment, including treatment with 2.5% DMSO and PBS controls and untreated sonificated lysed bacteria (562 nm). Protein calculations are based on known BSA standards and one trial was performed.*

The BCA protein assay kit was utilized to evaluate more precise protein content spillage in *E. coli*, revealing the most consistent results among the protein assays (Figure 20). Both control samples (DMSO and PBS) showed values below zero, supporting the conclusion that neither vehicle contributed to protein spillage under these conditions. While protein concentration did not directly correlate with increasing NEO concentration, all treated samples exhibited a clear increase in protein leakage from 1 h to 2 h. These results indicate that NEO treatment causes noticeable protein leakage in *E. coli*, suggesting the formation of temporary or permanent disruptions in the bacterial membrane large enough to allow proteins to escape.

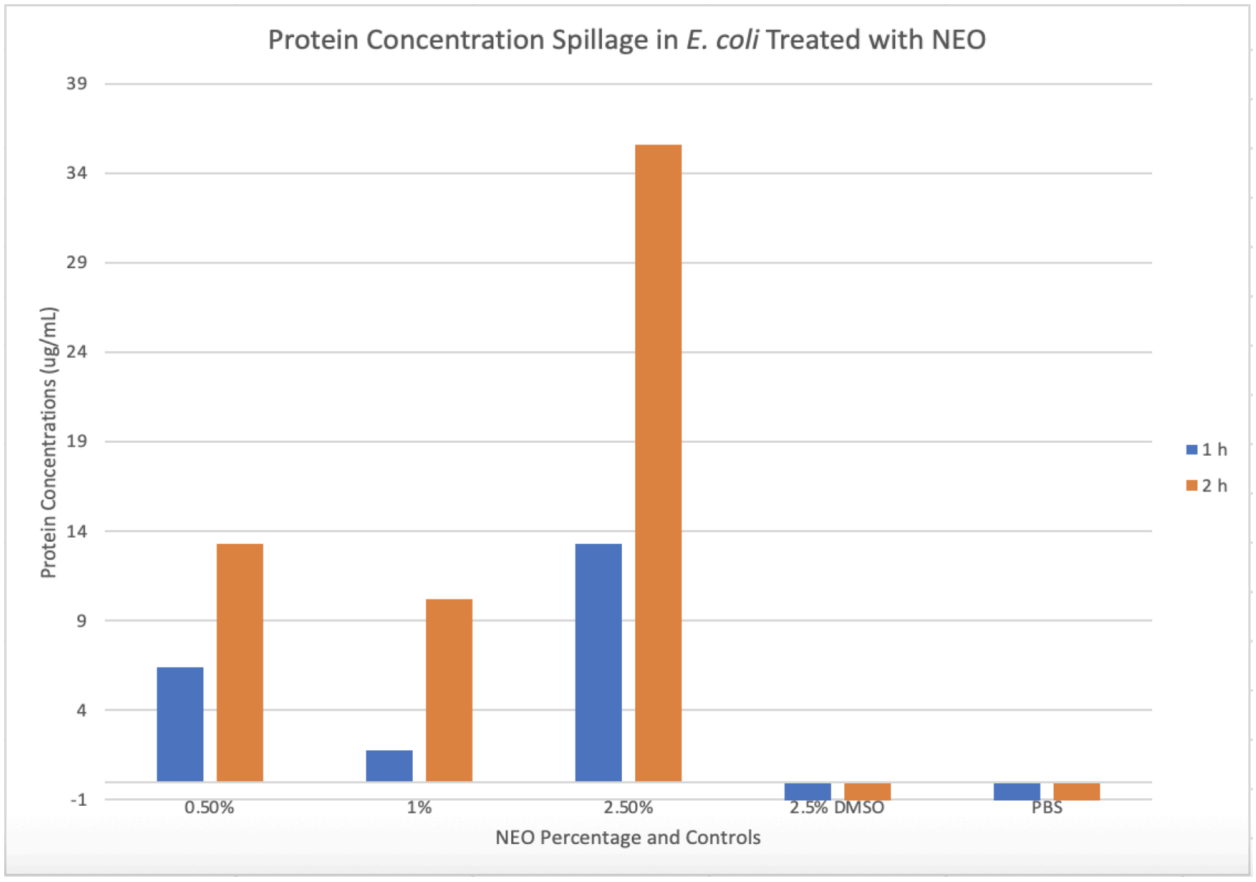


Figure 20: *BCA Protein Kit Assay Measurement of nonspecific protein spillage from E. coli persisters after NEO treatment, including treatment with 2.5% DMSO and PBS controls (562 nm). Protein calculations are based on known BSA standards and one trial was performed.*

