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THE EFFECTS OF CRUDE AND PURE GREEN TEA POLYPHENOLS ON
BACTERIA BIOFILM FORMATION AND ENDOSPORE GERMINATION

by

Hassan Tahir

A Master's Thesis Submitted to the Faculty of

Montclair State University

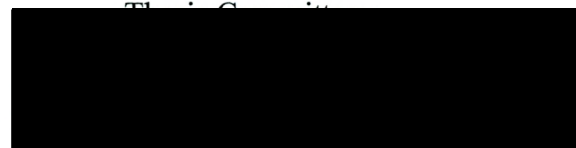
In Partial Fulfillment of the Requirements

For the Degree of

Master of Science August 2015

College of Science and Mathematics

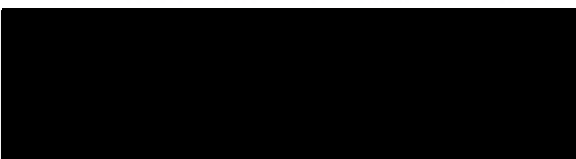
Biology and Molecular Biology



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BACTERIA BIOFILM FORMATION AND ENDOSPORE GERMINATION

A THESIS

Submitted in partial fulfillment of the requirements

For the degree of M.S. Molecular Biology

By

Hassan Tahir

Montclair State University

Montclair, NJ

August 2015

Abstract

Over the past couple of decades humans have been combating the problem of multi-drug resistant microorganisms. Multi-drug resistant strains of microorganisms have been causing not only problems for medical settings, but the world as well. Because of the continuous use of antibiotics within our time and age, there has been a huge increase in antibiotic resistant bacteria. In addition to the rise in multi-drug resistant bacteria over time, endospore cells have been causing increased levels of food-borne illnesses. Thus, there is a need for novel antimicrobial agents from nature derived products as an alternative way to treat and control these microorganisms. This specific study has been focused on the effects of green tea polyphenols (GTP) and a modified derivative, lipophilic tea polyphenol (LTP), Epigallocatechin Gallate (EGCG), and the modified derivative, Epigallocatechin Gallate – Stearate (EGCG-S) as antimicrobial agents against biofilm formation and endospore germination. The selected bacteria for this study were the gram negative bacteria *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* for the biofilm study and the endospore forming bacteria *Bacillus cereus*, *Bacillus megaterium*, and *Bacillus subtilis* for the endospore study. For the biofilm formation study, air-liquid interface assays, crystal violet assays, and Congo red assays were used to analyze the ability of the tested bacteria to form biofilm when treated with tea polyphenols. The results indicated that each of the tea polyphenols used in this research have strong inhibitory effects and LTP and EGCG-S had the strongest effects on inhibiting biofilm formation. Concentrations of 50 μ g/mL-100 μ g/mL were enough to inhibit biofilm formation from both bacteria. The fluorescent and scanning electron microscopic observations suggested that tea polyphenols affect the cell surface integrity, which was shown by damaged bacteria cells when treated. For the endospore germination study, colony-forming unit (CFU) studies and time kill studies indicated that concentrations of LTP and EGCG-S at 1% and 5% treated for 15 minutes and up to 2 hours were both able to inhibit endospore germination up to 100%. Transmission electron microscope images suggested that the inhibitory effect of EGCG-S might be due to its ability to damage the endospore coat and cause agglutination of endospores. These results suggest the potential beneficial effects of using each tea polyphenol to work as a nature derived compound to inhibit biofilm formation and endospore germination. It can be concluded from these results that each of the polyphenols, GTP, LTP, EGCG, and EGCG-S, can be used as potential antimicrobial agents.

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Introduction

1. Modern Threat from Antibiotic Resistant Bacteria

Recently within the modern era of microbiology there has been an increasing problem that has been affecting many food industries and medical facilities. This problem is the increase in microorganisms that are pathogenic and can cause severe human infections. These microorganisms are able to cause these severe human infections by contaminating many food industries and medical facilities. They are able to cause more and more diseases because they have become resistant to numerous antibiotics; making a treatment extremely difficult to find. By understanding how bacteria are rapidly evolving under specific antibiotic pressures, scientists can learn to control the development of these resistant organisms ^[4]. It was reported that in 2012 that there were 25,000 patients in Europe and 63,000 in America that died from multi-drug resistant bacterial infections ^[8]. Among these bacteria there are Vancomycin Resistant *Staphylococcus aureus* (VRSA), Multidrug Resistant *Staphylococcus aureus* (MRSA), and antibiotic resistant *Escherichia coli* (*E. coli*). Because bacteria like MRSA are becoming resistant to more and more drugs, due to the increased drug usage by humans, it has no longer been confined to typical intensive care units, acute care hospitals, and health care locations ^[28]. In addition to no longer being confined to specific locations, MRSA is becoming harder to treat. It has also been associated with increased mortality, increased health care and hospital costs, and longer stays for infected patients within hospitals ^[18]. These multidrug resistant bacteria are becoming extremely prominent because of their ability to evade

typical antibiotic treatments and are causing severe problems for humans and medical professionals. When bacteria start to become resistant to antibiotics they not only pose a huge threat to humans but they start to develop numerous defensive mechanisms to protect themselves. Virulence factors typically allow bacteria to become protected against a human cell's immune system, resulting in resistance ^[26]. In addition to variation in virulence factors and gene regulation, bacteria that are becoming multidrug resistant also start to up-regulate gene transcription for drug efflux mechanisms which contribute to the antibiotic resistance ^[25]. This up-regulation for genes involved in drug efflux proteins not only allows bacteria to start to develop a mechanism to move antibiotics or other drug compounds out of their body, but it allows them to persist and grow, spreading this defense mechanism.

The most prominent explanation as to how these multidrug resistant bacteria are rapidly increasing is the bacteria's ability to regenerate and replicate at relatively fast speeds ^[27]. Additionally, because a lot of modern medicine focuses on quick treatments for patients, antibiotics become prescribed continuously to patients. Over the past 5 years, dating back to 2010, antibiotic usage has shown a 75%-100% increase, further driving this antibiotic resistant era of bacteria ^[38].

Bacteria are able to easily gain resistance to drugs by genetic changes which occur from specific mutations which tend to become fixed within the evolved bacteria and makes them more deadly for patients that contract bacterial infections ^[11]. Because of all of these problems arising from the ability of bacteria to become resistant to multiple antibiotic drugs, a more recent therapeutic approach to targeting multiple drug resistant

bacteria includes focusing on compounds that are readily available to humans in nature. Since a more modern and nature derived approach is being taken, modern research focuses on using nature derived compounds to inhibit bacterial growth and decrease their abilities to cause diseases ^[24].

2. Nature Derived Products and Green Tea Polyphenols.

Several nature-derived products provided to humans are typically present within various nature inhabitants, such as the leaves of trees from natural environments. For centuries nature-derived products that can potentially work to kill multiple drug resistant bacteria have been available to combat bacteria. Focus should be put towards these nature-derived products to study their effects on bacteria that are drug resistant. Previous research has shown that the antimicrobial activity of compounds, such as essential oils derived from certain plants depends on the total content of specific compounds such as carvacrol ^[15]. Additionally, nature derived products are not toxic, easily accessible and modified, making them the most attractive option to be potentially used as an alternative treatment to combat multiple drug resistant bacteria.

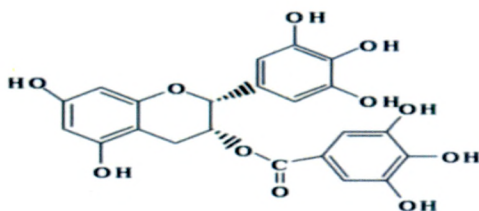
Among these compounds are compounds derived from plants known as polyphenols. Plant polyphenols have been studied for decades for their effects in environments on specific bacteria such as *Enterobacteriaceae* ^[7]. Polyphenols can be found within numerous types of plants and liquid beverages which typically range in concentration from 200-350mg per serving of beverage. ^[13], depending on plant type and processing style of the plant ^[21]. In addition to polyphenols other compounds that can be

found in plants are known as tannins ^[41]. These are nature-derived compounds that exhibit similar effects to catechins. Catechins are a subgroup of flavonoids and are also natural phenols which are classified as antioxidants ^[50]. It is suggested that along with catechins, there are numerous secondary metabolites that plants have developed over centuries as an evolutionary defense mechanism against herbivores and microorganisms, as well as a signaling compound to attract animals for pollen dispersal ^[43]. Since plants were able to develop these metabolites as a defense against microbes, their application could potentially be used to help fight the battle of multidrug resistant bacteria and other potentially pathogenic and harmful microorganisms ^[2]

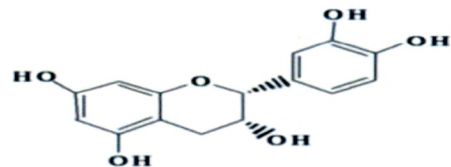
Antimicrobial secondary metabolites are present in the green tea plant, *Camillia sinensis* ^[13]. On average green tea has been shown to contain approximately 0.9 grams of total catechins within an average 8-ounce cup ^[20]. This indicates that within green tea there are typically high concentrations of catechins because of the evolutionary adaptation required of these plants to survive. In addition to the antimicrobial properties demonstrated by these catechins it has also been reported that they have been detected within the plasma membranes of human cells post consumption ^[32]. This signifies that upon consumption of tea catechins, the compounds tend to become absorbed within human cells and potentially work within the cells. Major catechins were classified as compounds known as Epi-Catechin (EC), Epigallocatechin (EGC) Epigallocatechin-Gallate (EGCG), and Epicatechin-3-Gallate (ECG)^[29]. The structures of these compounds are shown in Figure 1. These are the catechins that will be part of the study to look at their effects on biofilm formation and endospore germination. These catechins have demonstrated numerous pharmacological activities including antimicrobial, anti-

carcinogenic, anti-inflammatory, and antioxidant [47]. *Camillia sinensis* is classified as a green tea plant. In addition to green tea there are also oolong and black teas. The difference among these different types of teas is that black tea is completely fermented, oolong tea is semi-fermented, and green tea is not fermented [12]. The fermentation process leads to a change within the levels of tea polyphenols in each of the tea extracts. When tea is subjected to longer periods of fermentation, it becomes less potent in tea polyphenols. Because of this high concentration in tea polyphenols, green tea leaves have been used throughout history for numerous medical applications. Its potential medical applications includes its ability to inhibit particular viral replication such as HIV-1 by blocking HIV-1's attachment abilities to the glycoprotein gp120, on CD4 regions on white blood cells [51]. It was also reported that pure green tea polyphenols can inhibit HSV infection by blocking the adsorption of virus onto the host cell [32].

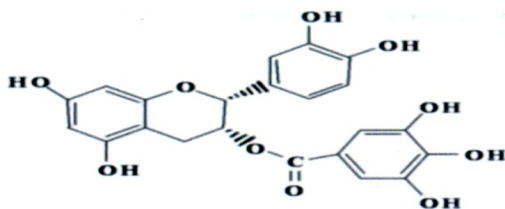
(-)-Epigallocatechin-3-gallate (EGCG)



(-)-Epicatechin (EC)



(-)-Epicatechin-3-gallate (ECG)



(-)-Epigallocatechin (EGC)

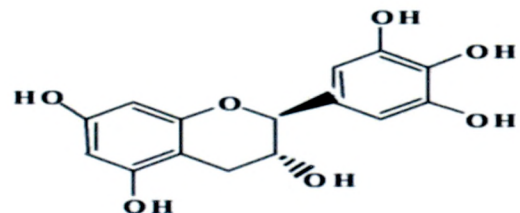


Figure 1: EGCG, EC, ECG, and EGC Molecular Structures [29].

In addition to working against some viruses these compounds also function to inhibit bacterial infections such as gastric cancer caused by *Helicobacter pylori* [38]. Based on its high concentration of catechins, green tea must be further evaluated and studied.

Although tea polyphenols derived from green tea, known as green tea polyphenols (GTP), demonstrate numerous beneficial properties, they tend to not be very stable within solution. Thus, these GTP's are chemically modified into a stable form, known as lipophilic tea polyphenols (LTP), GTP which has undergone esterification. [5]. In addition to this esterification process performed on GTP, EGCG also undergoes an esterification process to convert EGCG into EGCG-Stearate (Figure 2) to expand upon its applications so it is has enhanced cellular absorption and it becomes dissolvable within lipophilic media [51].

