# Essential Oils and The Ability to Inhibit Biofilm Formation of *Pseudomonas aeruginosa*

**Abstract**

Despite the constant innovation of science and technology, bacteria are outpacing the market for new and innovative antibiotics and antibiotic resistance is becoming a looming threat. With a decreasing number of antibiotic companies due to government legislation and the expensive Research and Development process. The first case of a superbug arose in Washoe County, Nevada in August, 2016. As a result, the present study explored the use of alternate substances to eradicate the potential superbug cases. The study focused on *Pseudomonas aeruginosa* and its notorious biofilm formation. The present research examined the ability of Peppermint, Roman Chamomile, and Lemongrass essential oils to inhibit biofilms. To evaluate the effectiveness of those test substances, a biofilm assay was prepared in a 96-microtiter plate. The plate was later run through spectrophotometer to indirectly assess bacterial growth and biofilm formation. The results show that Lemongrass has the most prominent reduction of both bacteria growth and biofilm formation. The same trend was observed for Peppermint, however, the correlation was weaker than the Lemongrass. Roman Chamomile essential oil did not show any effect on antimicrobial activity as both bacterial growth increased and biofilm formation stayed stagnant. Further studies are needed to justify bacterial growth patterns that were observed in this research.

*Keyword:* Essential oils, *Pseudomonas aeruginosa,* biofilm formation, biofilm assay, peppermint, roman chamomile, lemongrass

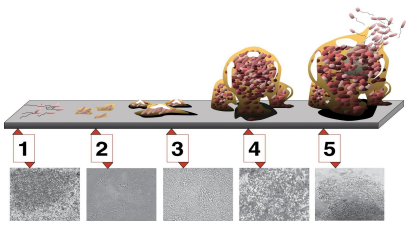
## Introduction and Literature Review

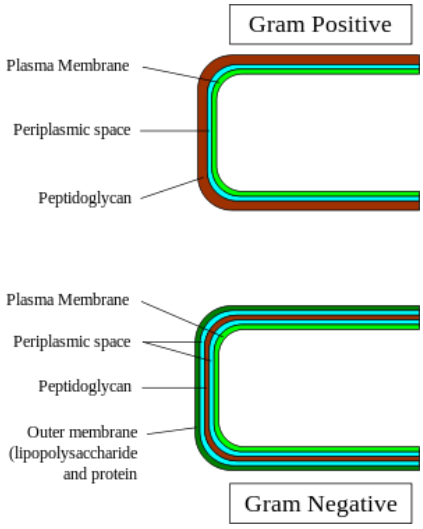
### Operational Definition

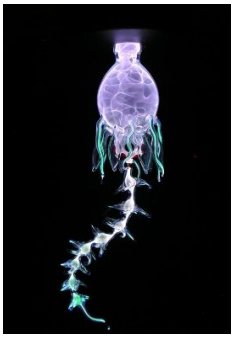
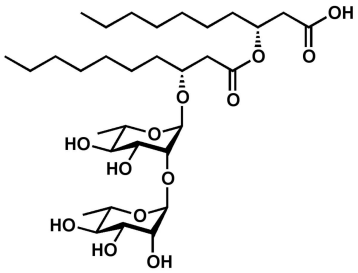
The following table contains words that critical to understand in the study.

Table 1.

*Definition of the paper's scientific terms*

| Term | Definition |
|---|---|
| 2X [1] | Positive control, a treatment with known response, so that the positive responses can be compared with the unknown. |
| Aliquot [2] | A technique that divides a larger amount of solution into small portion |
| Assay [3] | A laboratory technique that is used for qualitatively assessing or quantitatively measuring the presence, amount, or functional activity of a target entity. |
| Autoinducer [4] | Small chemical signaling molecule |
| Biofilm formation [5]   | An assemblage of surface-associated microbial cells that are irreversibly associated (not removed by gentle rinsing) and enclosed in a matrix of primarily polysaccharide material (Donlan, 2002) |

| | |
|---|---|
| Dehydrogenase [6] | An enzyme that catalyzes the removal of hydrogen atoms from a particular molecule, specifically in the electron transport chain reactions if cell respiration is in conjunction with the coenzymes NAD and FAD |
| Gram negative [7] | In the Gram staining method, the bacteria will display the color pink from the pink stain, safranin (*See Figure 1.2)*. Gram-negative contains an outer membrane, two periplasmic spaces, a layer of peptidoglycan, and a plasma membrane. (Campbell, 2012)   |
| Light absorbance [8] | A measure of the capacity if a substance can absorb light of a specific wavelength. It is equal to the logarithm of the reciprocal of the transmittance. |

| | |
|---|---|
| *lux, lasI/lasR, rhlI/rhlR* [9] | Genes on the bacteria |
| Microcentrifuge [10] | A centrifuge used in the laboratory to separate small particles in small biological sample. |
| Mono-flagellated [11] | Of a unicellular organism: Having a single bacterial tail  |
| *Pa* UCBPP-PA14 [12] | *Pseudomonas aeruginosa* strain isolated from a humans burn wound |
| Rhamnolipids [13] | Class of glycolipid produced by *Pseudomonas aeruginosa*   |
| Siderophores [14] | Molecules that bind and transport iron in a microorganism |
| Superbug [15] | A strain of bacteria that has become resistant to antibiotics |
| Supernatant [16] | The liquid lying above a solid residue after centrifugation |

| | |
|---|---|
| Swarming motility [17] | The ability to move the bacteria in an environment |
| Versatility [18] | Ability to move around |
| Vortex [19] | Whirling fluid at a high speed |
| Well [20] | A lot in the 96-microtiter plate |

**Introduction**

In September of 2016, the Centers for Disease Control and Prevention (CDC) reported the first case of superbug [15]. An anonymous woman in Washoe County, Nevada, was admitted with carbapenem-resistant Enterobacteriaceae (CRE) which is incurable by all available antibiotics. The 70-year-old patient broke her hip and received treatment in India. When she returned to the United States in August, she started to develop systemic inflammatory response syndrome which likely resulted from the hip fracture. After multiple hospitalizations, the patient passed away within a month (Chen, Todd, Kiehbaunch, Walters, & Kallen, 2016). The case highlights the danger of the unregulated usage of antibiotics. Antibiotics are commonly used to treat bacterial infections; however, people tend to utilize this medicine as a solution to all sicknesses. Common illnesses such as the cold, bronchitis, the flu, and sore throat, are most of the time caused by a virus, not bacteria. Antibiotics are not effective against viruses because these organisms insert its genetic material into a human cell as intracellular pathogens, while most bacteria builds up on the outside of the cell as extracellular pathogens (CDC, 2016). The misconception about antibiotics and the misuse of antibiotics has lead to the birth of

"superbugs," which are bacteria that are resistant to antibiotics. In the case of the patient from Nevada, the bacterial isolate that caused her death was successfully resistant to all of 26 types of available antibiotics.

This superbug case leads the public to question: how can pharmaceutical companies not invest more in the Researching and Development (R&D) process? The answer is simple: the market is not profitable. There has been a dramatic shift in the antibiotic business from the 1980s to 2010s. According to the European Federation of Pharmaceutical Industries and Associations (EFPIA), in 1983 to 1987, the United States Food and Drug Administration (FDA) approved a total of 16 antibiotics. Nevertheless, the number dropped to as low as two antibiotics in 2008 to 2011 (See*: Figure 1.1*). The R&D process requires companies to invest a large amount of capital into a drug that could be rendered ineffective with antibiotic resistance as soon as three months after becoming available to the public. Moreover, with increasing regulations from the governmental agency, private companies gradually abandoned the antibiotics market.