3.5 Proposed Mechanism for Neroli Oil's Ability to Disrupt Bacterial Membranes

In order to finalize the investigation into NEO's killing mechanism, a detailed theoretical molecular approach was taken to understand the precise mode of action against persister cells. Major components of NEO were researched to hypothesize how these molecules might interact with persister cell membranes (Figure 21). Most of the components contain an oxygen atom, allowing the oil to interact with aqueous or polar environments. This enables the components to

exist within the body or in a planktonic solution without being entirely rejected. However, the rest of each molecule is largely nonpolar, similar to a bacterial membrane. These membranes are primarily composed of amphipathic lipids, which arrange into a bilayer with a hydrophobic core that serves as a barrier to polar molecules and the surrounding aqueous environment. As a result of this lipophilicity, NEO components are strongly attracted to the membrane like a magnet, as they are also mainly nonpolar.

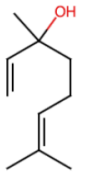
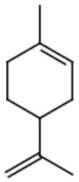
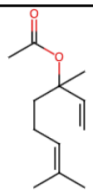
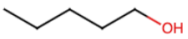
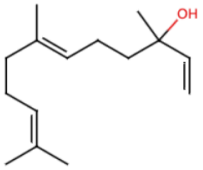
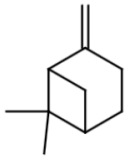
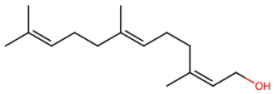
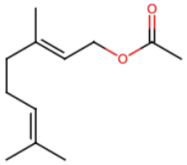
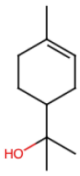
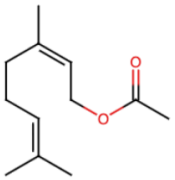
	**Linalool (28.5%)**		**Limonene (4.6%)**
	**Linalyl acetate (19.6%)**		**Pentanol (3.4%)**
	**Nerolidol (9.2%)**		**B-Pinene (3.0%)**
	**Z-E-farnesol (9.2%)**		**Geranyl acetate (2.8%)**
	**a-Terpineol (4.9%)**		**Neryl acetate (1.5)**

Figure 21: *Representation of the main molecular components of NEO according to concentration. Relative concentrations of components are shown on the left. Percentages of each component are shown on the right.*

The working hypothesis is that NEO seeks out other nonpolar molecules to interact with in an aqueous environment, such as the bacterial membrane. The oil components contain several oxygen and hydrogen atoms, which may form hydrogen bonds with teichoic acid chains in Gram-positive bacteria or lipopolysaccharide chains in Gram-negative bacteria (Figure 22). These components target anionic lipids found in bacterial membranes, including charged phosphate groups of teichoic acid or the polar heads of phospholipids in the plasma membrane.