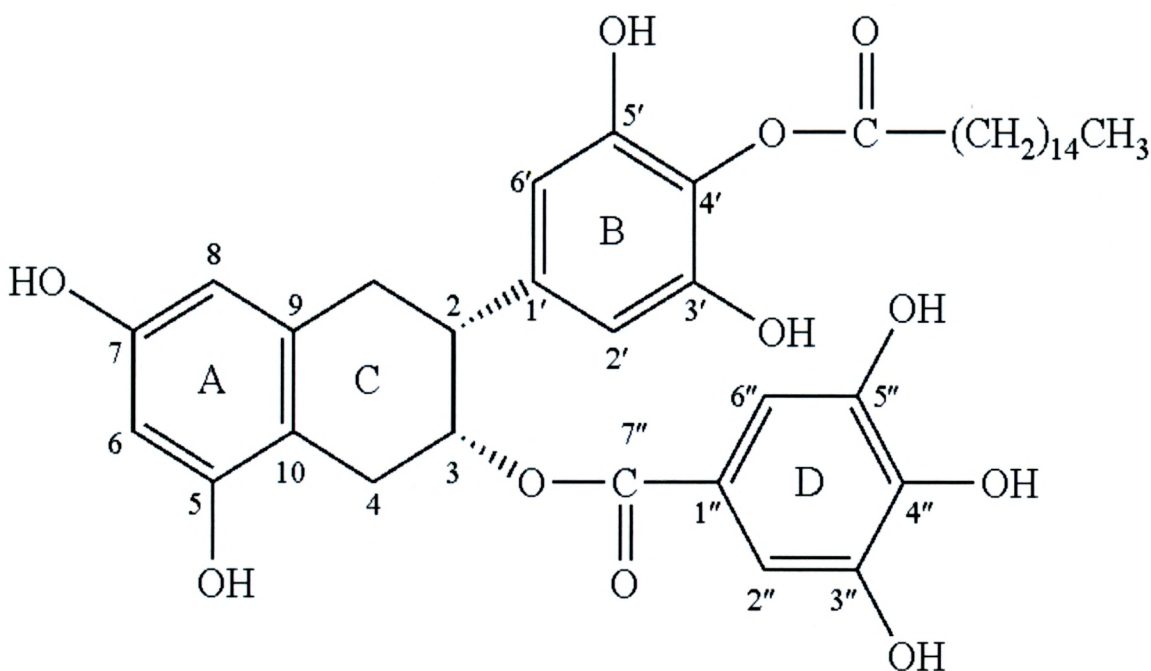


Figure 2: EGCG-Stearate Molecular Structure [22]

As seen by the structural composition in Figures 1 and 2 of each of these catechins, there are numerous hydroxyl (-OH) groups and aromatic rings attached and placed throughout the structure. Thus, when applying these compounds to bacteria they can potentially affect cellular functions because of their high concentration of hydroxyl groups. This high concentration of hydroxyl groups mimics those seen on β -lactam antibiotics. Previous research indicated that the potent tea polyphenols, EGCG and ECG, are able to disrupt pathogenic bacteria's membranes and they bind better to Gram positive bacteria in comparison to Gram negative bacteria [3]. In addition, it has been seen that EGCG is more effective on susceptible Gram positive bacteria than Gram negative bacteria because of the differences in the peptidoglycan layer's composition [6]. It is also reported that the crude tea polyphenols (GTP and LTP) have also shown some synergistic effects with certain antibiotics [17].

3. Microorganisms of Study

Selected bacteria for this study are *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Pseudomonas fluorescens* (*P. fluorescens*). *P. aeruginosa* is a bacillus (rod) shaped gram negative bacterium, a prevalent and opportunistic pathogen that can easily cause harm in human hosts if the proper conditions are present. *P. aeruginosa* is present within the normal flora of human's skin, large and small intestines, and lungs. *P. fluorescens* is also a bacillus (rod) shaped gram-negative bacterium. [46]. Thus, studying these bacteria will help to not only find ways to treat these bacteria, but also how to potentially reduce the number of bacteria that come in contact with humans.

Gram-negative bacteria are grouped based on several physiological characteristics that make them different from gram positive bacteria. These physiological characteristics include a thin peptidoglycan layer, inability to retain crystal violet dye, pink color post gram stain, and high lipopolysaccharide content (LPS) ^[44]. Bacteria are able to create biofilm when they are subjected to specific conditions. *P. aeruginosa* is related to food spoilage and biofilm formation, as well as wound infections ^[49]. *P. fluorescens* is also able to form biofilm, related to food spoilage, and able to fluoresce ^[46].

4. Potential Harm From Biofilm

Biofilm formation is a specific type of microbial mechanism that makes controlling bacteria extremely difficult. Biofilm form when bacterial cells develop a thin organized array of cells on top of either an inert or living surface and they tend to stay attached to that surface by adhering to it. Biofilm is known as extracellular polymeric substance (EPS), a bacterial secretion able to potentially grow as protection against external harsh conditions and medical treatments ^[19]. Within EPS there are bacterial DNA, proteins, carbohydrates, and other substances that provide structural and mechanical stability and protection for the bacteria cells within. The developed EPS matrix also works to provide extracellular enzymes within close proximity to bacteria cells to allow them to thrive inside the EPS layer ^[10]. This biofilm formation typically becomes extremely hard for an antibiotic to work against because antibiotics cannot easily penetrate the biofilm layer. Underneath this biofilm structure cells are constantly undergoing physiological changes by adhering to surfaces as well as adhering to other bacterial cells. Since biofilms are extremely difficult to treat, new techniques and

advances have been aimed towards nature-derived products such as tea polyphenols as an alternative treatment with promising results ^[10]. *P. aeruginosa* and *P. fluorescens* are biofilm forming bacteria that easily form thick layers of biofilm after only 24 hours ^[10]. This makes treating these bacteria a significant problem. Biofilm formation poses a risk for humans because there are numerous points where bacteria can enter the body, infect, and start to develop biofilm. These infections typically end up occurring in areas where prosthetic-related surgeries and joint replacement surgeries occurred, causing prosthesis-related infections ^[37]. When these regions become infected they are extremely difficult to treat with antibiotic therapy because of the location of the infections as well as the development of biofilm around the area. Thus, the potential use of tea polyphenols within medical settings should be studied to determine potential methods to reduce the incidence of infections and provide a better patient outcome following medical surgeries.

5. Identification and Characterization of Biofilm Growth in Bacteria

To study the possible ways that the tea polyphenols could work against biofilm formation, various assays were used to evaluate the biofilm formed by both bacterial species and to evaluate the effects of the tea polyphenols on formation of biofilm. It has been shown that bacteria biofilm formation grows very well at an air-liquid interface by allowing bacteria access to both media and space to grow biofilm. Numerous aerobic bacteria are able to grow at this air-liquid interface because they form multicellular communities known as pellicles by using their flagella to attach to the surface and grow ^[48]. The protocols have been established for studying biofilm from air-liquid interface as

described in Yamamoto et al. [48]. Thus in this research, an initial air-liquid interface assay was performed to study the growth of biofilm at an air-liquid interface on glass microscope slides and to study the effects of tea polyphenols in general on biofilm growth from *P. aeruginosa*.

It has been reported that Congo red agar assays can detect the presence and absence of biofilm in an environment. When bacteria biofilm formation occurs, the EPS secretes amyloid, which binds to the Congo red dye in the agar turning the plate or well from a blood red to a dark black precipitate [36]. The protocols and preparation of Congo red media were described in Shwartz et al. [36]. This is a preliminary assay for detection of biofilm in a qualitative manner. In addition to the Congo red agar and air-liquid interface assays, the crystal violet assay was also a standard assay to further quantitatively study the bacterial biofilm. The crystal violet assay is a way of separating out planktonic free floating bacterial cells to analyze strictly biofilm growth. This assay focuses on measuring the presence of biofilm cells by staining them separately from planktonic cells within a 24 well plate used, as described within the materials and methods [31]. These three methods, with some modifications, were used to carry out experiments to qualitatively and quantitatively measure biofilm growth inhibition with tea polyphenols.

BacLight stain is a fluorescent stain to characterize the cell's integrity. If the bacteria's cell surface is damaged, it shows red (propidium iodide) and if the cell surface is intact it shows green (SYTO 9) [42]. This live and dead fluoresce assay is useful to study the effect of tea polyphenols on the bacterial integrity. This assay was performed to explore the potential mechanism of the tea polyphenols working on the cell membrane of

bacteria cells. If tea polyphenols affect the cell membranes' integrity, then this stain would help view this and further elicit a mechanism as to how exactly the polyphenols are working against the production of biofilm and its growth.

6. Potential Harm from Endospores

In addition to the difficulty of trying to treat biofilm forming bacteria, there are bacteria that can easily form a protective cell state known as endospore. This is typically a state for a bacterial cell where it is protected within the cell by a spore coat. Cells enter the endospore state when they are subjected to harsh conditions such as extreme temperatures, chemicals or radiation. Once cells enter the endospore state they become protected from external factors. They are considered the most resistant living structures known because they have a high degree of resistance to heat and ultraviolet (UV) radiation ^[1]. Endospore poisoning from the genus *Bacillus* has been a huge problem within the food industry for decades because of the numerous food-borne diseases caused by the species within this genus ^[16]. General food processing with heat or radiation does not kill endospores and when the conditions favor, endospores will reactivate and germinate into vegetative cells. Vegetative cells are cells that are able to cause an infection and lead to a disease within people. This is mostly correlated with food poisoning because they are active, continuously dividing, and toxin secreting cells at this stage compared to the endospore stage ^[14]. In addition to the heat resistance, they are also antibiotic resistant; treating endospores is relatively difficult and requires more than antibiotics. Thus, modern advances should be pushing towards the use of nature derived

products such as tea polyphenols to combat endospores. Endospore cells have a relatively well developed spore coat which acts as a protective layer when these cells enter endospore form. This spore coat is made up of a proteinaceous layered "shield" which function as a barrier to allow certain compounds into the spore and block others out ^[53]. In addition, the endospore coat has been shown to hold numerous enzymes which have a direct role in both germination and detoxification ^[53]. Because of these spore related properties, treating endospores with compounds becomes relatively difficult and at times impossible. Thus, research aimed towards determining if endospores are susceptible to tea polyphenols must be performed. This research focused on utilizing green tea polyphenols and viewing their effects on endospore cells.

7. Genetic Regulation of Biofilm and Endospores.

When bacteria start biofilm formation they undergo genetic changes as well as physiological changes. Genetic changes that occur typically include activation of genes which promote the initial biofilm EPS formation, such as quorum sensing genes and 16S RNA genes ^[23]. In addition, other genes become easily unregulated, like the formation of pili to assist the bacteria to adhere to surfaces ^[23]. Other genes which play a role in biofilm development have been traced to those which aid in the formation of pili for bacteria ^[21]. One crucial operon known as the pel operon has been shown to also be essential for the formation of the glucose-rich matrix EPS ^[35].

Bacteria start to undergo physical changes and undergo numerous genetic changes turning them into endospores ,thus, they are dormant and not continuously undergoing

reproduction. It has been seen that sporulation within some species from the *Bacillus* genus undergo morphological and gene up regulation and down regulation when entering the endospore state. In addition to these changes a reason this occurs is because when vegetative cells start to become endospores they tend to be transcriptionally controlled by a new sigma factor known as sigma factor σ^E . This sigma factor gets transcribed by the *sigE* gene and can act as an inactive precursor to allow proper sporulation to occur [39]. Studying the effects of green tea polyphenols on both biofilm and endospore gene regulation can elucidate the mechanisms of tea polyphenols on their mode of action in inhibition of biofilm formation and spores germination.

8. Objectives of Study

With the problem of multidrug resistant, biofilm forming, and endospore forming bacteria, treatments to combat these bacteria are the major focus of this study. The objectives of this research are as follows:

1. Study and characterize the biofilm development in *P. aeruginosa*;
2. Analyze the effects of crude green tea polyphenols (GTP, LTP) and purified tea polyphenols (EGCG , EGCG-stearate) on the biofilm forming *P. aeruginosa* and *P. fluorescens*;
3. Observe the population density and biofilm forming properties through compound microscope, fluorescence microscope, and scanning electron microscope;
4. Study endospore germination and the effects of tea polyphenols on endospore germination to develop potential usage of tea polyphenols as a food preservative.

Materials and Methods

1. **Culturing Bacteria**

- a. Throughout the study each microorganism was constantly maintained and tested for purity prior to beginning each experiment. The bacteria strains were *P. aeruginosa* and *P. fluorescens*, Gram-negative bacteria. Each culture was kept within either Mueller Hinton agar plates, nutrient agar plates, Mueller Hinton broth, Tryptic Soy Broth, or Tryptic Soy Broth supplemented with 1-2% Sucrose. All broths and plates were sterilized prior to usage. Overnight cultures were prepared from stocks that were maintained at 4°C. The microorganisms were grown overnight prior to every experiment in a shaker at 37°C and 250rpm.
- b. Microorganisms were also stored in a -80°C freezer. To keep a frozen stock, the microorganisms were stored in 30% glycerol (v/v) solution). This was prepared by mixing 300µL of 30% glycerol to an autoclaved screw cap centrifuge tube along with 700µL of microorganism stock. Each of the tubes was placed into a microcentrifuge tube holder and placed in the -80°C freezer for storage.

2. **Media Preparation**

- a. All broths and plates; Mueller Hinton broth, Tryptic Soy Broth, and Tryptic Soy Broth supplemented with 1-2% Sucrose, Mueller Hinton, agar plates, and Nutrient agar plates (Difco) were prepared according to the instructions provided by the manufacture. To prepare Mueller Hinton broth and plates, thoroughly mix 38 g of the powder provided into 1 liter (L) of deionized water. When contents are thoroughly dissolved, autoclave the mixture at 121°C for 15 to 20 minutes. After

autoclaving, cool the medium for 30 minutes and then pipette 6 mL of broth into sterile culture tubes. For the Mueller Hinton agar plates, after autoclaving, the media was poured onto sterile agar plates and allowed to cool. The broth and agar plates were stored in a 4°C refrigerator until needed. Tryptic Soy Broth (TSB) was made by mixing 30 g of broth powder into 1 L of deionized water. The preparation procedure is the same as above. Tryptic Soy Broth and plates, supplemented with 1-2% sucrose, were made by mixing 30 g of Tryptic Soy Broth or Tryptic Soy Agar into 1 L of deionized water along with 10 - 20 g of sucrose. The preparation procedure is the same as the one provided above.

- b. Congo Red Agar was made by following the procedure outlined from the research performed by Schwartz et al. ^[34] which included first mixing 9.25 g of Brain Heart Infusion (BHF) broth powder with 12.5 g of sucrose, 5 g of bacto agar and 250mL of deionized water in a 500 mL flask and mix thoroughly on a hot plate until all ingredients were mixed, clear, and boiling. Congo red mixture was made using 0.4 g of Congo red powder and 10mL of deionized water (50X) ^[34]. Both preparations were autoclaved and set aside to cool to 55-60° C. Then 5 mL of the Congo red mixture was added to the broth and mixed well. After mixing both contents thoroughly and adding them into either 6 well plates (2 mL), 24 well plates (1 mL), or petri dish plates, they were placed aside and allowed to cool until solidification. Congo red media was made freshly before use in each experiment.

3. **Preparation of Green Tea Polyphenols**

All the green tea polyphenols, GTP, EGCG, LTP and EGCG-S were purchased from Camellix LLC, Augusta, GA.