In 2012, a law passed requiring companies to provide an additional five years of researching data about a potential drug before it can pass FDA regulation. Amanda Jezek, Vice-President of Public Policy and Government Relation from International Swaps and Derivative Association (ISDA), expressed her concern about the fate of antibiotics after the 2012 legislation passed. According to Ms. Jezek, the legislation, "could increase R&D spending on antibiotics and antifungals by over $1 billion over a 10-year-period" for something that companies could reach within five years (Sukkar, 2013). Antibiotics will soon cost more yet be less effective. As superbugs grow more prevalent, the future of patients with bacterial infections

may progress to hopeless. Thus, my research presents a crucial alternative solution to the growing resistance dilemma that could potentially cut down the cost of R&D processes.

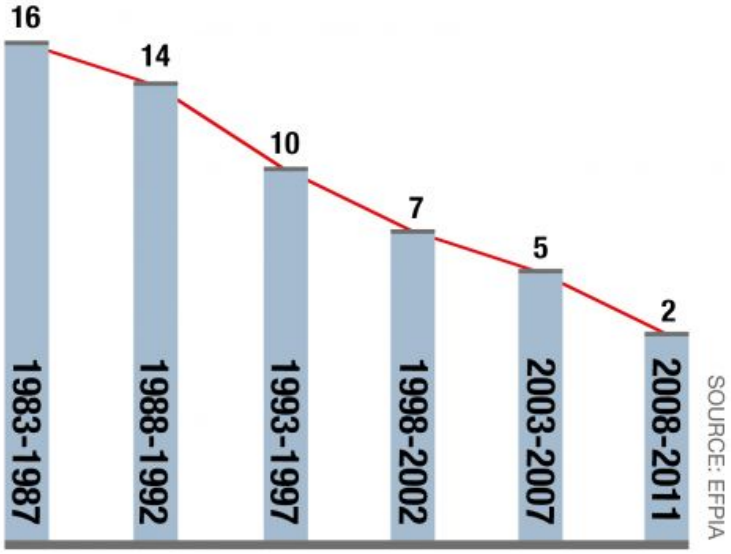



*Figure 1.1*: Number of antibiotics approved in the US (1983-2011)

***Pseudomonas aeruginosa***

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a bacillus that exists everywhere in the environment. The bacteria live civilly inside and outside of the human host. However, if the bacterial growth becomes uncontrollable, *P. aeruginosa* can evolve into a pathogenic organism, especially after exposure to contaminated water. For example, *P. aeruginosa* is the common cause of ear infections, skin rashes, or eyes infections from contact lenses that permeate in unsanitized saline solutions. Nonetheless, notoriety for this bacteria has come from resistance in the hospital setting. *P. aeruginosa* manifests into deadly diseases such as pneumonia, severe lung infections of cystic fibrosis patients, or severe post-surgical infection, which can be multidrug-resistant and potentially fatal. The CDC estimated that approximately 51,000 people

die from *P. aeruginosa*-related infections each year, 40% of which are cases of multidrug-resistance (*Pseudomonas aeruginosa in healthcare settings*, 2014).

*P. aeruginosa* grows incredibly tolerant against antibiotics due to its Gram-negative [4] characteristics. As a rod-shaped, mono-flagellated [11] bacterium, *P. aeruginosa* has an incredible nutritional versatility [18] (See: Table 1.1), (Adjidé et al., 2010). The Gram-negative bacteria is composed of an outer membrane, a thin layer of peptidoglycan, and a plasma membrane line in the innermost space. Due to membrane differences, bacterial responses differ (Campbell, 2012). The existence of an extra membrane in Gram-negative increases the antibiotic-resistant characteristic, which makes it more complex to treat *P. aeruginosa* infection.

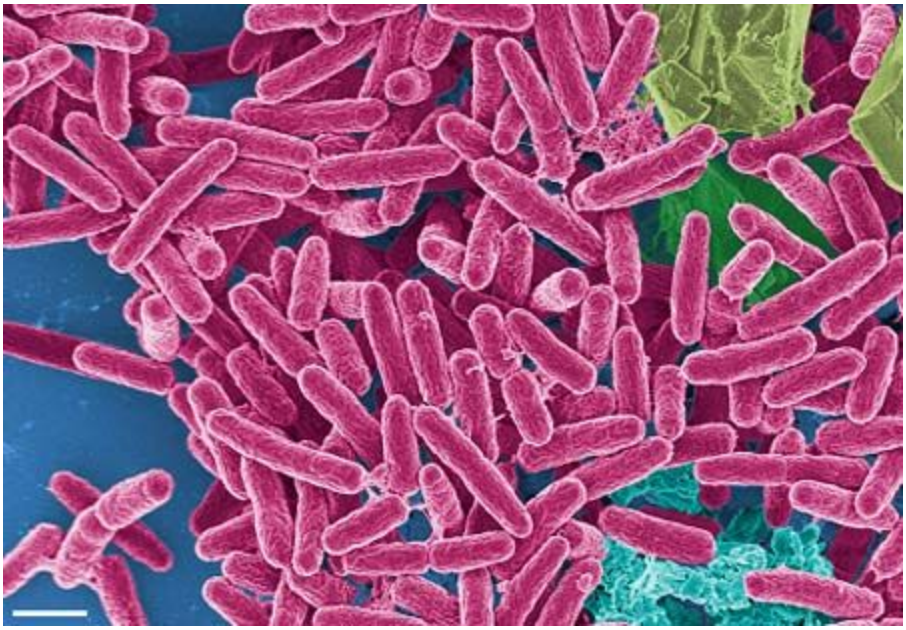


*Figure 1.2:* Three-dimensional visualization of *Pseudomonas aeruginosa*

In addition to these cell wall structures, some bacteria (e.g. *P. aeruginosa*) are capable of forming an extracellular layer called glycocalyx. This structure enables bacteria to adhere to both

surfaces as well as other bacteria. As the bacteria adhere to each other, they sometimes form communities called biofilms. Bacteria within these biofilms tend to be protected and more resistant to antibiotics.

**Biofilm formation in *Pseudomonas aeruginosa***

Biofilm formation [5] (See: Table 1.1) is an aggregate of bacteria that adheres well to a surface (Merritt, Kadouri, & O'Toole, 2005). The pathogens form a community that has food sources constantly available in the surrounding environment. Without the biofilm, the bacteria fights the antibiotics alone; however, with strong support from the neighboring bacteria, the biofilm is 1,000 times more resistant to antimicrobial drugs, which can lead to chronic inflammatory and cancer (Gordya et al., 2017). Cystic fibrosis patients tend to have the most severe complications from *P. aeruginosa* infection and its biofilm formation. Cystic fibrosis (CF) is an autosomal recessive genetic disease that increases mucous viscosity and obstructs an individual's airway (O'Brien et al., 2017). *P. aeruginosa* is hard to eradicate in CF patients due to its constant adaptation and mutation in the host (Perez, Costa, Freitas, & Barth, 2011). Various research studies have contributed to the discovery of the antibiofilm agents in R&D that could alleviate the burdens of the disease (Amoils, 2005; Brigmon, Berry, Singh, & Narayan, 2011; Donlan, 2001; Ferreira et al., 2015; Hentzer & Givskov, 2003; Hirakawa & Tomita, 2013; Kalia et al., 2015; Zeng et al., 2008). However, developing treatment requires a deep understanding of "bacterial biofilm-specific-physiological traits" (Ferreira et al., 2015; Vital-Lopez, Reifman, & Wallqvist, 2015). The present research aimed to document the effects of essential oils on *P. aeruginosa* growth and biofilm formation.