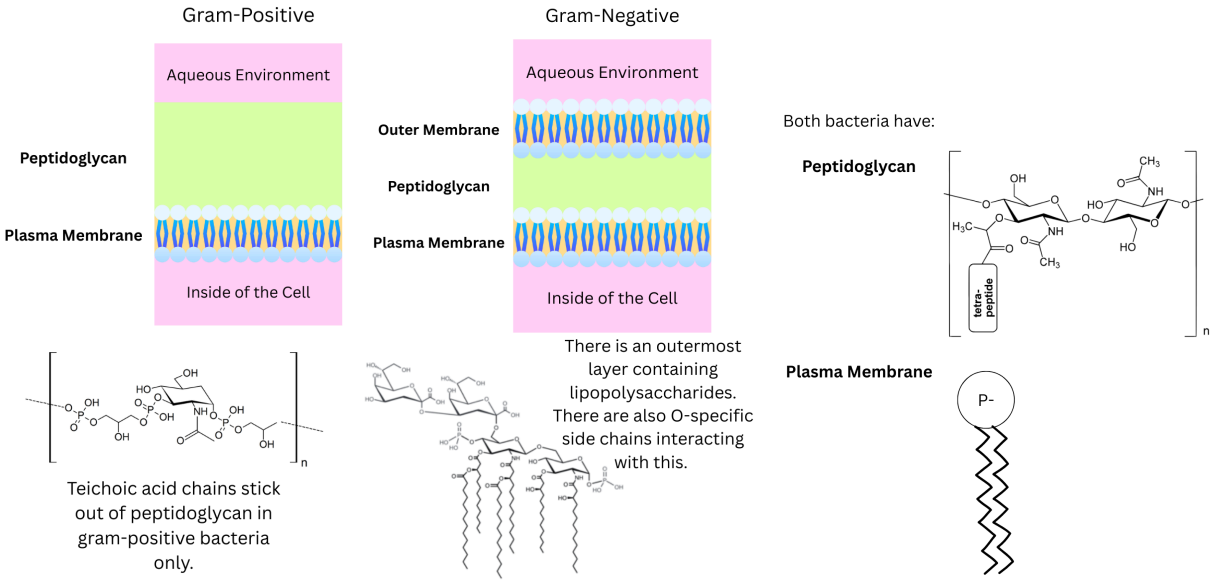


Figure 22: *Schematic representation of Gram-positive and Gram-negative bacterial membranes, including chemical structures.*

Once they interact with the outermost part of the cell, the oil components can insert themselves into the membrane and continue to reduce its fluidity. Since membrane fluidity is essential to membrane function, this disruption can compromise the membrane's integrity, leading to cell lysis. Additionally, the nonpolar nature of the membrane may promote strong intermolecular interactions with the oil components, weakening its interactions with the surrounding aqueous environment. These weakened interactions can create small holes in the membrane. This effect must surpass NEO's interaction with the surrounding aqueous environment, allowing it to strongly associate with the lipid bilayer, ultimately disrupting its structure and forming pores in what was once an intact cellular barrier. Although this effect may be relatively short-lived, if numerous NEO molecules attack the membrane simultaneously, the damage accumulates, forming additional holes and ultimately causing leakage of intracellular material.

4. Conclusions

As antibiotic resistance continues to be a relevant research topic, there is an urgent need to develop new methods against antibiotic resistance and persistence. In recent years, evidence shows that bacterial persisters play a role in relapse and recalcitrance of infections, and continues to be an ongoing problem.[3] This antibiotic tolerance is the result of phenotypic changes in bacteria, though antibiotic resistance, responsible for genetic mutations, contributes to tolerance by slowing metabolic growth.[3] To shift away from typical pharmaceutical antibiotics, attention is turning towards natural products and remedies.

This study investigates the effect of neroli essential oil (NEO) on bacterial cells, focusing on its potential as an antimicrobial agent. NEO is a potent essential oil known for its anti-inflammatory, antioxidant, and antiangiogenic properties.[61] The primary objective was to determine whether NEO can effectively kill persister cells in *S. epidermidis* and *E. coli*, thereby contributing to broader efforts to address antibiotic resistance. Essential oils like NEO, commonly used in skincare, offer accessibility and the potential to eliminate bacterial cells on the skin's surface before they enter the bloodstream. This study sought to answer the question of whether or not NEO can eradicate persister cells that survive antibiotic treatment. Using *S. epidermidis*, tests revealed that NEO completely eradicated planktonic persisters within 24 h, with over 99% of the killing occurring within the first hour. Similar results were obtained with *E. coli* and complete planktonic persister killing was observed after 24 h of treatment. These results are extremely comparable to those found when investigating tea tree essential oil (TTEO), that determined complete persister killing in both bacteria, though *S. epidermidis* persisters treated with NEO seem to lyse at a more rapid rate.[55] These findings demonstrate that NEO is highly

efficient at killing persister cells quickly, even at low concentrations, in the planktonic state. The rapid killing rate also offers insight into the potential mechanism of action. Since metabolic pathways typically require more time to activate, the speed of persister eradication suggests that NEO acts through a direct killing mechanism rather than one dependent on cellular metabolism.

In order to further investigate biofilm-related infections, NEO was evaluated as a potential anti-biofilm agent. Multiple trials using *S. epidermidis* produced results with no consistent pattern, suggesting that NEO may cause a similar level of biofilm killing as the DMSO control under the tested conditions. Although a substantial reduction in persister cells was observed over 48 hours, the specific component responsible for this effect remains unclear. NEO treatment of *E. coli* biofilms yielded variable experimental results, with some concentrations reducing CFU/mL and others showing increased growth. Despite an unclear dose response relationship, NEO demonstrated greater antibacterial activity than the minimal killing of the DMSO control, especially when compared to its effect on *S. epidermidis*. This result suggests that the observed biofilm reduction is primarily due to NEO rather than the solvent.