- a. Both GTP and EGCG were prepared using deionized water. Based on the concentration required, the stock solution was prepared accordingly by adding the necessary amount of extracted powder to deionized water in a sterile microcentrifuge tube or conical tubes. The solution formed was then vortexed for 1-2 minutes at maximum speed to allow all contents to dissolve to form a clear solution. Concentrations of stock solutions, based on the experiment, are described within the results.
- b. Both LTP and EGCG-S were prepared using 200 proof ethanol. The stock solution was prepared based on the concentration required for the experiment. The necessary amount of tea polyphenol powder was added to 200 proof ethanol in a sterile microcentrifuge tube or conical tubes. The solution formed was then vortexed for 1-2 minutes at maximum speed to allow all contents to dissolve to form a clear solution. Based on the experiment, detailed stock solutions are described within the results.

4. **Overnight Broth and Agar Bacterial Culture Preparation**

- a. To prepare an overnight broth culture of bacteria initial bacteria used for overnight culture and sterile culture broth tubes were removed from 4° C and placed at room temperature for 0.5 to 1 hour. Each broth culture tube was taken and labeled according to date and bacterial name. Aseptically, bacteria were taken

from a freshly flamed culture tube or an agar plate slide, using a sterile cotton swab. The swab was placed in a culture tube that had been aseptically flamed upon opening. The swab was swirled around within the broth culture tube and aseptically flamed and closed. This tube was placed within either a 37°C incubator shaker set to 250 rpm or a 37°C incubator for 24 hours. This entire procedure was performed using aseptic technique.

- b. To prepare an overnight agar plate of bacteria, initial bacteria used for agar plate was swabbed on the agar plate aseptically.

5. **Monitoring the growth of *Pseudomonas aeruginosa***

- a. The overnight cultured bacteria was initially taken and diluted to an optical density (OD) of 0.1 in its respective media and the OD was taken at 600nm absorbance. This absorbance reading was used as the initial start time reading. The diluted bacteria were then placed in a shaking incubator set to 37°C and 250 rpm. The growth was monitored every hour for a period of 15 hours to obtain the OD reading at 600nm. The readings were repeated three times and a growth curve was generated for *P.aeruginosa* using the mean and standard déviation (SD).

6. ***Pseudomonas aeruginosa* Air-Liquid Interface Study for Bacterial Biofilm Production**

- a. Since bacteria are present as biofilm at the air-liquid interface ^[46], the air-liquid interface was studied to view biofilm. Sterile microscope slides were placed vertically in sterile 50 mL conical tubes. Overnight cultures were prepared and

diluted in Tryptic Soy Broth (TSB) to an OD₆₀₀ of 0.1. After diluting the bacteria, each tea polyphenol used for the experiment was prepared in a master stock as described previously. This consisted of mixing 1g of the compound being tested within 1mL of either deionized water or 200 proof ethanol to create a master stock of 1g/mL. This was performed on the day of every experiment to ensure all tea polyphenols were freshly prepared for that experiment. For this experiment the tea polyphenols were prepared within percentages based on weight/volume ratios. Both 0.5% and 1% of the tea polyphenols were prepared by taking a master stock and diluting it within broth. After properly preparing all tea polyphenols, five 50 mL conical tubes were taken and labeled accordingly; Control, 0.5% GTP, 1% GTP, 0.5% LTP, and 1% LTP. Afterwards a sterile microscope slide was placed vertically inside each tube with 24 mL of the diluted bacteria mixture. From the master stock, 1mL was taken and added to the corresponding labeled tubes. Each tube was then placed in a shaking incubator set to 37°C and 250 rpm for a period of 24 hours. After 24 hours, a simple stain was performed on each slide at the air liquid interface area and cover slips were added to each slide. Finally, images were taken using ZEISS Axioscope (Axiovision 3.0) microscope equipped with a camera at 1,000x magnification. Images were specifically taken at the area of the air-liquid interface, the location where the liquid inside the conical tube meets the air. This experiment was carried out under aseptic conditions.

7. **The Effects of GTP and LTP on *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* Biofilm Formation by Analyzing the Population Density on a Glass Surface**

- a. Overnight cultures of both bacteria species were prepared the day prior to starting this experiment. Both bacteria from the overnight cultures were taken and diluted in TSB to an OD₆₀₀ of 0.1. After diluting each bacterial culture, each tea polyphenol used for the experiment was prepared in a master stock. The diluted overnight bacteria cultures were plated within 6 well plates. First each well within a 6 well plate was labeled and positive and negative controls were added as well for each type of bacteria. Then 300 µL of bacteria were added to each well that required bacteria. Each master stock of tea polyphenols was added to reach the desired concentration of treatment. The rest of the well was filled with Tryptic Soy Broth to a total volume of 2mL as seen in Table 1 below. After all proper media, bacteria, and treatment were added to each well, a sterile cover slip was then added. Plates were then taped shut and placed in a shaker at 85 rpm at room temperature for 5 days. After 5 days, the plates were removed from the shaker and a simple stain was performed on each coverslip in each well. After performing a simple stain the cover slips were placed on top of a sterile microscope slide inverted. Images of each slide were taken using a ZEISS Axioscope (Axiovision 3.0) microscope equipped with a camera; at 100x, 400x, and 1000x oil. This was done for both *P. aeruginosa* and *P. fluorescens*. The entire experiment was performed aseptically.

6 well Plate (Time = 5 Days) Slides (Microscopic observation)

1.7 mL Broth 300 μ l <i>P. aeruginosa</i> 100 μ l of DI water sterile cover-slip	1.6 mL Broth 300 μ l <i>P. aeruginosa</i> 100 μ l of 60-EGCG stock sterile cover-slip	1.6 mL Broth 300 μ l <i>P. aeruginosa</i> 100 μ l of 15-EGCG stock sterile cover-slip
1.7 mL Broth 300 μ l <i>P. aeruginosa</i> 100 μ l of ETOH sterile cover-slip	1.6 mL Broth 300 μ l <i>P. fluorescens</i> 100 μ l of 100-EGCG-S stock sterile cover-slip	1.6 mL Broth 300 μ l <i>P. fluorescens</i> 100 μ l of 25-EGCG-S stock sterile cover-slip

Table 1: 6 Well plate templates for preparation for microscopic observation.

8 Congo Red Agar Assay for Bacterial Biofilm Formation from *Pseudomonas aeruginosa*

- a. Because Congo red agar assays are quick and essential assays to determine if a certain bacteria forms biofilm^[36] this assay is a crucial first experiment for biofilm formation. Overnight cultures of both bacteria were produced and diluted in Tryptic Soy Broth, similar to previous experiments. Each tea polyphenol was prepared as a master stock of 1mg/mL, similar to previous experiments. Each treatment for the experiment was combined with the diluted bacteria within sterile 1.5mL microcentrifuge tubes and incubated at 37°C for 1 hour with one tube only containing bacteria as a positive control and one containing only broth for a

negative control. After 1 hour of incubation, 500 μL of the cultures were added to the Congo red agar in the 6 well plates. The cultures were evenly spread over the agar using a sterile loop. After insuring the solution was dry on the surface of the Congo red plates, the plates were incubated at 37°C for 24 hours. After an incubation period of 24 hours, the color of the plates was observed and images were taken of both the top and bottom of the agar. The experiment was performed under aseptic conditions.

9 Dose-Dependent Congo Red Agar Assay for Biofilm Production and Biofilm Reversal Assay

- a. Overnight cultures of the bacteria were produced and diluted within Tryptic Soy Broth, similar to previous experiments. Each tea polyphenol was prepared as a master stock of $1\text{mg}/\text{mL}$, similar to previous experiments. Different volumes of bacteria culture ($5\ \mu\text{L}$, $10\ \mu\text{L}$, $15\ \mu\text{L}$, $20\ \mu\text{L}$, $25\ \mu\text{L}$, and $50\ \mu\text{L}$, respectively) were pipetted into each well of a six well plate. After pipetting each volume within its respective well in the Congo red agar plates, the cultures were allowed to dry on the surface of the agar. After the cultures had dried on the surface of each well, the plates were sealed and incubated at 37°C for 24 hours. After an incubation period of 24 hours, the plates were observed, and images were taken of both the top and bottom of the plates. For the reverse biofilm assay, $500\ \mu\text{L}$ of $250\ \mu\text{g}/\text{mL}$ of EGCG-S were added to each well and allowed to dry. After the solution had dried completely the plates were sealed and incubated at 37°C for 24 hours. After

an incubation period of 24 hours, images of both the top and bottom of the plates were taken. This experiment was carried out under aseptic conditions.

10. Crystal Violet Assay with GTP, LTP, EGCG, and EGCG-S

- a. Crystal violet assays are experiments which can be performed to view the effects that tea polyphenols have on only biofilm growth from bacteria ^[32]. Overnight cultures of the bacteria were produced and diluted within Tryptic Soy Broth, similar to previous experiments. Each tea polyphenol was prepared as a master stock of 1mg/mL, similar to previous experiments. After properly labeling the 24 well plates, 800 μ L of TSB and 600 μ L of bacteria were added to each well. Afterwards, the tea polyphenols were added to each well with specific concentrations of 25, 50, 100, and 200 μ g/mL as seen in Table 3. The plates incubated at 37°C for 5 days. Following incubation, the cell suspension within the wells was aspirated and the wells were washed once with PBS, aspirated and air-dried. After aspiration and wash, 2mL of 0.1% w/v crystal violet stain was added into each well and allowed to sit for 20 minutes. After 20 minutes the fluid was aspirated again and a quick wash with deionized water was performed. After washing, the fluids within the wells were aspirated again and the plates were inverted to dry for at least 15-20 minutes to overnight. Images were taken and 2 mL of 30% acetic acid was added to each well. Finally each wells' content was measured for absorbance using a spectrophotometer set to a wavelength of 595 nm.

Crystal Violet Assay in 6 Well Plates for *P. aeruginosa* and *P. fluorescens*

1.7 mL Broth 300 µl <i>P. aeruginosa</i> 100 µl of DI water	1.6 mL Broth 300 µl <i>P. aeruginosa</i> 100 µl of 60µg/mL EGCG stock	1.6 mL Broth 300 µl <i>P. aeruginosa</i> 100 µl of 15 µg/mL EGCG stock
1.7 mL Broth 300uL <i>P. aeruginosa</i> 100 µl of ETOH	1.6 mL Broth 300uL <i>P. fluorescens</i> 100 µl of 100 µg/mL - EGCG-S stock	1.6 mL Broth 300 µl <i>P. fluorescens</i> 100 µl of 25 µg/mL - EGCG-S stock

Table 2: 6 Well plate templates for Crystal Violet Assay

Crystal Violet Assay in 24 Well Plates for *P. aeruginosa* and *P. fluorescens*

Positive Control 800 broth 600uL Bacteria Only + 200 ul ETOH	Positive Control 800 broth 600uL Bacteria Only + 200 ul ETOH	Positive Control 800 broth 600uL Bacteria Only + 200 ul ETOH	Blank	Negative Control 800 broth 600uL GTP Stock	Negative Control 800 broth 600uL LTP Stock	Negative Control 800 broth 600uL EGCG Stock	Negative Control 800 broth 600uL EGCG-S Stock
800 broth 600uL Bacteria 25 ul of stock + 175 ul of ETOH 25ug/mL GTP	800 broth 600uL Bacteria 50ul of stock + 150 ul of ETOH 50ug/mL GTP	800 broth 600uL Bacteria 100ul of stock + 100 ul of ETOH 100ug/m L GTP	800 broth 600uL Bacteria Take 200 ul of stock 200ug/ mL GTP	800 broth 600uL Bacteria 25 ul of stock + 175 ul of ETOH 25 ug/mL LTP	800 broth 600uL Bacteria 50ul of stock + 150 ul of ETOH 50 ug/mL LTP	800 broth 600uL Bacteria 100ul of stock + 100 ul of ETOH 100ug/mL LTP	800 broth 600uL Bacteria Take 200 ul of stock 200ug/m L LTP
800 broth 600uL Bacteria 25 ul of stock + 175 ul of ETOH 25ug/mL EGCG	800 broth 600uL Bacteria 200ul of stock +150 ul of ETOH 50ug/mL EGCG	800 broth 600uL Bacteria 100ul of stock + 100 ul of ETOH 100ug/m L EGCG	800 broth 600uL Bacteria Take 100 ul of stock 200ug/ mL EGCG	800 broth 600uL Bacteria 25 ul of stock + 175 ul of ETOH 25ug/mL EGCG-S	800 broth 600uL Bacteria 50ul of stock + 150 ul of ETOH 50ug/mL EGCG-S	800 broth 600uL Bacteria 100ul of stock + 100 ul of ETOH 100ug/m L EGCG-S	800 broth 600uL Bacteria Take 200 ul of stock 200ug/m L EGCG-S
Postive control_2 800 broth 600 ul of cells + 200 ul of broth	Postive control_2 800 broth 600 ul of cells + 200 ul of broth	Postive control_2 800 broth 600 ul of cells + 200 ul of broth					

Table 3: 24 Well Template for Crystal Violet Assay

11. **BacLight Live and Dead Assay**

- a. A predicted mechanism as to how the tea polyphenols were effecting bacteria cells dealt with potential damage occurring to bacteria cell membranes. Thus, a BacLight live and dead assay was performed to analyze the effects of tea polyphenols on the cell membrane ^[42]. The experiment set up is the same as previously described in section 10 except no coverslip was added to the wells. The Live and Dead BacLight (Life Technologies, Carlsbad, CA) fluorescence stains were prepared using SYTO 9 and propidium iodide powder according to the manufacturers' protocol. The dyes were prepared as 2X stock solutions then diluted with deionized water to a final concentration of 15 μM for SYTO 9 and 30 μM for propidium iodide. To stain the cells, equal amounts of bacteria and mixed dye were added into a sterile 1.5mL micro centrifuge tube and allowed to incubate at room temperature in the dark for a period of 15 minutes. After 15 minutes, 5 to 10 μL of the stained suspension was taken and added to a sterile microscope slide and a sterile cover glass slip was added on top. Finally, the slides were viewed under a ZEISS Axioscope (Axiovision 3.0) fluorescent microscope equipped with a camera.