**Basic Genome of *Pseudomonas aeruginosa* biofilm.** According to Li Zhang et al., in *P. aeruginosa* UCBPP-PA14 [12], the three main genes or operons with biofilm-specific regulators are *ndvB,* PA1875-1877, and *tssC1* (See: Table 1.1). These components are not directly involved in biofilm formation; however, they play an active role in biofilm-specific antibiotic resistance. Similarly, encoders such as PA2070 and PA5033 also play an essential part in the antibiotic resistance. As a result, by deleting PA0756, PA2070, and PA5033, the lethality significantly reduces (Zhang et al., 2013). By attacking these genes, future medicines hold a promise to reduce the virulence in CF patients.

***Pseudomonas aeruginosa* quorum-sensing circuit**. Just like other bacteria, *P. aeruginosa* performs a newly discovered activity among bacteria, quorum sensing. Quorum sensing (QS) is the process of how bacteria communicate with each other. Bacteria produce specialized autoinducers [4] (See: Table 1.1) that can bind bacteria within its species, or bacteria outside of its species (Tomlin, Coll, & Ceri, 2001). The autoinducer itself varies based on the task the bacteria wants to perform. Once enough autoinducers become present in the environment, the bacteria will carry out the task that it intended (Taillefumier & Wingreen, 2015; Wilder, Diggle, & Schuster, 2011). Bacteria do not work alone--they work as a community. Just like humans use language to communicate and understand each other, bacteria use autoinducers to do the same task.

***Autoinducers in Pseudomonas aeruginosa biofilm.*** Currently, there are three type of autoinducers that have been discovered: acetylated homoserine lactones (AHLs--only found in Gram negative [7] bacteria), *N-3-oxohexanoyl-L-homoserine lactone* (AI-1) (only found in Gram positive bacteria), and autoinducer-2-compounds (AI-2s) (found in both Gram positive and Gram negative) (González & Keshavan, 2006; Raffa et al., 2005). *P. aeruginosa* contains two AHL *lux* [9]-like systems that regulate the bacterial virulence factor, *lasI/lasR* [9] and *rhlI/rhlR* [9] (See: Table 1.1) (Monnet & Gardan, 2015; Papenfort & Bassler, 2016; Sifri, 2008). Along with regulating swarming motility [17], siderophores [14], and rhamnolipids [13] (See: Table 1.1). QS plays an additional role in regulating biofilm formation (Kalia et al., 2015; Merritt, Kadouri, & O'Toole, 2005). Previous studies have shown that by applying an anti-QS therapeutic regulator derived from natural remedies to a growing biofilm, the growth is reduced but not eliminated (Costerton, Caldwell, Korber, Lewandowski, & Lappin-Scott, 1995; Zeng et al., 2008). The purpose of the present study is to verify the therapeutic benefits that were demonstrated in prior studies and to identify which natural remedy is the best to suppress biofilm virulence.

**Essential Oils**

Beáta et al., defines essential oils (EOs) as, "aromatic hydrophobic liquids originated from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots)" (1970). Companies produce mass manufactured EOs by acquiring the natural ingredients and extracting them through the fermentation process or through effleurage steam. By injecting EOs into the bacterial media, the pH level and osmosis in the environment becomes disrupted. Bacterial response differs at different EOs concentration. One of the most prominent EO

characteristics is the ability to inhibit the mitochondrial ATPase activity and reduce the mitochondria of the cell. EOs affect the mitochondrial dehydrogenase [6] (See: Table 1.1) in fungi and inhibit "glucose-induced reduction of external pH in a time and concentration-dependent manner" (Beáta et al., 1970; Harjai, Gupta, & Sehgal, 2014; Koh et al., 2013).

The present study contributes to the field of Microbiology, particularly into antimicrobial activities of a bacterial specie, *P. aeruginosa.* The present study examined how EOs inhibit biofilm formation of *P. aeruginosa*. *Chamaemelum nobile* (Roman Chamomile) EO, *Mentha piperita* (Peppermint) EO, and *Cymbopogon* (Lemongrass) EO were tested in different concentration. Currently, no research has been conducted to reckon the effectiveness of these EOs against *P. aeruginosa* biofilm with bacterial growth. A laboratory strain of *P. aeruginosa* was used throughout the study. *P. aeruginosa* was grown in a 96-wells microtiter plate that contained nutrient broth and various EO concentrations ranging from 0.1% to 20%. The study used biofilm formation assay [3] to observe bacterial adherence to an abiotic surface. The plate was run through spectrophotometry to evaluate light absorbance[8] (See: Table 1.1). The outcomes were analyzed according to the Stephanovic's assessment for biofilm production--if the biofilm formation of the test results is lesser than the positive control, it indicates a linkage between EOs and QS in biofilm reduction. The positive result also indicates EOs do, in fact, interrupt the environment significantly and should be used in future antibiotic manufacturing. One can further examine if EO molecules act as AHLs and inhibit bacteria from communicating with each other. Every procedure was done in biological triplets.

From the review of the literature, the following question was formulate to drive the research: Can Essential Oils (e.g. *Chamaemelum nobile, Mentha piperita,* and *Cymbopogon)* inhibit *Pseudomonas aeruginosa* biofilm formation and bacterial growth?

## Materials and Methods

### Nutrient Broth

*P. aeruginosa* was grown in Tryptic Soy Broth (TSB). TSB casein digest and soybean digest which acts as a food source to allow the bacteria to survive and grow. A stock solution was made as per manufacturer's requirements and autoclaved at 121°C for 15 minutes. The sterile stock solution was used to make the working solutions. The working solutions were made as follows.

Table 2.1:

*PBS Powder (in gram) and water (in millimeter) ratio for different concentration*

| Concentration | TSB Powder (g) | Water (ml) |
|---|---|---|
| 2X [1] (positive control) | 1.6 g | 100 ml |
| 0.1% | 1.6 g | 99.9 ml |
| 0.5% | 1.6 g | 99 ml |
| 1% | 1.6 g | 98 ml |
| 5% | 1.6 g | 95 ml |

| | | |
|---|---|---|
| 10% | 1.6 g | 90 ml |
| 20% | 1.6 g | 80 ml |

### ***Pseudomonas aeruginosa*** **culture**

Proper aseptic techniques were used to culture and propagate the *P. aeruginosa.* A flame was used to sterilize the surrounding air and ethanol to clean the work area. A modified 4-quadrant streak method was used to isolate the bacteria on stock agar plates. Briefly, instead of using the inoculating loop, 3 thirds method was used. This method was performed by using sterile 1-ml micropipette tips to spread the bacteria on half of the agar plate. This tip was then discarded and a new tip was obtained. The bacteria was then dragged from the contaminated area to second third of the agar plate. Again, the used tip was discarded and a new tip was used to spread the bacteria from the previous third to the last third of the plate. The bacteria was spread until it covered the plate (See: *Figure 2.1*). After culturing the bacteria, the plate was incubated at 36.5-37°C for 24 hours. The bacteria was then inoculated into a broth culture (liquid-based) and after incubation placed at 4°C until used.