Given the increasing clinical relevance of biofilm-associated infections, further research is warranted to evaluate NEO's ability to penetrate and act within the biofilm environment. One potential explanation for the limited efficacy observed in this study is that the experimental methods may not have successfully produced a robust or uniform biofilm, a known challenge for this laboratory. Alternatively, it is possible that NEO lacks the capacity to effectively kill persister cells within biofilms due to limitations in its chemical composition. While the lipophilic nature of NEO's constituents favors interaction with bacterial lipid membranes, the structural complexity and chemical composition of the biofilm matrix may hinder its penetration. The

extracellular matrix, which is full of polysaccharides made up of repeating units with alcohol and ether groups, might not interact well with the mostly nonpolar nature of NEO. While some oxygen-containing parts of the essential oil could help with interaction, the matrix's dense and water-rich structure is likely a tougher barrier to get through than the bacterial membrane itself. These structural factors may limit how well NEO can reach the persister cells hidden inside.

It remains unclear exactly how, at the molecular level, the membrane of dormant persister cells can be disrupted to cause cell lysis. Although many studies have demonstrated that this disruption is possible, the precise interactions between certain molecules and the membrane that lead to its breakdown are still not well understood. This study aims to address several key questions surrounding the mechanism of action: Is the cell membrane being disrupted, leading to leakage of intracellular contents? What specific components are leaking out of the bacterial cells? And how large are the molecules escaping through the damaged membrane?

Trials were first conducted using a colorimetric assay to assess bacterial membrane integrity and gather information on the overall direct killing mechanism. Previous attempts with this EB dye assay on *S. epidermidis* yielded inconclusive results, leading to the explanation that the procedure required optimization. After one trial with the revised method, results still lacked a clear pattern. Incomparable to the previous study, Gram-positive and Gram-negative bacteria treated with tea tree essential oil (TTEO) clearly showed absorbance values higher than one absorbance unit. The controls of this study, in contrast, had low absorbance values, showing that there was direct killing through disrupting the cell membrane.55 NEO-treated *S. epidermidis* appears less effective at direct killing compared directly to the TTEO EB assays, however protein and DNA trials in this project still highlight cell membrane disruption as the killing

mechanism. NEO-treated *E. coli* exhibited more promising results, with all concentrations producing absorbance values around 0.3 units, while control values remained minimal. Since EB dye binds to cells with compromised membranes, it is possible that NEO directly disrupted the *E. coli* cell membrane, leading to cell lysis. The TTEO investigation recorded EB dye absorbances around 2 absorbance units for *E. coli* samples, which is notably higher than NEO-treated *E. coli*.[55] This form of comparison may not be practical, but it may show that TTEO more effectively disrupts *E. coli* cell membranes than NEO. Given the structural differences in the membrane of *S. epidermidis*, alternative mechanisms may contribute to the rapid killing of Gram-positive bacteria. Overall, it is concluded that the rapid killing of NEO-treated *E. coli* is likely a result of membrane disruption, although further research is needed to clarify the mechanisms involved in Gram-positive bacterial death.

Now that it is established that persister cell death occurs rapidly and the EB assay confirms membrane disruption as the likely cause, the second research question can be addressed: what is leaking out of the cell and how big are these molecules? An assay measuring the absorbance of DNA yielded very consistent and revealing results. As the concentration of NEO increases, so does the absorbance of DNA after 1 h and 2 h of treatment of both Gram-positive and Gram-negative bacteria. This outcome provides strong evidence that DNA leakage occurs early in the killing process and stands as the second most compelling result of this investigation, next to the 100% persister eradication from planktonic persister cells. Since this part of the study focused on the first two hours of rapid killing, it confirms that significant amounts of DNA are released during that time. Given the essential role of DNA in cellular function, its leakage likely contributes directly to cell death caused by NEO. Structurally, DNA is approximately 3.4 Å per base pair along its axis and about 20 Å in width.[62] While the exact

length of the bacterial DNA studied is unknown, the molecule's size and complexity suggest that substantial membrane disruption must occur to allow its escape, implying that the pores formed in the persister cell membrane are large enough to accommodate intact or fragmented DNA.