Live and Dead BacLight assay on *P. aeruginosa* and *P. fluorescens*

1.7 mL Broth 300 μ L <i>P. aeruginosa</i> 100 μ L of DI water	1.6 mL Broth 300 μ L <i>P. aeruginosa</i> . 100 μ L of 60 μ g/mL-EGCG stock	1.6 mL Broth 300 μ L <i>P. aeruginosa</i> 100 μ L of 15 μ g/mL-EGCG stock
1.7 mL Broth 300 μ L <i>P. aeruginosa</i> 100 μ L of ETOH	1.6 mL Broth 300 μ L <i>P. fluorescens</i> 100 μ L of 100 μ g/mL - EGCG-S stock	1.6 mL Broth 300 μ L <i>P. fluorescens</i> 100 μ L of 25 μ g/mL - EGCG-S stock

Table 4: 6 Well plate templates for BacLight Live and Dead Fluorescence Study

12. Scanning Electron Microscopy (SEM)

- a. Overnight cultures of the bacteria were prepared and diluted in Tryptic Soy Broth, similar to previous experiments. Each tea polyphenol was prepared as a master stock of 1mg/mL, similar to previous experiments. Bacteria were then treated using 50 μ g/mL and 100 μ g/mL of LTP and EGCG-S. Specific cover slips purchased from neuVitro (Vancouver, WA) were used for Scanning electron microscopy (SEM) imaging. Cover slips were put in the well containing treated or untreated bacteria and incubated in different time periods according to the experimental design (from 6 hours to 60 hours) at 37°C. A long-term incubation experiment with 5 days was also carried out to look at the morphology and the biofilm of the samples.

The preparation of SEM samples is described as follows:

1. Rinse samples (cells or tissues) with 1x PBS (pH 7.2) or 0.1 M sodium cacodylate buffer [$\text{Na}(\text{CH}_3)_2 \text{AsO}_2 \cdot 3\text{H}_2\text{O}$] three times for 5 minutes each.
2. Fix samples with 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C or 30 min at room temperature.
3. Rinse samples with 0.1 M sodium cacodylate buffer three times for 10 minutes each.
4. Post fix samples in 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes at 4°C.
5. Rinse samples three times in 0.1M cacodylate buffer for 10 minutes each.
6. Dehydrate samples in a series of ethanol (30%, 50%, 70%) for 10 minutes each.
7. Store overnight in 70% ethanol at 4°C.
8. Continually dehydrate samples in series of ethanol (80%, 90%, 2x 100%) for 10 minutes each.
(Avoid allowing samples to become dry in each step)
9. Transfer samples into microporous vials and immerse the vials in ethanol. Keep samples and vials in 100% ethanol at all times.
10. Put the vials in Denton Critical Point Dryer, and then dry samples using liquid CO_2 passing its critical point at 1072 psi and 31 °C.
11. Mount samples on a stub and coat a thin layer of copper metal film using Denton IV Sputter Coater.

13. Endospore Enrichment and Purification

Three endospore-forming bacteria, *Bacillus cereus*, *B. megaterium* and *B. subtilis* were used in this study.

Three different methods were used to check the efficiency of enrichment of endospores and described as follows:

- a. Wuytack method involves first preparing a media of sterile CM3 nutrient agar containing 0.6g of $MgSO_4$ and 0.25g of KH_2PO_4 per liter of deionized water ^[45]. After mixing all contents within a 2 L Erlenmeyer flask until contents were clear and boiling; they were autoclaved, poured, and given time to solidify. The desired bacteria was inoculated on the plates and sealed with parafilm. Plates were incubated at 37°C for 7-10 days to starve cells and force endospore formation to occur. After the incubation period ended plates were observed to determine the amount of endospores that formed by performing a spore stain and visualized at 1000x oil magnification.
- b. Thermophilic method involves first preparing a sterile sporulation media consisting of 5g of bactopectone, 3g of yeast extract, 2g of NaCl, 1g of glucose, 0.5g of $MnSO_4$, and 20g of agar at a pH of 7.2 ^[33]. Plates were incubated at 55°C for 3days to starve cells and force endospore formation. After the incubation period ended plates were observed to determine the amount of endospores that formed by performing a spore stain and visualized at 1000x oil magnification.
- c. The Mustafa method involves initially making brain heart infusion tubes and modified nutrient agar made with 0.3% $MnSO_4$ per liter ^[30]. An overnight culture of bacteria was created in the brain heart infusion broth and 0.1mL aliquots of the

culture was taken and spread on the agar plates. These plates were then enclosed in polyethylene bags to avoid drying out and they were incubated for 7-10 days in an incubator set to 27°C to starve cells forcing endospore formation to occur. After the incubation period ended plates were observed to determine the amount of endospores that formed by performing a Schaeffer-Fulton stain and a microscope at 1000x oil magnification.

14. Endospore Purification and Treatment

- a. Transfer ½ of a plate or a whole plate of bacteria with plastic loop from spore plate to autoclaved test tube with DI water. Start with a larger volume (4-8 mL), and dilute/ add more bacteria as needed to get an OD_{650nm} reading of 1.00.

Spore culture is then purified as described as follows:

- 1.1. Pipette culture into locking centrifuge tubes in 750 µL aliquots. Centrifuge culture at 10,000 rpm for 10 minutes.
- 1.2. Set aside 1 tube for boiled control with ethanol (BC-EtOH). Purify with ethanol, not water.
- 1.3. Pour supernatant and resuspend pellet in 200 µl DI water, vortex. Resuspend tube of BC-EtOH in 200 µL ethanol.

d) Repeat centrifugation and resuspension 3x.

- b. Purified spores were boiled for at 100°C for 20 minutes (make sure to set aside a number of them for the unboiled control (2xGTP/LTP).

Centrifuge for 5 minutes at 10,000 rpm. Pour supernatant and resuspend spore tubes meant for hydrophilic tubes in 200 μ L DI water and lipophilic treatments in 200 μ L ethanol.

Treat spores for 2 hours at 37°C

- o 120 μ l cells control unboiled
- o 120 μ l cells control boiled
- o 120 μ l cells + 0.006 g EGCG/GTP (5%)
- o 120 μ l cells + 0.0012 g EGCG/GTP (1%)
- o 120 μ l cells + 0.006 g EGCG-S/LTP (5%)

120 μ l cells + 0.0012 EGCG-S/LTP (1%)

Perform serial dilutions, vortex briefly after each pipetting.

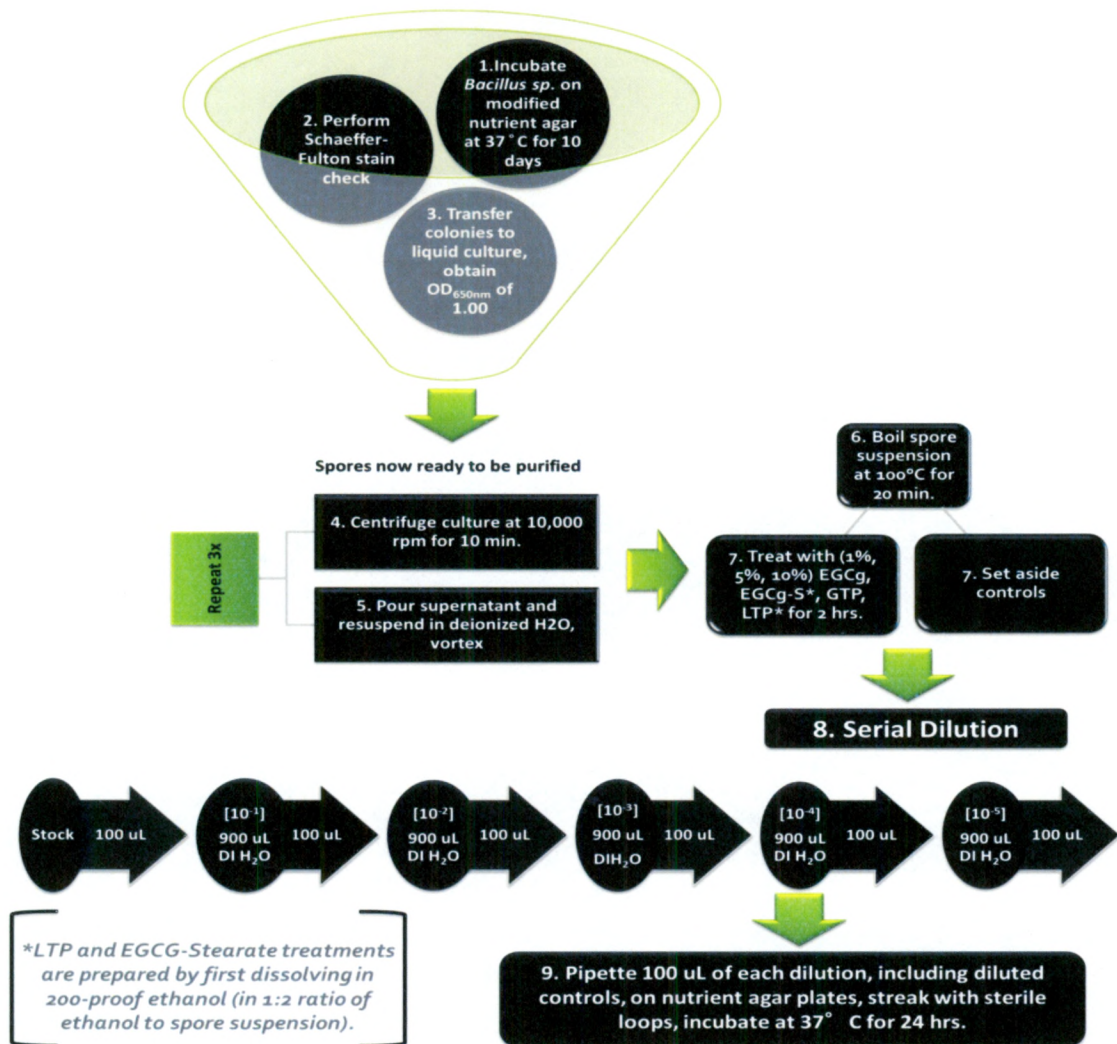
- **For hydrophilic treatments**

- o 100 μ l from stock + 900 μ l DI-H₂O (10^{-1})
- o 100 from previous + 900 μ l DI-H₂O (10^{-2})
- o 100 from previous + 900 μ l DI-H₂O (10^{-3})
- o 100 from previous + 900 μ l DI-H₂O (10^{-4})
- o 100 from previous + 900 μ l DI-H₂O (10^{-5})

- **For lipophilic treatments**

- o Same as above, replace DI with ethanol
- **For boiled and unboiled controls**
 - o 100 ul from stock + 900 μ l DI-H₂O (10^{-1})
 - o 100 from previous + 900 μ l DI-H₂O (10^{-2})
 - o 100 from previous + 900 μ l DI-H₂O (10^{-3})
 - o 100 from previous + 900 μ l DI-H₂O (10^{-4})
 - o 100 from previous + 900 μ l DI-H₂O (10^{-5})

Plate 100 μ l of each dilution on nutrient agar plates. Spread using sterile disposable loops evenly over plate. Incubate plates for 24 hours at 37°C and count colonies the next day.



Flowchart of Endospore Germination and Purification Protocol

15. Endospore Time-Kill Study

For this study, *B. cereus* was used and the protocols are the same as previously described. In order to determine the minimum time used to kill the spores, a time-

kill experiment was carried out. Instead of treating the spores with tea polyphenols for 2 hours, 5, 10, 15, and 30 minutes and 1 hour were used to treat the spores with different concentrations (1% and 5%) of tea polyphenols LTP and EGCG-S. After boiling, perform serial dilutions on all the samples and plate 100 μ l of each dilution on nutrient agar plates. Spread using sterile disposable loops evenly over plate. Incubate plates for 24 hours at 37°C and count colonies the next day on each plate as described previously.

16. Transmission Electron Microscopy (TEM)

The endospores were prepared and treated with EGCG-S for two hours and then prepared for TEM observations. One mL of *B. cereus* spores suspension was spun down by centrifugation at 14,000 rpm for 5 minutes and the samples resuspended in 5% glutaraldehyde for 1 hour to process the primary fixation of cells. The spores were then pelleted down by centrifuging at 14,000 rpm and then resuspended in 50 μ l of 4% sodium cacodylate agarose (2 grams of agarose dissolved in 50-mL of 0.1 M sodium cacodylate buffer). After the solution had solidified, the spore agarose was dehydrated by incubating the samples in the processing machine under the following conditions: 1) 0.1 M sodium cacodylate buffer for 10 minutes; 2) 0.1 M sodium cacodylate buffer for 10 minutes/0.1 M sodium cacodylate buffer for 10 minutes; 3) 2% osmium tetroxide (in 0.2 M sodium cacodylate buffer) for 120 minutes; 4) 0.1 M sodium cacodylate buffer for 10 minutes; 5) 0.1 M sodium cacodylate buffer for 10 minutes; 6) 8 % tannic acid (0.05 M sodium cacodylate buffer) for 20 minutes. 7) 0.1 M sodium cacodylate

buffer wash for 15 minutes; 8) 0.1 M sodium cacodylate buffer wash for 10 minutes; 9) 50% ethanol for 10 minutes; 10) 70% ethanol for 10 minutes; 11) 95% ethanol for 10 minutes; 12) 95% ethanol for 10 minutes; 13) 100% ethanol for 10 minutes; 14) 100% ethanol for 10 minutes; 15) 50% ethanol/50% propyleneoxide mixture for 10 minutes; 16) 100% propyleneoxide for 10 minutes; 17) 100% propylene oxide for 10 minutes; 18) 2 : 1 propylene oxide : Epon mixture for 3 hours; 19) 1:1 propylene oxide : Epon mixture for 6 hours; 20) 1 : 2 propylene oxide : Epon mixture for 9 hours; 21) pure Epon for 3 hours; 22) pure Epon for 3 hours. After dehydration, the samples were transferred into pure Epon and cured at 60°C vacuum oven for 48 hours. The solid samples were sliced into 1 μ m thick sections in a Porter Blum MT-2, and then 120 nm thin sections in RMC MTXL Ultramicrotome. The uranylacetate and lead citrate stains were used for sample staining. All the samples were examined in a Hitachi H 7500 Transmission Electron Microscope, and photographed by AMT 1 Meg CCD Camera.