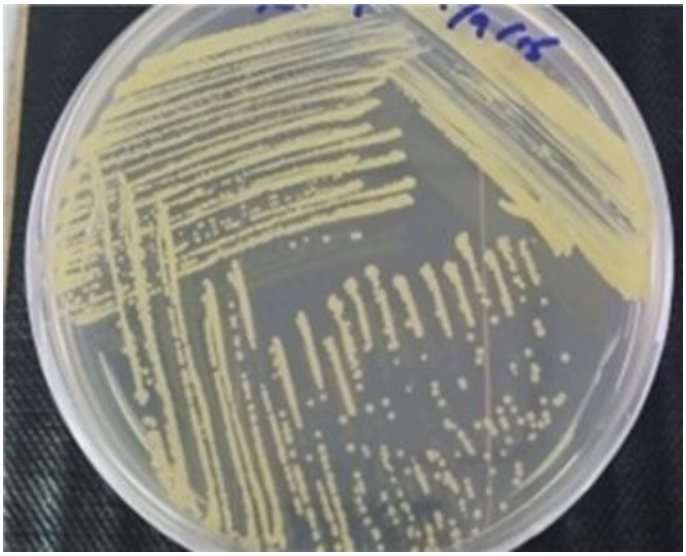


*Figure 2.1:* 3/3 Quadrant Demonstration *(*Note: This is an example of the method, not the actual picture during the experiment)

**Microtiter Plate Biofilm Assay**

To build the biofilm assay, the stock media was aliquotted [1] into microcentrifuge tubes. Following that, EOs were added at various concentrations to make a total final volume of 1,200 microliters (µl). The microcentrifuges tubes were color-coded to differentiate between EOs. Purple represents Roman chamomile (RC) EO; Green depicts Peppermint (PP) EO; and Orange portrays Lemongrass (LG) EO. Each mixed tube was vortexed [19] to make the solution as homogenous as possible. The ratio of media and EOs is outlined in the following table:

Table 2.2:

*Media (in µl) and essential oils (in µl) ratio*

| Concentration | Media (µl) | Essential Oils (µl) |
|---|---|---|

| | | |
|---|---|---|
| 0.1% | 1198.8 µl | 1.2 µl |
| 0.5% | 1194 µl | 6 µl |
| 1% | 1188 µl | 12 µl |
| 5% | 1140 µl | 60 µl |
| 10% | 1080 µl | 120 µl |
| 20% | 960 µl | 240 µl |

Before building the assay, the 2X [1] solution was diluted to 1X. Next, the bacteria needed to be suspended in 1X PBS from the bacteria broth that was previously made. Four 0.5 ml *P. aeruginosa* tubes were made and placed in a microcentrifuge [10]. The tubes were spun at 10,000 rpm for 5 minutes to pellet the bacteria at the bottom of the tube. The supernatant [16] was removed and discarded from the tube containing the bacteria pellet. The pellet was then resuspended in 1,500 µl of 1X PBS. The tube was vortexed to homogenize the culture.

The microtiter plate was labeled as the following:

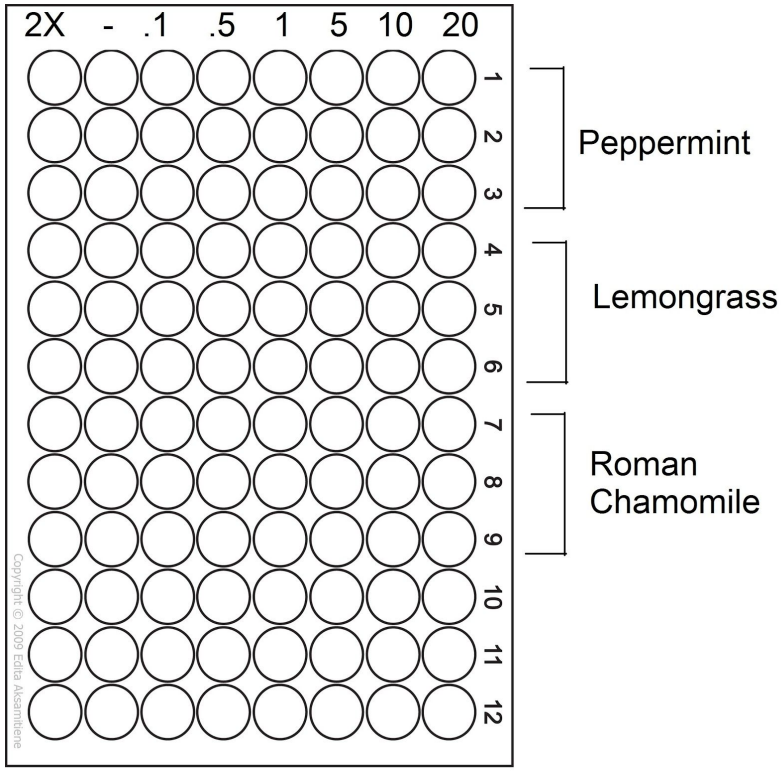



*Figure 2.2*: Labeled microtiter biofilm assay

Each well[20] contained 100 µl of *P. aeruginosa* and 100 µl of the prepared media (i.e. EO and 2X stock TSB), except for the 2X and negative control columns. The 2X well contains 100 µl 2X TSB and 100 µl of *P. aeruginosa* alone. For the negative control, 100 µl of 1X PBS and 100 µl of 2X TSB media were injected into each well for the optical standard. Each EO concentration was assayed in triplets to ensure uniformity and statistical relevance. After the microtiter plate was built, the bacteria was incubated for 24 hours at 37°C. After the incubation period, the content of the wells was transferred into a new microtiter plate for evaluation of bacterial culture turbidity (i.e. cloudiness). Higher turbidity corresponds to higher light absorption which indicates more growth. To assess biofilm formation, the empty wells of the

first microtiter plate were washed twice with 1X PBS to remove the planktonic cells. Next, to visualize the microbial cells, 125 µl of 0.1% crystal violet was added to each well. The microtiter plate was incubated at room temperature for 10 minutes. The plate was then washed and air dried for several days. The final steps included solubilizing the surface associated dye by adding 200 µl of 30% acetic acid for 10 to 15 minutes at room temperature. Then, 125 µl of the crystal violet/acetic acid solution was pipetted into a new microtiter plate. The biofilm formation was indirectly calculated using a spectrophotometer that measures light absorption at 490 nm and 630 nm.