In order to build on this finding, several assays were conducted to investigate protein content leakage, as proteins are another essential component of cellular function. While generally smaller than DNA, proteins are still relatively large molecules, and their presence outside the cell further supports significant membrane disruption. The Bradford assay was used to assess nonspecific protein leakage as a result of NEO treatment. In the case of *S. epidermidis*, the results suggest a clear increase in protein concentration from 1 h to 2 h across all NEO-treated samples. The control groups showed lower protein levels, indicating that the observed leakage may be due to the essential oil, though the difference is not significant enough to rule out the possibility that it could be the solubilizing agents. By contrast, the *E. coli* trial yielded less interpretable results. All treated samples displayed slightly elevated protein concentrations at 1 h, followed by a decline at 2 h. This is an unexpected result and may reflect a methodological error, as once proteins are released from cells, they cannot re-enter or degrade within such a short timeframe under these conditions. These assays, however, were performed with different supernatant volumes, deeming the *E. coli* assay less reliable. While the results for *S. epidermidis* provide supportive evidence for membrane disruption by NEO, further optimization and replication are needed to confirm these findings in *E. coli*.

In an attempt to improve upon the variability seen in the Bradford assay, the BCA protein kit assay was utilized because it relies on a more specific protein-binding reagent. These assay results support the idea that NEO induces membrane disruption in both bacteria, though with

some key differences between the species. While *S. epidermidis* showed higher protein leakage overall, the DMSO control yielded similar values. It is difficult to completely rule out the solvent as a contributing factor. Once again, the lysed bacteria sample gave inconclusive results, leaving no true "baseline" for comparison. In *E. coli* however, a clear pattern, while inconsistent compared to the increasing concentration of NEO, was observed where NEO caused protein leakage and the controls did not. The concentration of leaked protein was much lower than that of the Gram-positive bacteria. This finding can be a result of the more resilient outer membrane or simply a less efficient membrane interaction with the essential oil. Collectively, the protein and DNA leakage experimental results reinforce the idea that NEO acts quickly and directly on the membrane, though its efficacy may depend on species-specific cell envelope properties. The holes induced from the NEO-membrane interaction must be large enough to fit a complex DNA molecule through, showing that this effect is quite significant.

Ultimately, NEO shows strong evidence of successfully eradicating persister cells in *S. epidermidis* and *E. coli* in the planktonic state, making it a promising candidate for development as a broad-spectrum anti-biofilm agent. Additionally, spectrophotometric analysis of DNA and protein leakage showed positive absorbance readings, indicating that cell death occurred through a direct killing mechanism. These findings suggest that NEO likely disrupts the bacterial membrane, forming small pores that cause the leakage of vital cellular components and ultimately lead to cell lysis. More research is needed to better understand its killing mechanism, particularly in biofilm-associated environments, where persister cells are shielded and harder to eliminate.

The proposed chemical mechanism, centered around membrane disruption, remains theoretical and requires further experimentation to confirm its effects at the molecular level. Future studies could utilize 3D modeling of NEO's interactions with the cell membrane, or assays could be repeated with the pure major components of NEO. Major components of TTEO were investigated and revealed that terpinen-4-ol, which comprises 42.59% of TTEO, was the leading cause of cell death.[55] While there is no direct overlap of TTEO molecules in the main components of NEO, α-Terpineol (5% in NEO) has an extremely similar chemical structure to terpinen-4-ol, which is responsible for TTEO killing in persister cells. It may be interesting to compare NEO essential oil components amongst known anti-persister essential oils to identify a trend. Additionally, testing against a wider range of bacterial species and under physiologically relevant conditions will be essential for evaluating its therapeutic potential. A future pharmaceutical investigation into drug delivery is needed to translate these findings into practical medical treatments. Overall, NEO demonstrates encouraging potential as a broad-spectrum therapeutic agent targeting both persisters and biofilm-related infections.

5. Supplementary

Table 2: *Abbreviations used and corresponding definitions*

Abbreviation	**Definition**
NEO	Neroli essential oil
S. epidermidis	*Staphylococcus epidermidis*
E. coli	*Escherichia coli*
MDR	Multi-drug resistant
hip	Highly persistent
TA	Toxin-antitoxin
TTEO	Tea tree essential oil
DMSO	Dimethyl sulfoxide
MIC	Minimum inhibitory concentration
PBS	Phosphate-buffered saline
OD	Optical density
CFU	Colony forming unit
EB	Erythrosin B
BCA	Bicinchoninic acid assay

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