Results/Discussion

A. Study the Effect of Tea Polyphenols on Biofilm Formation

1. Monitoring the Growth of *Pseudomonas aeruginosa*

To properly view the growth of bacteria, a growth curve was generated. Bacterial growth was monitored over a period of 15 hours using a spectrophotometer at 600nm, Figure 3 is the growth curve for *P. aeruginosa*, showing its growth stages at its optimal growth temperature at 37°C. A standard growth curve consists of four individual phases; lag phase, exponential growth, stationary phase, and death phase. A short lag phase and followed by a rapid increase in OD reading in the bacteria's exponential growth phase. As shown in Figure 3 the stationary phase is indicated by the steady OD readings around the later hours 10-15 hours. This indicates the number of cell growth is equivalent to cell death. Since the bacteria were grown within close culture tubes they have access to only the amount of resources provided within the media placed inside the tubes. Thus, over time when the microorganisms reach a maximum number they slowly start to die because of lack of nutrients and other environmental factors. The information from the growth curve helps determine changes in growth and times of exponential growth in control microorganisms when comparing the growth of green tea polyphenol treated bacteria. From the growth curves, generation time of the tested bacteria can be determined. At the optimal growth condition, the generation time for *P. aeruginosa* was determined by using the equation: $g = (\log_{10} N_t - \log_{10} N_0) / \log_{10} 2$. Using this formula to calculate generation time, it was seen that it takes *P. aeruginosa* about 40 minutes to double in population.

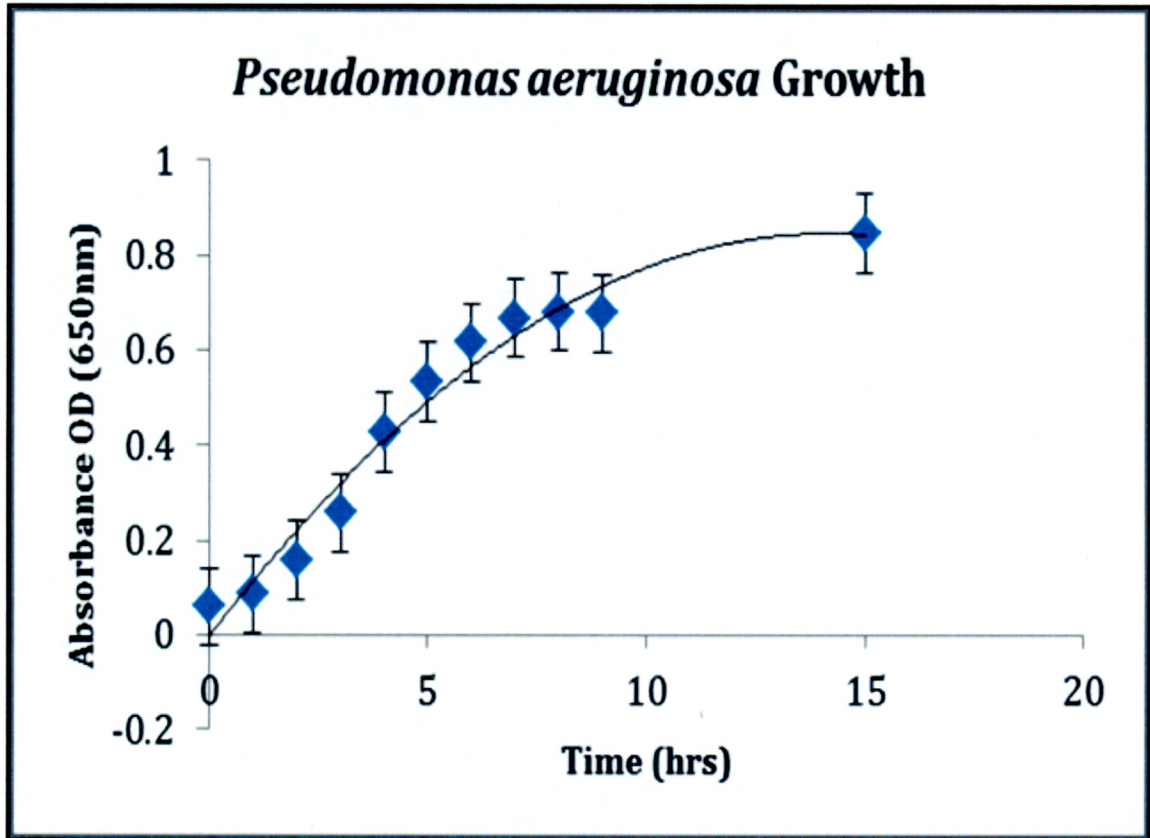


Figure 3: The growth curve of *P. aeruginosa*. The growth is monitored by turbidity study at OD 600nm. The growth curve is resulted from the mean of three replicas and y arrow bar represents the standard deviation (SD). Growth occurred at 37° C in a shaking incubator at 250 rpm.

2. Air-Liquid Interface for Bacterial Biofilm Production in *P. aeruginosa*

An air-liquid interface experiment was carried out first to observe biofilm formation by *P. aeruginosa* and view if the crude tea polyphenols (GTP and LTP) had an effect on population density at this interface. Bacteria were grown in culture tubes containing media as well as tea polyphenols (0.5 % and 1% of GTP and LTP) for a period of 24 hours. A vertical microscope slide was added in each culture tube, half exposed to air and half immersed in liquid media, the area of air liquid interface was observed under microscope for biofilm formation. An untreated sample was used as control. This initial study was to view the potential effects of the tea polyphenols on population density at the biofilm forming area. The results of different treatments are shown in Figure 4; the images indicated that at the air-liquid interface the treatment of 0.5% and 1% of GTP and LTP on *P. aeruginosa* decreases the cell density in a dose dependent pattern at the air-liquid interface when compared with the control. The results suggest that the tea polyphenols have an effect on biofilm formation and can reduce the population density in the treated area.

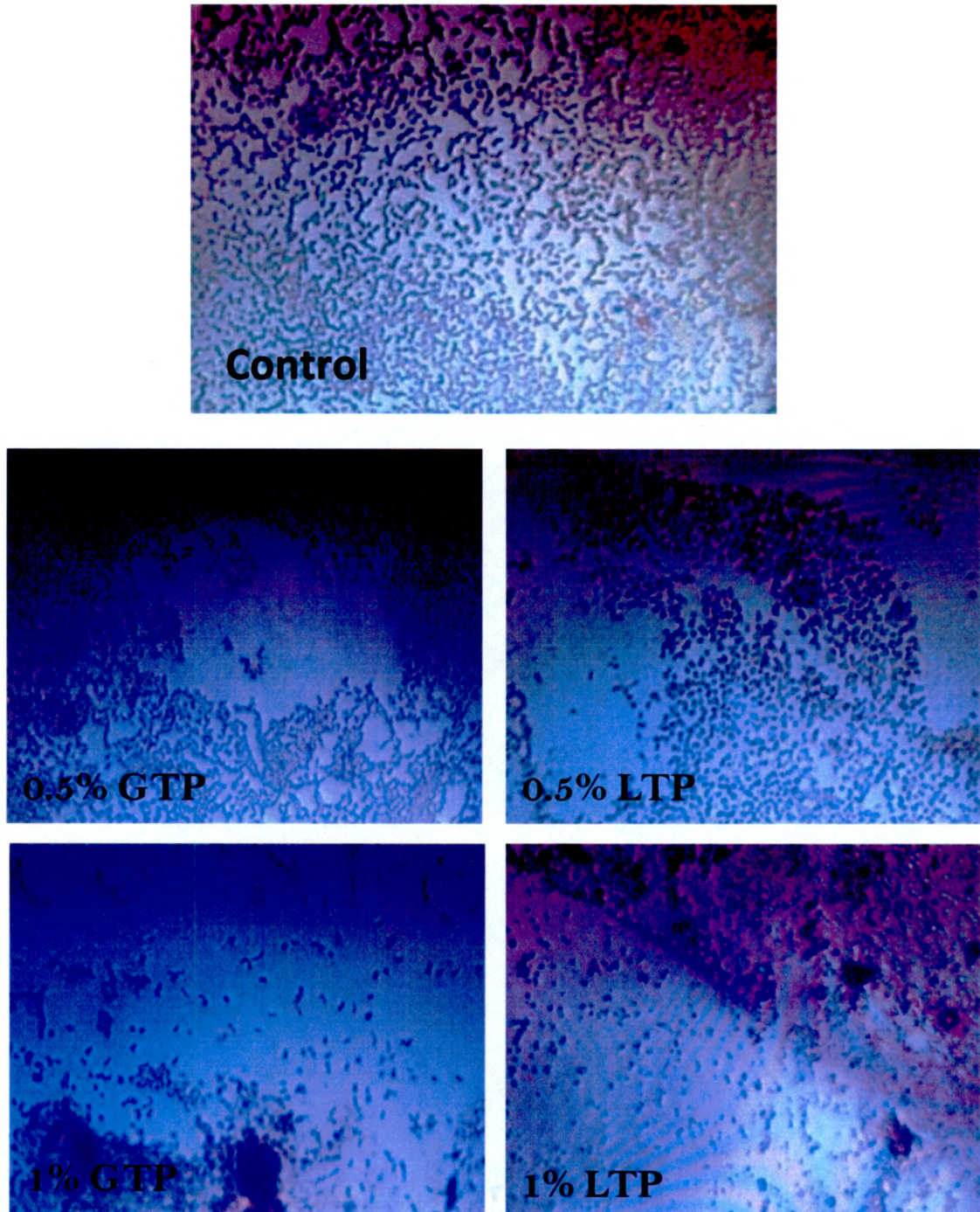


Figure 4: Microscopic observation of Air-Liquid Interface Study (1000X) of polyphenol treated (0.5% GTP and LTP; 1% GTP and LTP) *Pseudomonas aeruginosa*

3. The Effects of GTP and LTP on *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* Biofilm Formation by Analyzing the Population Density on a Glass Surface

Upon viewing the effects of both GTP and LTP on bacteria population through the air-liquid interface experiment, further microscopy studies were performed to study the effects of the tea polyphenols on biofilm formation of both *P. aeruginosa* and *P. fluorescens* by observing the population density on a glass surface. These experiments involved growing both bacteria treated and not treated in 6 well plates. Growing the bacteria in 6 well plates with cover-glass slips was described in Material and Methods section 7. After allowing the bacteria to grow at their optimal temperature for 5 days they were stained and viewed. By using this experiment further data was gathered on the effects of the tea polyphenols on population density as well as on morphology on a submerged glass area placed at the bottom of the well. Results shown in Figures 5 and 6 show that when comparing the images of the 30 μ g/mL and 50 μ g/mL GTP and LTP treatments to the controls for both microorganisms there is a significant decrease in bacteria cell population. A decrease in population density could be correlated with the tea polyphenols effecting bacteria cells' adherence abilities. This suggested a decrease in adhered cells and less biofilm accumulation.

Pseudomonas aeruginosa

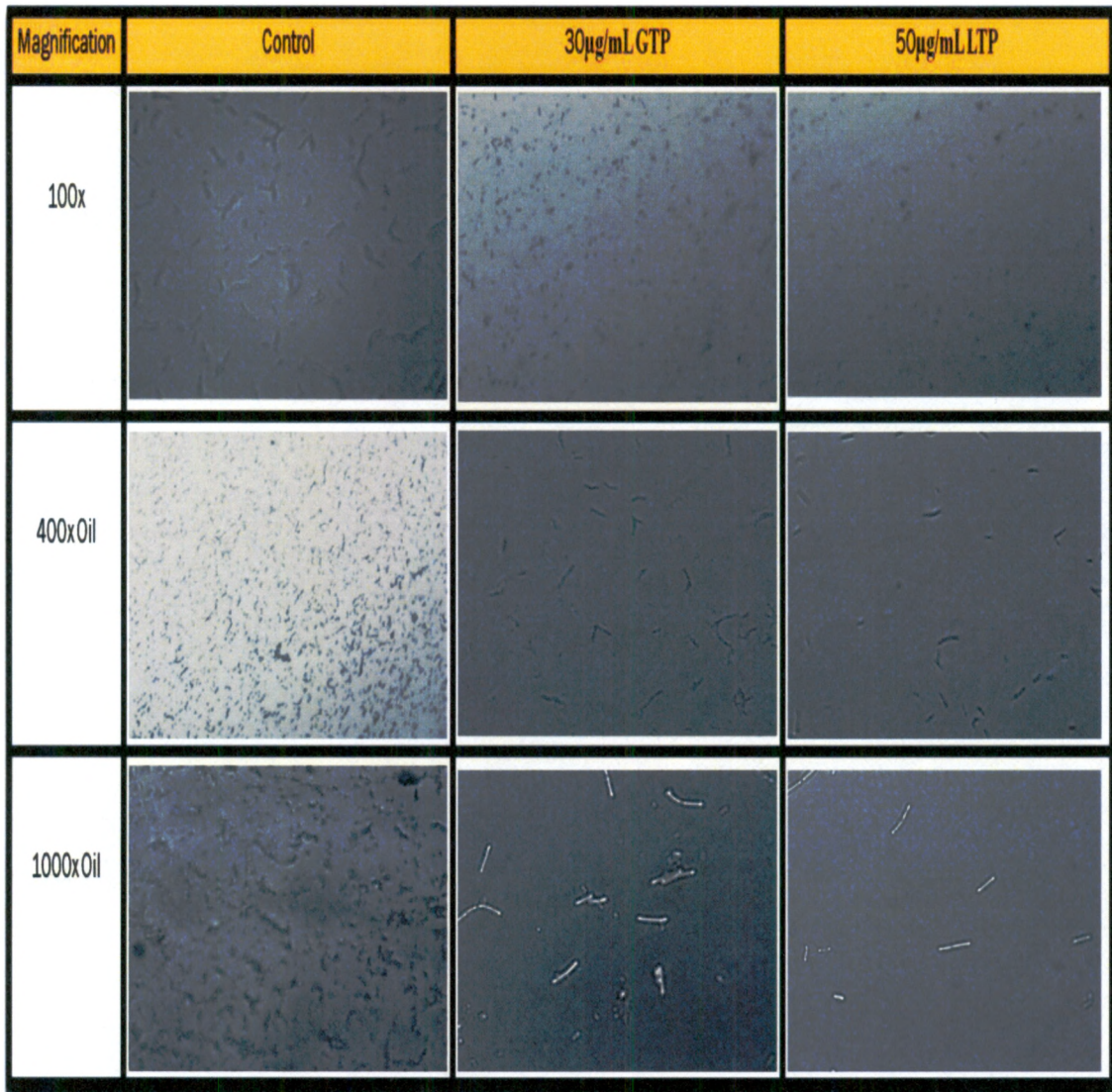


Figure 5: Microscopic observation of bacteria population density in *Pseudomonas aeruginosa* with 30 μ g/mL and 50 μ g/mL GTP and LTP and Control