## Results

To analyze the results, two microtiter plates were evaluated: (1) the new microtiter plate that contained overnight growth of *P. aeruginosa*, was put under a plate reader and scanned at the light intensity of 490 nm and 630 nm to examine *P. aeruginosa* biofilm formation through light absorbance [5] (See: Table 1.1) and (2) the microtiter plate that was solubilized with acetic acid and scanned with the nano-droplet spectrophotometer to evaluate absorbance at the light intensity of 555 nm. Both data sets were analyzed by creating a trendline to demonstrate the progression of the data. Because there are two sets of biofilm formation data available from 2 separate wavelengths, the dataset at the light intensity of 630 nm were chosen to correlate with the bacterial growth at 555 nm. The results at 630 nm give a clearer trend compare to the results at 490 nm. The rate of change of the trendlines answers will correlate whether reduction in bacterial growth with biofilm formation

Table 3.1

*Average light absorbance (nm) at 630 nm of Pseudomonas aeruginosa (biofilm growth):*

| Essential Oil Concentration | 0 | 0.1 | 0.5 | 1 | 5 | 10 | 20 |
|---|---|---|---|---|---|---|---|
| Peppermint | 0.466 | 0.342 | 0.231 | 0.208 | 0.204 | 0.223 | 0.248 |
| Lemongrass | 0.383 | 0.365 | 0.372 | 0.290 | 0.271 | 0.224 | 0.237 |
| Roman Chamomile | 0.451 | 0.430 | 0.507 | 0.382 | 0.460 | 0.441 | 0.455 |

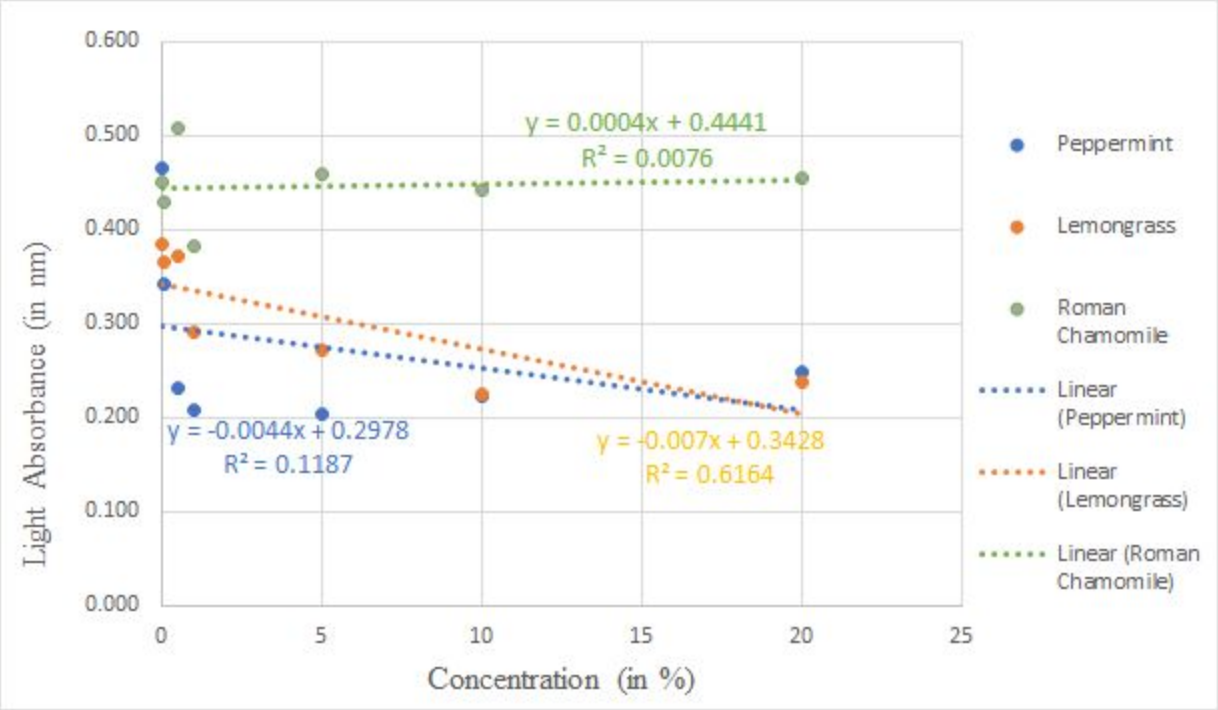



*Figure 3.1:* Effects of Essential Oils on Biofilm Formation in *Pseudomonas aeruginosa*

**Biofilm formation**

Lemongrass showed the strongest antimicrobial activity out of the three EOs. The positive control, which only contained bacteria and nutritious media, had the light absorbance started at 0.383 nm. As the concentration increased, the light absorbance of the biofilm reduced with the rate of 0.007 light absorbance/nm. At the highest concentration, the light absorbance is at 0.237 nm, 0.146 less than the positive control. The best fit line suggested a moderate correlation. The data and the trendline indicated that Lemongrass was the most effective EO to inhibit *P. aeruginosa* biofilm growth. Similarly, Peppermint EO also demonstrated a strong antibiofilm activity by reducing the biofilm formation at a rate of 0.0044 light absorbance/nm. The most significant drop in biofilm formation ranged from positive control to 0.5% concentration. This means that the optimal in concentration lies between 0.1% to 0.5% concentration. The variance between the highest concentration and the lowest concentration (positive control) of the Peppermint EO is 0.218 light absorbance/nm. Despite the average line is not as strong as the Lemongrass EO trendline, the effectiveness of Peppermint EO was undeniable. As for Roman Chamomile EO, the data was inconclusive. The biofilm was suppressed when the concentration of the EO increased from 0% to 0.1%. However, there was a sharp growth from 0.1% to 0.5% concentration, 0.077 light absorbance/nm. After the augmentation, the trend stayed stagnant with a small variation in growth. The average biofilm formation of the *P. aeruginosa* in Roman Chamomile EO is 0.0004 light absorbance/nm.

Table 3.2:

*Average light absorbance (in nm) at 555 nm of Pseudomonas aeruginosa (bacterial growth):*

| Essential Oils | 0 | 0.1 | 0.5 | 1 | 5 | 10 | 20 |
|---|---|---|---|---|---|---|---|
| Peppermint | 0.206 | 0.255 | 0.276 | 0.251 | 0.238 | 0.192 | 0.22 |
| Lemongrass | 0.236 | 0.217 | 0.199 | 0.191 | 0.197 | 0.187 | 0.160 |
| Roman Chamomile | 0.285 | 0.225 | 0.208 | 0.186 | 0.200 | 0.218 | 0.268 |

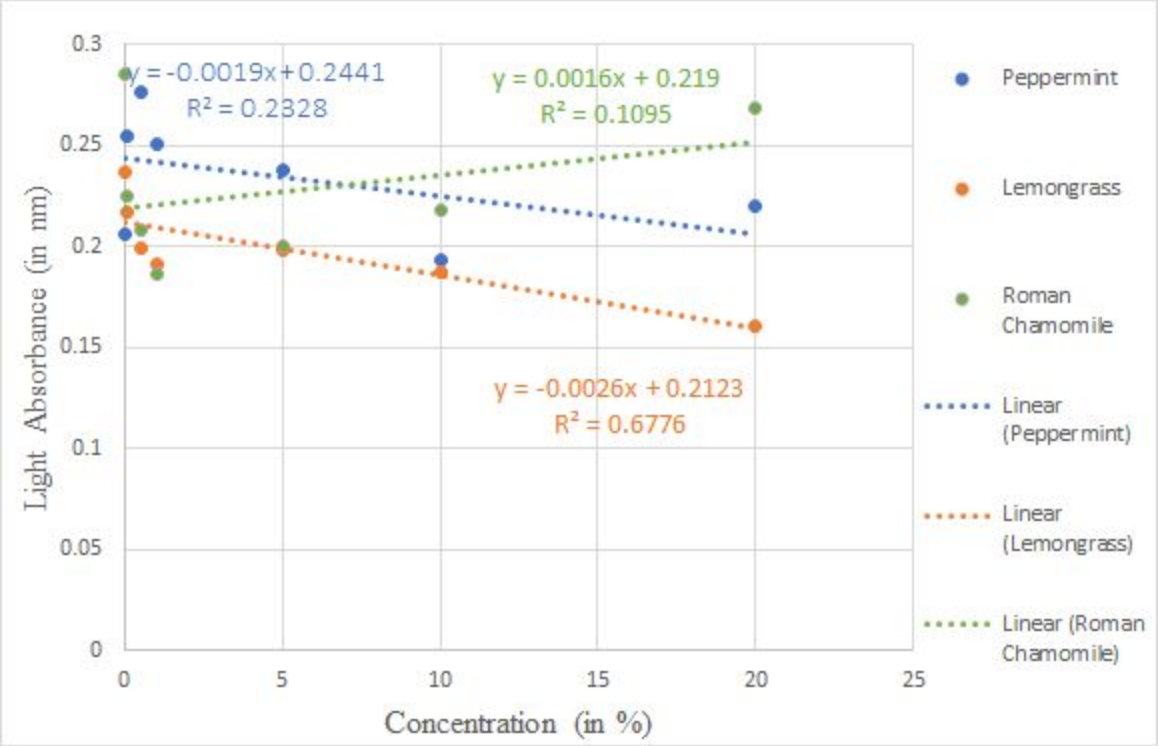



*Figure 3.2:* Effects of Essential Oils on *Pseudomonas aeruginosa* Growth

**Bacteria growth**

The same plate that was solubilized with the acetic acid was used to evaluate the bacteria growth. Compare the positive control, the highest concentration reduces the light absorbance to 0.076 nm. Just like the biofilm formation, Lemongrass EO demonstrated the most prominent anti-microbial activity. The EO has a decrease in bacterial growth at a rate of 0.0026 light absorbance/nm. Bacteria that grew in Peppermint EO also decreased, however, the overall trend is not as strong as Lemongrass EO. The bacteria experienced a surge in growth as Peppermint EO was introduced at 0.1% concentration. The light absorbance increases 0.049 nm from the positive control to the 0.1% concentration for the Roman Chamomile EO. This weak correlation indicates that Peppermint EO was not as effective as Lemongrass EO. Especially at 20% concentration, the light absorbance for bacteria growth is at 0.220 nm, 0.014 nm more than the positive control. The rate of bacterial growth of Peppermint EO decreases at 0.0019 light absorbance/nm. As for Roman Chamomile, the bacterial growth increased much more than biofilm formation. The bacterial growth rate is at 0.0016 light absorbance/nm. The 20% concentration is almost as equal to the positive control, 0.206 nm. The data is not as conclusive as Lemongrass due to the fact that the best fit line is 0.1095 which equated a weak correlation.

## Discussion

There are two datasets available for the biofilm growth available--490 nm and 630 nm. The results at the light intensity of 630 nm was chosen to compare with the biofilm growth at 555 nm because the higher light intensity exhibited clearer changes among the data. By looking

at the two set of data, one can say that Lemongrass exhibited the most effective antimicrobial activity. The EO inhibited bacterial growth and biofilm formation at the same time. The data of different concentration showed a significant difference to the positive control data. Peppermint EO illustrated the same behavior as Lemongrass, nonetheless, the correlation is weaker. Peppermint EO diminished biofilm formation capabilities, but bacterial growth results were not as conclusive. The potential of Peppermint antimicrobial activity needs further investigation. As for Roman Chamomile, the results found contrasted to the Rasamiravaka et al. study (2015). In that study, Roman Chamomile exhibited a strong antimicrobial effect. However, in this study, Roman Chamomile demonstrated little to none antimicrobial effects on the *P. aeruginosa*. In fact, and interestingly, it seemed to enhance both growth and biofilm formation.

The present study might also confirm the hypothesis that EOs disrupt the external bacterial pH level and mitochondrial ATPase activity that were observed in previous studies (Beáta et al., 1970; Harjai, Gupta, & Sehgal, 2014; Koh et al., 2013). Furthermore, essential oil molecules might act as AHLs inhibition by competitive binding with AHLs molecules, the autoinducers that were used to communicate among the bacterial population. By doing so, the study also confirms the EO characteristic as anti-quorum sensing medicine, which also was proven in multiple other research.

**Limitation**

Throughout the study, multiple obstacles could have affected the observed results. This is the first study that the primary investigator has ever done, thus the laboratory techniques and general lab etiquette might be called into question. Furthermore, while replicating the experiment, the number of bacteria used for the biofilm assay and growth assay was different.

The bacterial growth assay had significantly more bacteria, hence the bigger data points. Moreover, the primary investigator might not have washed the microtiter plate after staining it with crystal violet correctly, leading to the above results as it went through the plate reader.

## Conclusion and Future Directions

The present study shows the potential antimicrobial effect of EOs through the results of the Lemongrass EOs. This statement coalesces with Kalia et al., study, where they prove the effectiveness of cinnamon oils against the bacterial and biofilm growth. Notwithstanding, unlike other studies, the present research ties bacterial and biofilm growth together. Unlike many found in the literature, Peppermint EO antibiotic behavior toward biofilm formation and bacterial growth has never been investigated before, to the best of primary investigator's knowledge. The Peppermint EO demonstrates that it is possible that bacteria death increases its virulence. However, the randomness of the data intrigues some questions about its validity. In addition, the Roman Chamomile EO contradicts the *Bai* et al.*,* study. In their study, Roman Chamomile shows a strong antimicrobial activity of the essential oils, especially in Gram-negative bacteria like *P. aeruginosa*. Nonetheless, my research shows a different scenario where the EO not only promotes the growth, but also the formation of biofilm. Perhaps, *P. aeruginosa* might consider Roman Chamomile EO as a source of glucose that it can convert into metabolism food source. *P. aeruginosa* has the ability to live anywhere in the environment. As stated previous studies, due to its quick ability to adapt, the bacteria can survive at the extreme essential oil concentration. This explained the unexpected growth of the bacteria. Future studies should replicate the exact same experiment without the limitations to validate the results of this study and confirm the

hypotheses. Lemongrass has the most prominent antimicrobial features. It successfully suppressed both bacterial and biofilm growth in *P. aeruginosa*. Moreover, this finding is critical to the current scientific knowledge. No studies have been conducted to investigate the *P. aeruginosa* biofilm and growth inhibition. This research fills in the gap of essential oil antimicrobial behavior, especially of Peppermint EO and Lemongrass EO. The study also reports on the inability to replicate the published results. Roman Chamomile does not contain any antibiotic effects, instead of strong correlation as stated in previous studies (Amoils, 2005; Brigmon, Berry, Singh, & Narayan, 2011; Donlan, 2001; Ferreira et al., 2015; Hentzer & Givskov, 2003; Hirakawa & Tomita, 2013; Kalia et al., 2015; Zeng et al., 2008).

**Future Direction**

The need for further investigation of these EOs is critical. Future studies should delve into the component of the Peppermint EO and Roman Chamomile EO and identify the specific chemical compound that triggers the results found in this study. Lemongrass seems like a potential component to integrate into antibiotic manufacture. The chemical substance offers a cheaper solution that the drug companies are seeking. The study is a response to Ms. Jezek (Sukkar, 2013) statement of expensive R&D process, thus could further intrigues pharmaceutical companies to take interest in natural remedies. With CF patients, they can start consuming food that is enriched with Lemongrass to limit the internal biofilm formation of *P. aeruginosa* in the lung. More importantly, the new component in essential oils could help eradicate superbugs along with other chemical syntheses. Ideally preventing cases like Washoe County from ever occurring again (Chen, Todd, Kiehbaunch, Walters, & Kallen, 2016).