Pseudomonas fluorescens

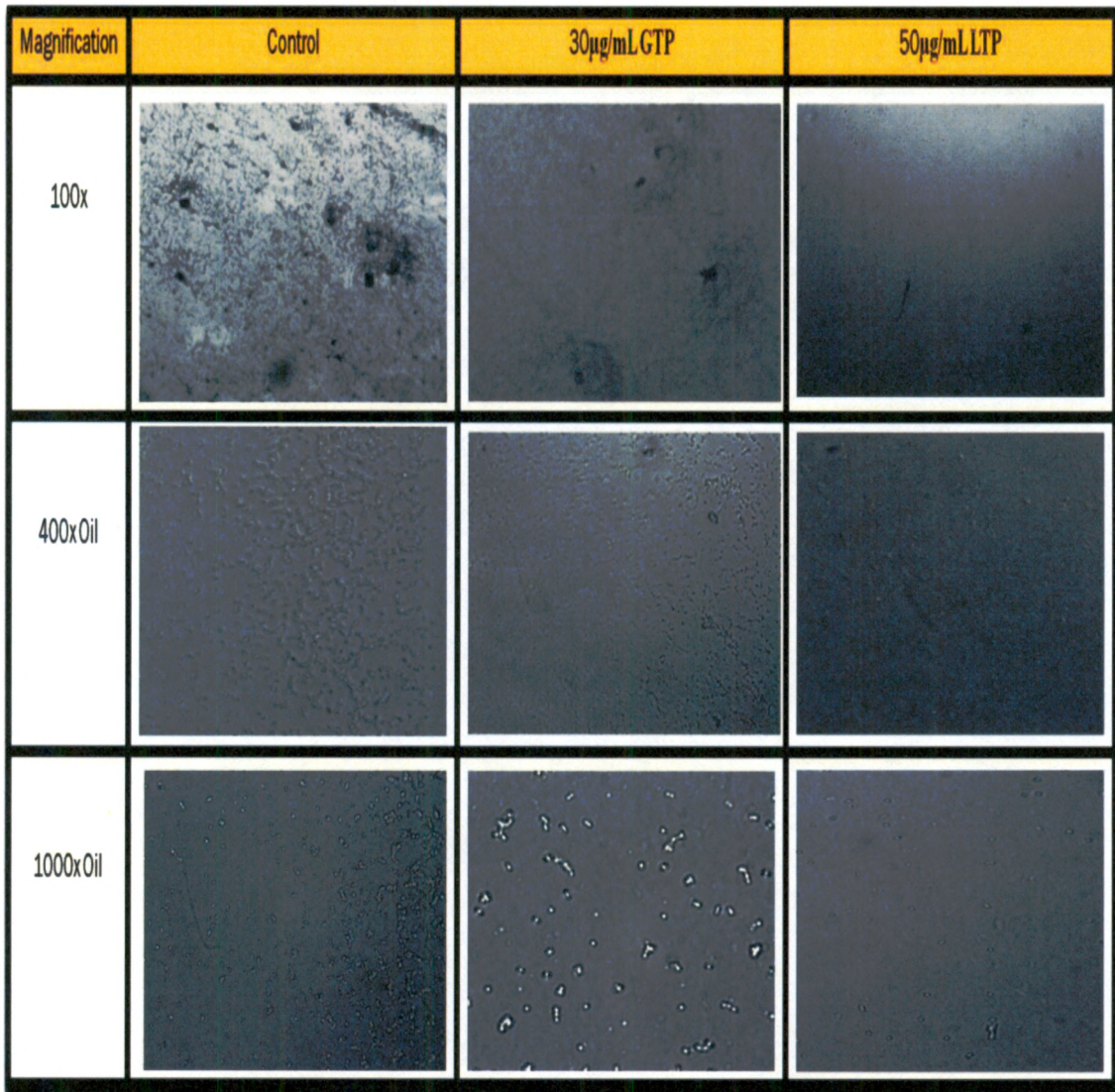


Figure 6: Microscopic observation of bacteria population density in *Pseudomonas fluorescens* with 30µg/mL and 50µg/mL GTP and LTP and Control

4. Short Term Microscopic Study of Tea Polyphenols on *Pseudomonas aeruginosa*

After viewing that the tea polyphenols used in the previous experiments were able to lessen the overall cell population density, a short-term microscopic study was performed to view the effects of the potent tea polyphenols on *Pseudomonas aeruginosa*. This study was set up similar to the previous experiment "The Effects of GTP and LTP on *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* Biofilm Formation by Analyzing the Population Density on a Glass Surface". However, for this study bacteria were only treated for 2 hours and 4 hours. Treatment concentrations used within this study were 15 µg/mL, 60 µg/mL, and 100 µg/mL. Results from the study shown in Figure 7, illustrated that bacteria cells agglutinated with one another after 4 hours of treatment. These results further support the hypothesis that tea polyphenols can affect the ability of bacteria cells to bind to surfaces by either affecting their external structures or inhibiting another mechanism for binding. Although bacteria cells were only treated for 4 hours there was an exhibited effect on their ability to bind. As compared to the control cells, which are more clumped and overlaid within treated slides than the control. The control slide demonstrates a more even spreading of bacteria cells over a surface than treated slides. Additionally, at 4 hours post treatment there is a lot more agglutination than at 2 hours. The reason for this could be that the amount of time the tea polyphenols were exposed to the bacteria also play a huge role in how effective the tea polyphenols are in effecting the bacteria cells.

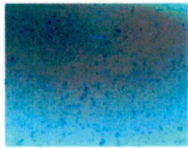
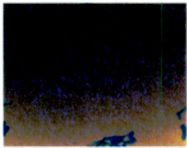
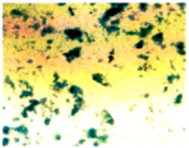
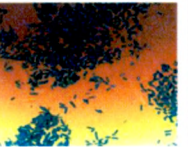
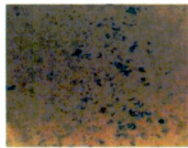
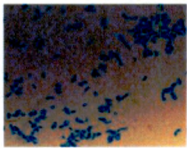
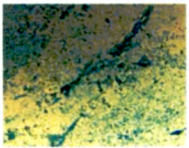
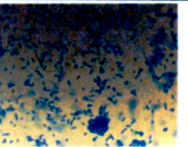

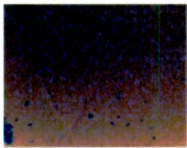
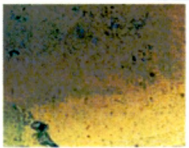
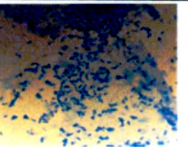
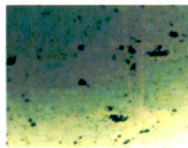
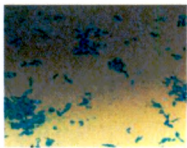
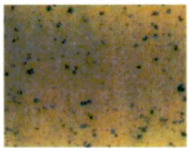
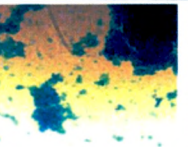
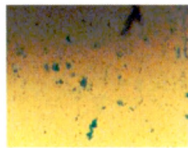
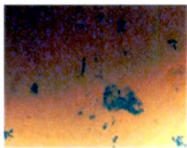
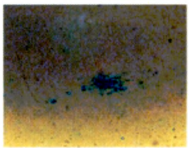
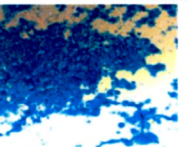
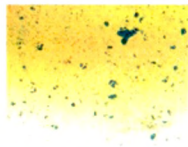
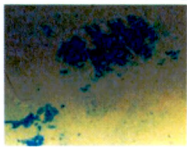
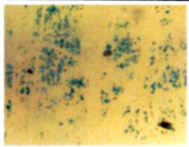
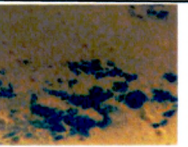
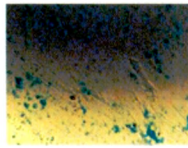
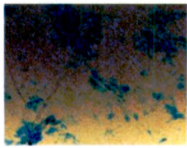
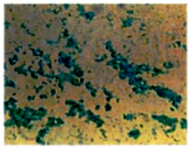

	2 hr 100x	2 hr 1000x	4 hr 100x	4 hr 1000x
Control				
15 µg/mL EGCG				
15 µg/mL EGCG-S				
60 µg/mL EGCG				
60 µg/mL EGCG-S				
100 µg/mL EGCG				
100 µg/mL EGCG-S				

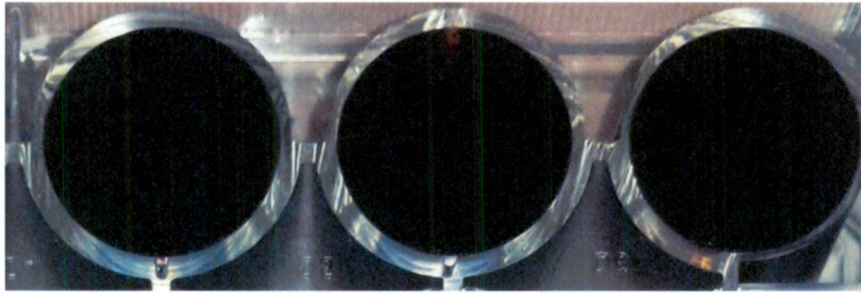
Figure 7: Short term microscopic images of *Pseudomonas aeruginosa* after treatment with both EGCG and EGCG-S. (100x & 1000x Oil)

5. Congo Red Agar Assay for Bacterial Biofilm Formation from *Pseudomonas aeruginosa*

Congo red agar is used as a direct test to determine whether bacteria are able to form biofilm by whether the agar changes color^[36]. The Congo red dye that is present within the agar can bind to the amyloid produced by biofilm forming bacteria and produce dark black precipitate. The black precipitate can be viewed from the top and bottom of the agar to illustrate biofilm formation. To determine whether *P. aeruginosa* forms biofilm in the presence and absence of tea polyphenols, the bacteria were inoculated onto a Congo red agar plate with and without treatment. Initially, positive and negative controls were grown to demonstrate the difference in dye colors as shown in Figure 8. Biofilm positive plates are easily distinguished from biofilm negative plates. These results are used to further evaluate the effect of the tea polyphenols on the biofilm presence and absence. The time kill study was carried out by treating the bacteria with four different tea polyphenols (GTP, EGCG, LTP and EGCG-S) at two different concentrations (50 and 100 µg/mL) for 1 hour, 4 hours, 8 hours and 24 hours, respectively. The results are shown in Figures 9-12. The results from this preliminary study suggested that different tea polyphenols worked when they were used in concentrations similar to the previously used concentrations of 50µg/mL to 100µg/mL. EGCG-S and LTP worked better than GTP and EGCGS and EGCG-S has the best effect on inhibiting biofilm formation in *P. aeruginosa*. If the biofilm was formed, the biofilm grew significantly at day 2. Treatment for 24 hours provided the best results for all the treatments. In further experiments only EGCG-S was used and instead of testing the tea polyphenol for an extended period of time, one hour treatment was used. The dose

response (50, 100, 150, and 200 $\mu\text{g}/\text{mL}$ of EGCG-S) studies were carried out. The results are reported in Figure 13 and indicated that wells containing 50, 100, 150 $\mu\text{g}/\text{mL}$ of EGCGS developed a dark black precipitate, which is the same as seen in non-treated positive control and the bottom of the well, indicating bacteria biofilm formation. Both the negative control and the well that was treated with 200 $\mu\text{g}/\text{mL}$ of EGCG-S remained their bright red color suggesting that there is no biofilm formation at this concentration. These results indicated that a 200 $\mu\text{g}/\text{mL}$ concentration of EGCG-S treated for 1 hour is needed to completely inhibit biofilm formation.

(1) Positive



(2) Negative

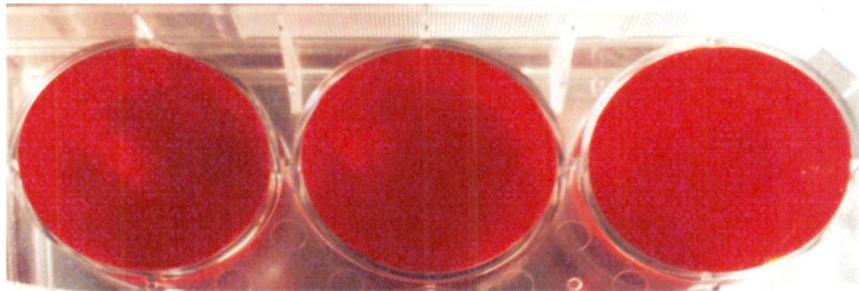


Figure 8: Positive and Negative controls for *Pseudomonas aeruginosa* grown on Congo Red Agar.