## References

Adjidé, C., De Meyer, A., Weyer, M., Obin, O., Lamory, F., Lesueur, C., . . . Ganry, O. (2010, April). Évaluation des risques microbiologiques hydriques associés à stenotrophomonas maltophilia et pseudomonas aeruginosa au chu d'amiens. *Pathologie Biologie, 58*(2), E1-E5. doi:10.1016/j.patbio.2009.07.006

Amoils, S. (2005). Bacterial physiology: Signal interference. Retrieved March 20, 2017, from http://www.nature.com/nrmicro/journal/v3/n12/full/nrmicro1308.html

Bai, S., Buchbaue, G., Jirovetz, L., Denkova, Z., Slavchev, A., Stoyanova, A., . . . Geissler, M. (2009, May). Antimicrobial Activities of Roman Chamomile Oil From France and Its Main Compounds. *Journal of Essential Oil Research,21*(3), 283-286. Retrieved March 22, 2017, from Academic Search Complete.

Beáta, K. E., Anita, V., Julianna, T. J., Csilla, G., Miklós, T., M., C., . . . Csaba, V. (1970, January 01). Essential oils against bacterial biofilm formation and quorum sensing of food-borne pathogens and spoilage microorganisms. Retrieved March 19, 2017, from http://publicatio.bibl.u-szeged.hu/6469/

Brigmon, R. L., Berry, C., Singh, R. N., & Narayan, R. J. (2011, March). Applications of biotechnology in development of biomaterials: Nanotechnology and biofilms. *Journal of the South Carolina Academy of Science, 9*(1), 33-36. Retrieved March 20, 2017, from Academic Search Complete.

Campbell, N. A. (2012). Chapter 27: Bacteria and archaea. In *Campbell biology* (9th ed.). San Francisco, CA: Benjamin-Cummings.

CDC. (2016, November 14). Antibiotics aren't always the answer. Retrieved March 17, 2017, from https://www.cdc.gov/features/getsmart/

Chen, L., Todd, R., Kiehbaunch, J., Walters, M., & Kallen, A. (2016, September). *Notes from the field: Pan-resistant new delhi metallo-beta-lactamase-producing klebsiella Pneumoniae — Washoe County, Nevada, 2016* (United States, U.S. Department of Health and Human Services, Centers for Disease Control and Prevention). Retrieved March 17, 2017, from https://www.cdc.gov/mmwr/volumes/66/wr/mm6601a7.htm

Coffey, B. M., & Anderson, G. G. (2014). Biofilm formation in the 96-well microtiter plate. Retrieved March 19, 2017, from https://www.ncbi.nlm.nih.gov/pubmed/24818938

Costerton, J. W., Caldwell, D. E., Korber, D. R., Lewandowski, Z., & Lappin-Scott, H. M. (1995). Microbial biofilms. Retrieved March 19, 2017, from http://www.annualreviews.org/doi/pdf/10.1146/annurev.mi.49.100195.003431

Donlan, R. M. (2001, October 15). Biofilm formation: A clinically relevant microbiological process. *Clinical Infectious Diseases, 33*(8), 1387-1392. Retrieved March 20, 2017, from Academic Search Complete.

Donlan, R. M. (2002, September). Biofilms: Microbial life on surfaces. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2732559/

Ferreira, J. A., Penner, J. C., Moss, R. B., Haagensen, J. A., Clemons, K. V., Spormann, A. M., . . . Stevens, D. A. (2015, August 07). Inhibition of aspergillus fumigatus and its biofilm by pseudomonas aeruginosa is dependent on the Source, phenotype and growth Conditions of the bacterium. *PLoS ONE, 10*(8), 1-27. doi:10.1371/journal.pone.0134692

González, J. E., & Keshavan, N. D. (2006, December). Messing with bacterial quorum sensing. *Microbiology & Molecular Biology Reviews, 70*(4), 1-1. doi:10.1128/MMBR.00002-06

Gordya, N., Yakovlev, A., Kruglikova, A., Tulin, D., Potolitsina, E., Suborova, T., . . . Chernysh, S. (2017, March 09). Natural antimicrobial peptide complexes in the fighting of antibiotic resistant biofilms: Calliphora vicina medicinal maggots. *PLoS ONE, 12*(3), 1-19. doi:10.1371/journal.pone.0173559

Harjai, K., Gupta, R. K., & Sehgal, H. (2014, March). Attenuation of quorum sensing controlled virulence of pseudomonas aeruginosa by cranberry. *Indian Journal of Medical Research, 139*(3), 446-453. Retrieved March 20, 2017, from Academic Search Complete.

Hentzer, M., & Givskov, M. (2003, November 01). Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. Retrieved March 20, 2017, from http://www.jci.org/articles/view/20074

Hirakawa, H., & Tomita, H. (2013, May 13). Interference of bacterial cell-to-cell communication: A new concept of antimicrobial chemotherapy breaks antibiotic resistance. Retrieved March 20, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3652290/

Kalia, M., Yadav, V. K., Singh, P. K., Sharma, D., Pandey, H., Narvi, S. S., & Agarwal, V. (2015, August 11). Effect of cinnamon oil on quorum sensing-controlled virulence factors and biofilm formation in pseudomonas aeruginosa. *PLoS ONE, 10*(8), 1-18. doi:10.1371/journal.pone.0135495

Koh, C., Sam, C., Yin, W., Tan, L. Y., Krishnan, T., Chong, Y. M., & Chan, K. (2013, May). Plant-derived natural products as sources of anti-quorum sensing compounds. *Sensors (14248220),13*(5), 6217-6228. doi:10.3390/s130506217

Merritt, J. H., Kadouri, D. E., & O'Toole, G. A. (2005, July 6). Growing and analyzing static biofilms. Retrieved March 20, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4568995/

Monnet, V., & Gardan, R. (2015, July 15). Quorum-sensing regulators in gram-positive bacteria: 'cherchez le peptide. *Molecular Microbiology, 97*(2), 181-184. doi:10.1111/mmi.13060

O'Brien, S., Williams, D., Fothergill, J. L., Paterson, S., Winstanley, C., & Brockhurst, M. A. (2017, February 03). High virulence sub-populations in Pseudomonas aeruginosa long-term cystic fibrosis airway infections. *BMC Microbiology, 17*, 1-8. doi:10.1186/s12866-017-0941-6

Papenfort, K., & Bassler, B. L. (2016). Quorum sensing signal-response systems in Gram-negative bacteria. Retrieved March 20, 2017, from https://www.ncbi.nlm.nih.gov/pubmed/27510864

Perez, L., Costa, M., Freitas, A., & Barth, A. (2011, May 17). Evaluation of biofilm production by *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis and non-cystic fibrosis

patients. Retrieved March 20, 2017, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3769813/

Raffa, R. B., Iannuzzo, J. R., Levine, D. R., Saeid, K. K., Schwartz, R. C., Sucic, N. T., . . . Young, J. M. (2005, February 01). Bacterial communication ("quorum sensing") via ligands and receptors: A novel pharmacologic target for the design of antibiotic drugs. Retrieved March 19, 2017, from http://jpet.aspetjournals.org/content/312/2/417.long