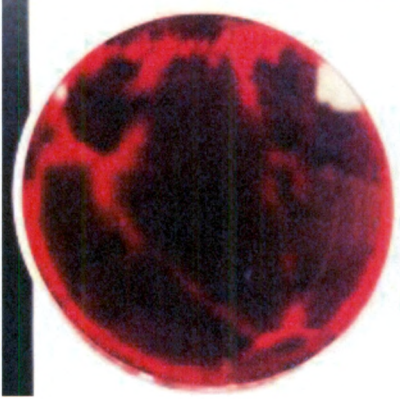
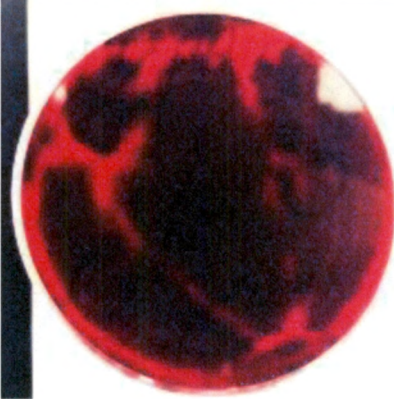
<i>Pseudomonas aeruginosa</i>	Hour 1 50 ug/mL	Hour 1 100 ug/mL
Day 1		
Day 2		

Figure 9: 1 Hour Treatment of 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ of GTP, LTP, EGCG, and EGCG on *Pseudomonas aeruginosa*.

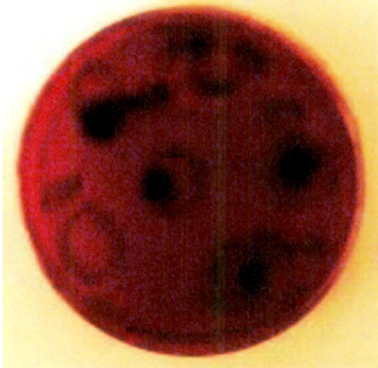
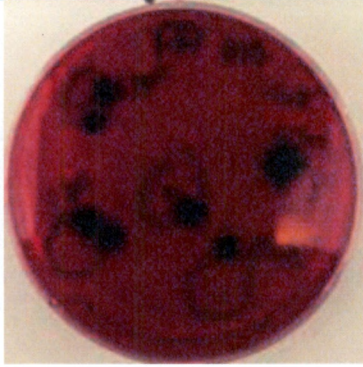


<i>Pseudomonas aeruginosa</i>	Hour 4 50 ug/ml	Hour 4 100 ug/ml
Day 1		
Day 2		

Figure 10: 4 Hour Treatment of 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ of GTP, LTP, EGCG, and EGCG on *Pseudomonas aeruginosa*.





<i>Pseudomonas aeruginosa</i>	Hour 8 50 ug/ml	Hour 8 100 ug/ml
Day 1		
Day 2		

Figure 11: 8 Hour Treatment of 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ of GTP, LTP, EGCG, and EGCG on *Pseudomonas aeruginosa*.

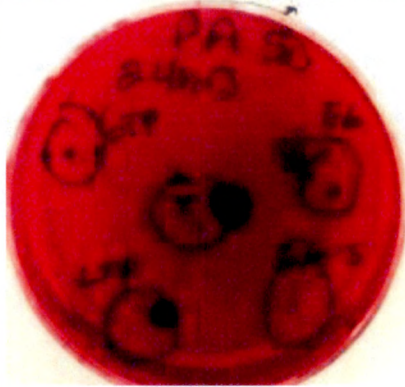

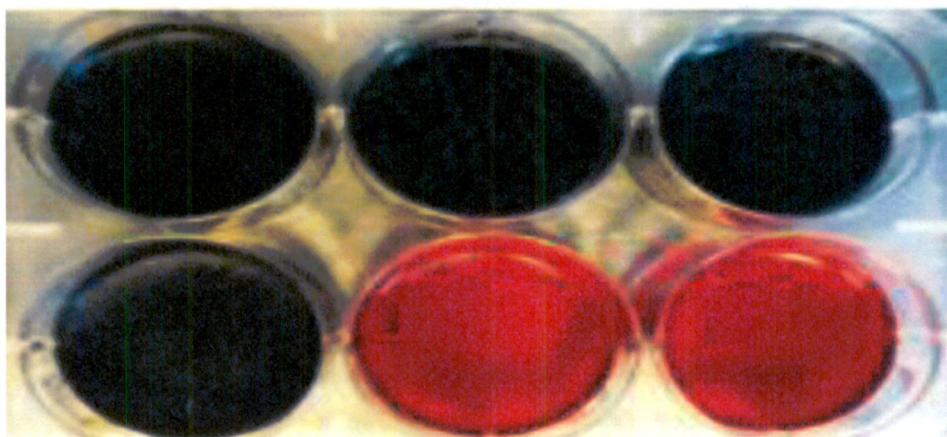
<i>Pseudomonas aeruginosa</i>	Hour 24 50 ug/ml	Hour 24 100 ug/ml
Day 1	Treated for 24 hours	Treated for 24 hours
Day 2		

Figure 12: 24 Hour Treatment of 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ of GTP, LTP, EGCG, and EGCG on *Pseudomonas aeruginosa*

50µg/mL EGCG-S	150µg/mL EGCG-S	Positive Control
100µg/mL EGCG-S	200µg/mL EGCG-S	Negative Control

(1) Top agar view



(2) Bottom agar view

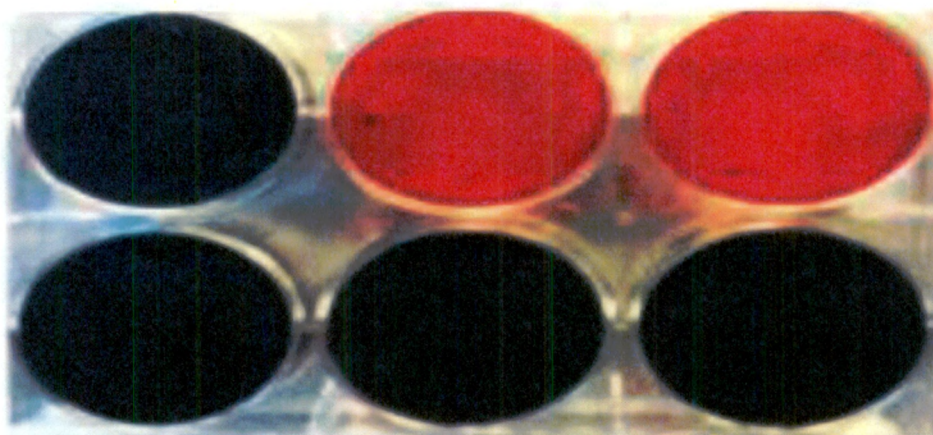


Figure 13: Congo Red Agar Assay for determining if *Pseudomonas aeruginosa* produces biofilm under certain treatment concentrations of EGCG-S.

6. Dose-Dependent Congo Red Agar Assay for Biofilm Production and Biofilm Reversal Assay

This assay tested if the bacteria required a specific density of cells to initiate bacterial biofilm formation. This was a population-dependent study using different amounts of *P. aeruginosa* (5, 10, 15, 20, 25 and 50 μ L) on to the Congo red agar. After 24 hours of incubation at 37°C, the images were taken as shown in Figure 14. The results indicated that each volume of cultures from 5 μ l to 50 μ l were able to form biofilm in one day. This indicates that *P. aeruginosa* even at a low concentration of 5 μ l was able to form biofilm in 24 hours. The density required for biofilm formation may be lower than 5 μ l as shown by Figure 14. Further experiments are needed to further dilute the culture and calculate the known population of bacteria to provide more accurate information.

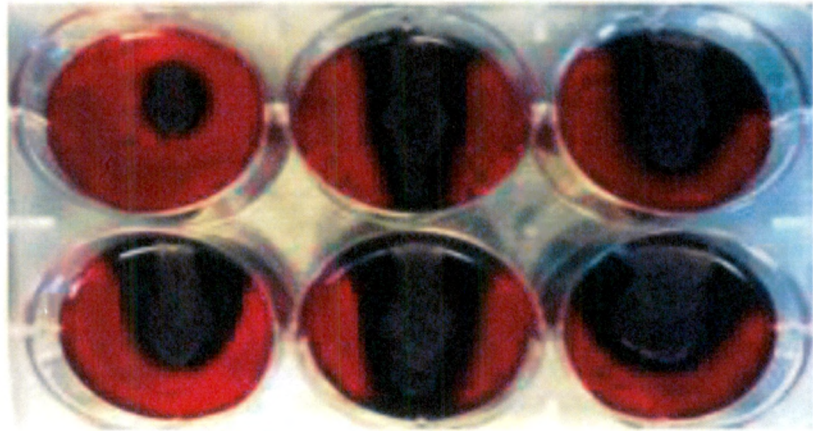
Biofilm reversal assay was carried out on day 2; a concentration of 250 μ g/mL of EGCG-S was applied to each of the wells and incubated overnight to observe if EGCGS is able to reverse the already formed biofilm. This would be indicated by the changing of colors from a dark black precipitate to a bright red color, similar to the original color of Congo red. After adding 250 μ g/mL of EGCG-S on formed biofilm, it can be seen from the top view of the agar that the biofilm was formed in black is reversible in red. This indicated that EGCG-S is not only able to inhibit the formation of biofilm at certain concentrations, as seen from previous experiments, but it is able to reverse the formed biofilm. Further experiments are needed to support this finding.

5 μL	10 μL	15 μL
20 μL	25 μL	50 μL

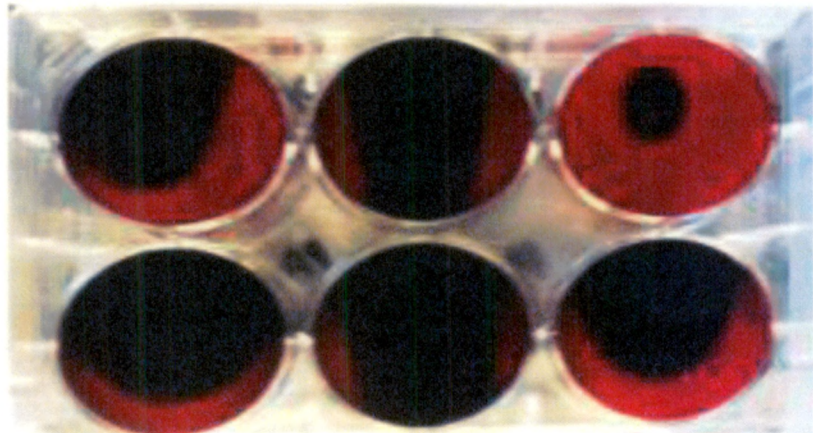
[1]

Day 1

Top Agar View



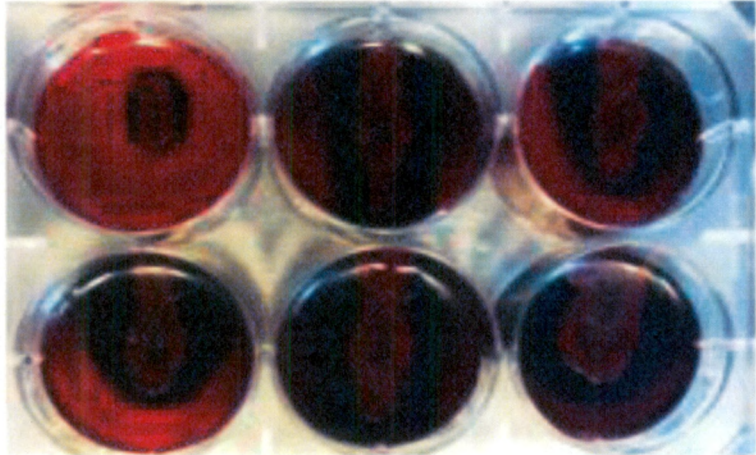
Bottom Agar View



Reverse Congo Red study in day 2

[2]

Top Agar View



Bottom Agar View

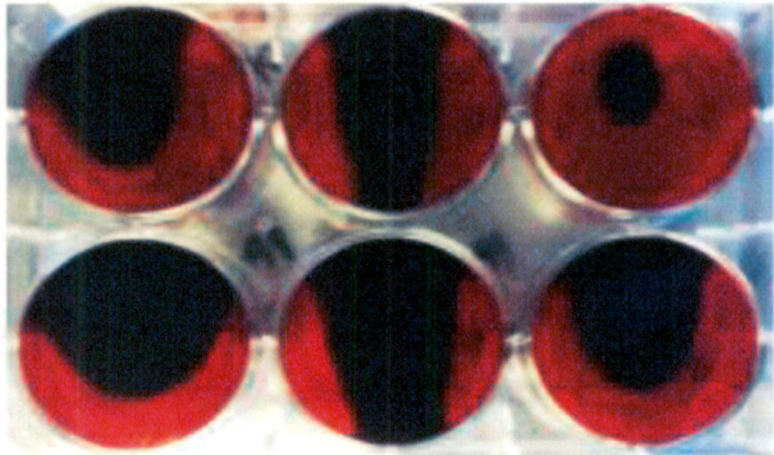


Figure 14

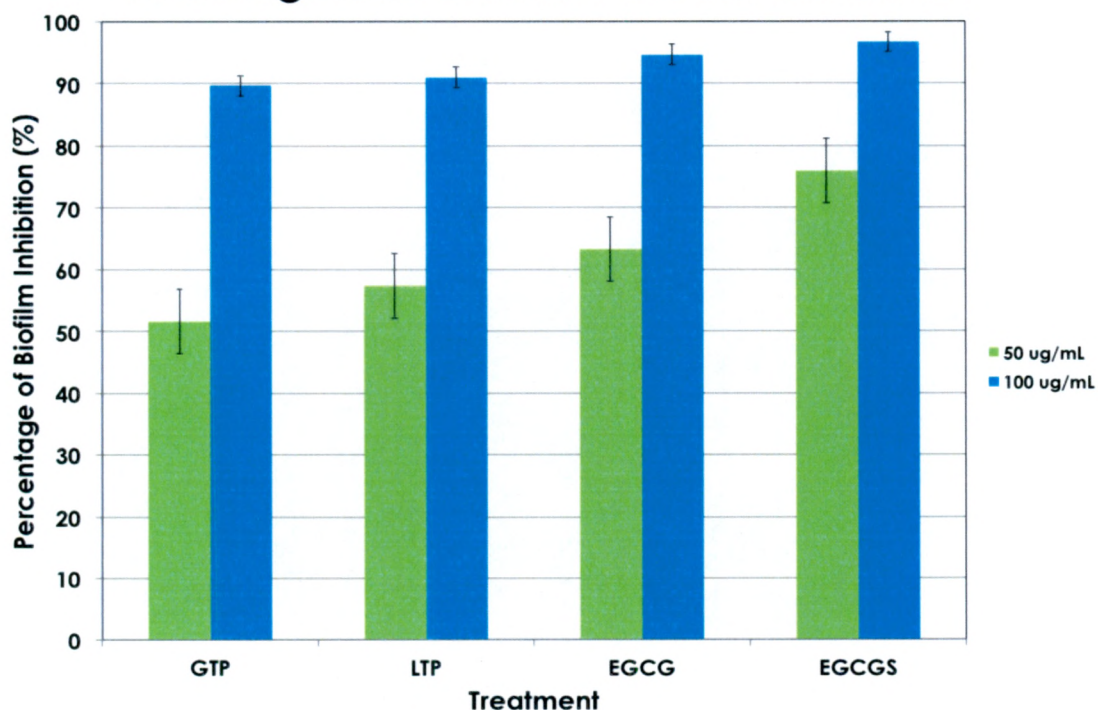
(1): Dose Dependent Biofilm Formation on Congo Red Agar After 1 Day of Incubation.