Rasamiravaka, T., Vandeputte, O. M., Pottier, L., Huet, J., Rabemanantsoa, C., Kiendrebeogo, M., . . . Jaziri, M. E. (2015, July 17). Pseudomonas aeruginosa Biofilm Formation and Persistence, along with the Production of Quorum Sensing-Dependent Virulence Factors, Are Disrupted by a Triterpenoid Coumarate Ester Isolated from Dalbergia trichocarpa, a Tropical Legume. Retrieved March 27, 2017, from http://journals.plos.org/plosone/article?id=10.1371%2Fjournal.pone.0132791

Sifri, C. D. (2008, October 15). Quorum sensing: Bacteria talk sense. *Clinical Infectious Diseases,47*(8), 1070-1076. doi:10.1086/592072

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Ćirković, I., & Ruzicka, F. (2007, August). Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *Apmis, 115*(8), 891-899. doi:10.1111/j.1600-0463.2007.apm_630.x

Sukkar, E. (2013, November 13). Why are there so few antibiotics in the research and development pipeline? Retrieved March 17, 2017, from http://www.pharmaceutical-journal.com/news-and-analysis/features/why-are-there-so-few-antibiotics-in-the-research-and-development-pipeline/11130209.article

Taillefumier, T., & Wingreen, N. S. (2015, May). Optimal census by quorum sensing. *PLoS Computational Biology, 11*(5), 1-20. doi:10.1371/journal.pcbi.1004238

Tomlin, K. L., Coll, O. P., & Ceri, H. (2001, October). Interspecies biofilms of Pseudomonas aeruginosa and Burkholderia cepacia. *Canadian Journal of Microbiology, 47*(10), 949-954. Retrieved March 20, 2017, from Academic Search Complete.

United States, Centers for Disease Control and Prevention, Division of Healthcare Quality Promotion (DHQP). (2014, May 07). *Pseudomonas Aeruginosa in healthcare settings*. Retrieved March 17, 2017, from http://www.cdc.gov/hai/organisms/pseudomonas.html

Vital-Lopez, F. G., Reifman, J., & Wallqvist, A. (2015, October 02). Biofilm formation mechanisms of Pseudomonas aeruginosa predicted via genome-scale kinetic models of bacterial metabolism. *PLoS Computational Biology, 11*(10), 1-24. doi:10.1371/journal.pcbi.1004452

Waite, R. D., Qureshi, M. R., & Whiley, R. A. (2017, March 16). Modulation of behaviour and virulence of a high alginate expressing Pseudomonas aeruginosa strain from cystic fibrosis by oral commensal bacterium Streptococcus anginosus. *PLoS ONE, 12*(3), 1-18. doi:10.1371/journal.pone.0173741

Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., & Greenberg, E. P. (2008). Gene expression in Pseudomonas aeruginosa biolms. *NATURE, 413*, 860-864. Retrieved March 19, 2017, from http://www.ou.edu/microarray/oumcf/whiteleyetal.pdf

Wilder, C. N., Diggle, S. P., & Schuster, M. (2011, August). Cooperation and cheating in Pseudomonas aeruginosa: The roles of the las, rhl and pqs quorum-sensing systems. *ISME*

*Journal: Multidisciplinary Journal of Microbial Ecology, 5*(8), 1332-1343. doi:10.1038/ismej.2011.13

Zeng, Z., Qian, L., Cao, L., Tan, H., Huang, Y., Xue, X., . . . Zhou, S. (2008, May). Virtual screening for novel quorum sensing inhibitors to eradicate biofilm formation of Pseudomonas aeruginosa. *Applied Microbiology & Biotechnology, 79*(1), 119-126. doi:10.1007/s00253-008-1406-5

Zhang, L., Fritsch, M., Hammond, L., Landreville, R., Slatculescu, C., Colavita, A., & Mah, T. (2013, April 24). Identification of genes involved in Pseudomonas aeruginosa biofilm-specific resistance to antibiotics. Retrieved March 19, 2017, from http://journals.plos.org/plosone/article?id=10.1371%2Fjournal.pone.0061625

*Words Count: 4106*

# APPENDIX A

## Raw Data

Average light absorbance at 490 nm of Pseudomonas aeruginosa:

| Essential Oils (in nm) | (+) | 0.1% | 0.5% | 1% | 5% | 10% | 20% |
|---|---|---|---|---|---|---|---|
| Peppermint | 0.005 | 0.012 | 0.011 | 0.013 | 0.020 | 0.030 | 0.045 |
| Lemongrass | 0.005 | 0.002 | 0.027 | 0.088 | 0.056 | 0.034 | 0.021 |
| Roman Chamomile | 0.007 | 0.004 | 0.008 | 0.012 | 0.028 | 0.042 | 0.054 |

Light absorbance at 490 nm of Pseudomonas aeruginosa:

| Essential Oils | 0 | 0.1 | 0.5 | 1 | 5 | 10 | 20 |
|---|---|---|---|---|---|---|---|
| Peppermint | 0.005 | 0.01 | 0.011 | 0.012 | 0.018 | 0.028 | 0.045 |
| | 0.005 | 0.012 | 0.012 | 0.012 | 0.02 | 0.032 | 0.039 |
| | 0.006 | 0.015 | 0.011 | 0.016 | 0.022 | 0.029 | 0.05 |
| | | | | | | | |
| Lemongrass | 0.006 | 0.0002 | 0.027 | 0.085 | 0.056 | 0.034 | 0.023 |
| | 0.003 | 0.004 | 0.023 | 0.091 | 0.055 | 0.034 | 0.021 |
| | 0.007 | 0.003 | 0.032 | 0.087 | 0.057 | 0.034 | 0.019 |
| | | | | | | | |
| Roman Chamomile | 0.007 | 0.004 | 0.008 | 0.013 | 0.026 | 0.041 | 0.0504 |
| | 0.008 | 0.005 | 0.006 | 0.011 | 0.028 | 0.042 | 0.058 |
| | 0.006 | 0.004 | 0.01 | 0.011 | 0.03 | 0.043 | 0.055 |

Light absorbance at 630 nm of Pseudomonas aeruginosa:

| Essential Oil Concentration | 0 | 0.1 | 0.5 | 1 | 5 | 10 | 20 |
|---|---|---|---|---|---|---|---|
| Peppermint | 0.512 | 0.397 | 0.262 | 0.228 | 0.211 | 0.231 | 0.254 |
| | 0.465 | 0.302 | 0.211 | 0.191 | 0.192 | 0.211 | 0.241 |
| | 0.42 | 0.328 | 0.22 | 0.206 | 0.209 | 0.226 | 0.248 |
| Lemongrass | 0.423 | 0.313 | 0.359 | 0.26 | 0.249 | 0.225 | 0.289 |
| | 0.363 | 0.45 | 0.366 | 0.276 | 0.276 | 0.236 | 0.215 |
| | 0.364 | 0.331 | 0.392 | 0.334 | 0.288 | 0.212 | 0.208 |
| Roman Chamomile | 0.429 | 0.39 | 0.456 | 0.335 | 0.663 | 0.446 | 0.546 |
| | 0.467 | 0.407 | 0.562 | 0.36 | 0.335 | 0.442 | 0.401 |
| | 0.456 | 0.492 | 0.503 | 0.451 | 0.381 | 0.434 | 0.418 |