(2): Biofilm Reversal Assay with 250 μ g/mL of EGCG-S Applied for 24 hours.

7. Quantitative Study of Biofilm using Crystal Violet Assay

The previous experiments were performed to view the general effects of both crude and pure tea polyphenols on biofilm formation qualitatively. After viewing that the tea polyphenols can inhibit biofilm formation, the next set of experiments using crystal violet assays were carried out to quantitatively study the tea polyphenols effects on biofilm formation in *P. aeruginosa*. GTP, LTP, EGCG, and EGCG-S of different concentrations (0, 50 and 100 $\mu\text{g}/\text{mL}$) were used to treat bacteria for 1 to 5 days to allow the bacteria to form biofilm in either 6 well or 24 well plates. A crystal violet assay is performed to quantitatively study the amount of biofilm and repeated three times in each condition. The results reported in Figure 15 indicated that 100 $\mu\text{g}/\text{mL}$ of all tea polyphenols inhibited the biofilm significantly with the percentage of inhibition ranging from 89% to 97%. Percentage of inhibition for 50 $\mu\text{g}/\text{mL}$ ranged from 52% to 76%. The pure compounds EGCG and EGCG-S demonstrated a significantly higher inhibition effect than the crude extracts. EGCG-S at 100 $\mu\text{g}/\text{mL}$ was the best and was able to inhibit the biofilm to 97%. This study suggested that with increasing concentration of each tea polyphenol, there is a directly correlated increase in biofilm inhibition, thus it was dose dependent.

***P. aeruginosa* Biofilm Percent Inhibition**



Treatment	Concentration	Percentage Biofilm Inhibition (%)
GTP	50µg/mL	52
	100µg/mL	89
LTP	50µg/mL	57
	100µg/mL	91
EGCG	50µg/mL	63
	100µg/mL	94
EGCG-S	50µg/mL	76
	100µg/mL	97

Figure 15: Crystal Violet Assay for measuring biofilm percent inhibition on *Pseudomonas aeruginosa* using both crude, GTP & LTP, and pure, EGCG & EGCG-S, tea polyphenols.

8. BacLight Live and Dead Assay on EGCG and EGCE-S Treated Biofilm forming

P. aeruginosa

After quantitatively viewing that each of the tea polyphenols used were able to inhibit biofilm formation and that the use of the pure tea polyphenols inhibits biofilm formation better than the crude extracts, the possible mechanism as to how these tea polyphenols are working was studied. Some reports looked at the damaging effects of compounds on the cell membrane and integrity of *P. aeruginosa* cells^[44]. To view this possible mechanism of damage to the cells, a live and dead fluorescent microscopic observation was performed on treated cells with 1/4 the minimum inhibitory concentration (MIC) and the MIC of the pure tea polyphenols. Concentrations of 15 and 60 µg/mL of EGCG and 25 and 100 µg/mL of EGCG-S were used in this study. To perform this assay the bacteria was grown with various treatment concentrations within 6 well plates containing glass cover slips. After the required incubation period of 24 hours the live and dead assay was performed and results were viewed at 1000X magnification. Cells that are green indicate cells that have intact cell membranes and alive; cells that are red indicate cells that have damaged cell membranes thus are not alive. The results showed in Figure 16 indicated that a majority of the bacteria cells present in the control with no treatment were alive and showed green in color with intact cell membranes. However, when viewing the treatment images with different concentrations and types of tea polyphenols the cell density was significantly reduced and the majority of cells were red in color. This suggested that one potential mechanism as to how both these compounds work is through effecting the integrity of cell membrane of *P. aeruginosa*.

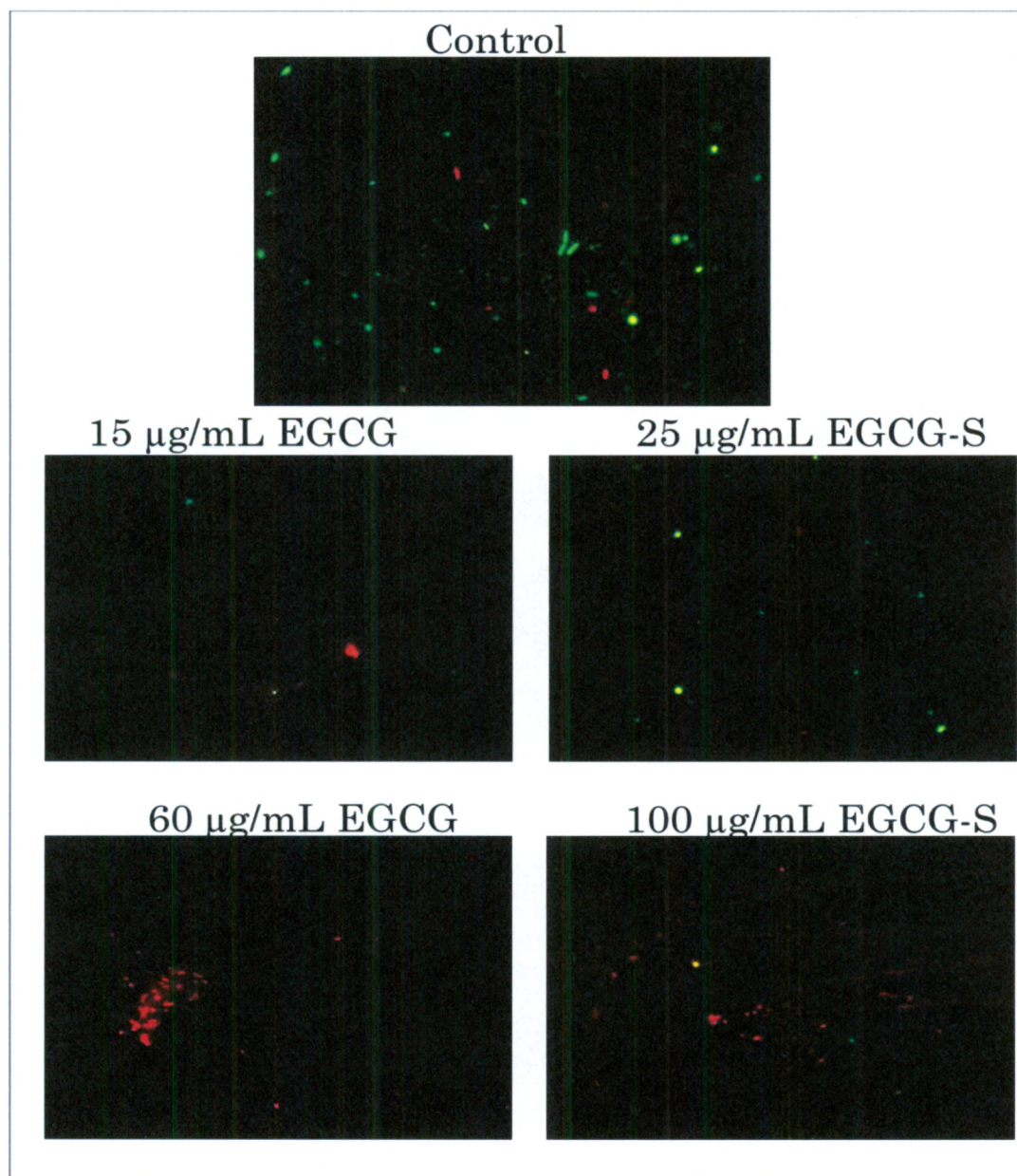
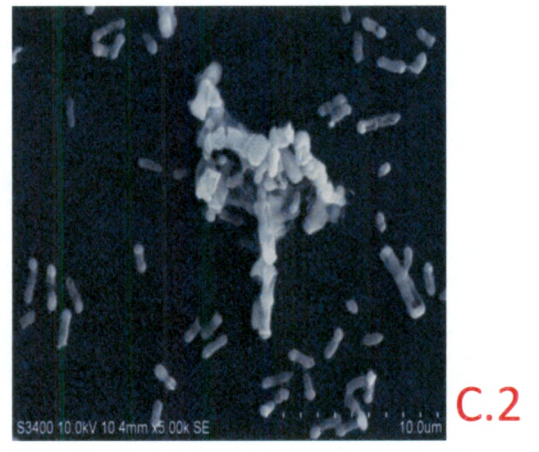
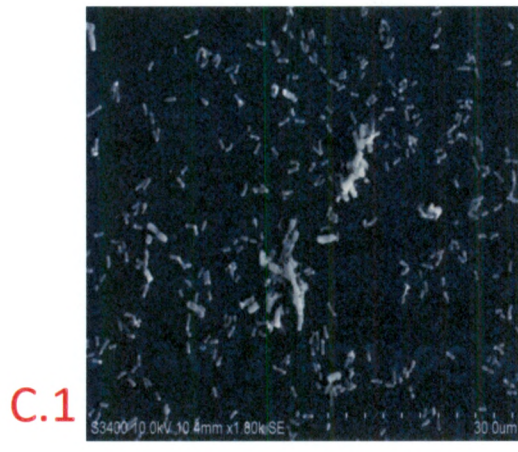
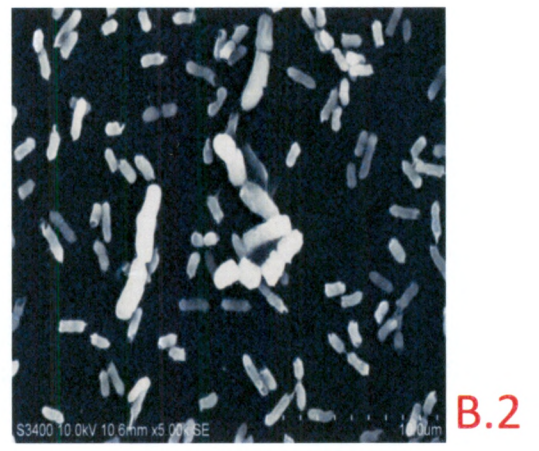
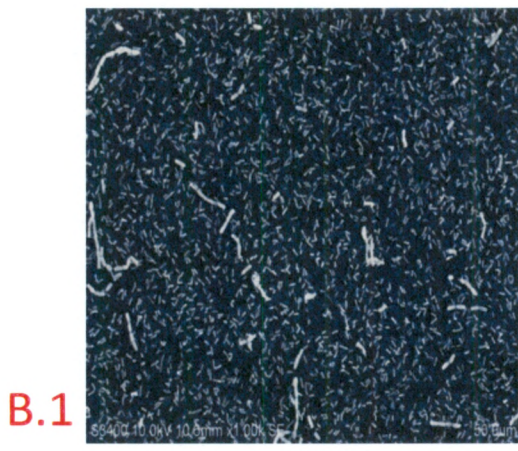
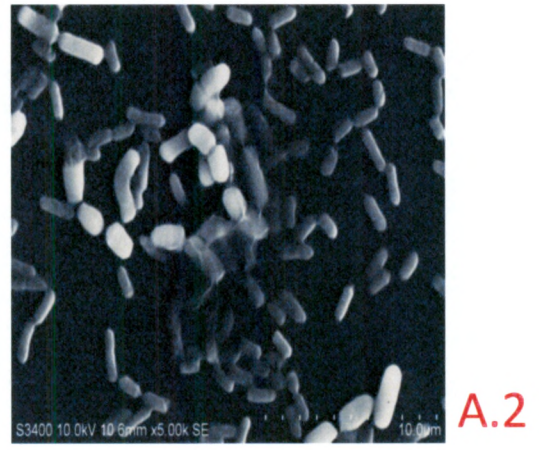
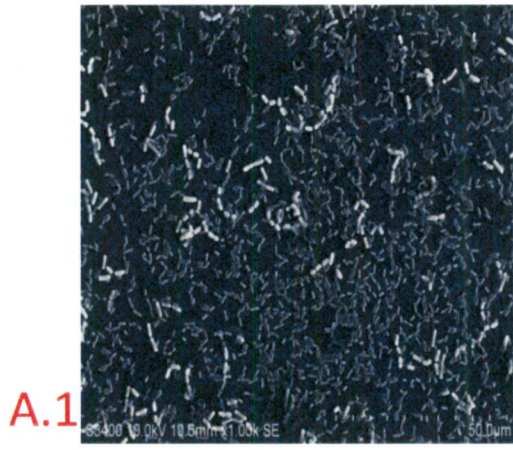


Figure 16: BacLight Live and Dead stain on *Pseudomonas aeruginosa* to determine cell wall integrity using pure tea polyphenols EGCG and EGCG-S at 1/4 the MIC and the MIC concentration found for each compound. (1000x Oil Immersion)

9. Time Course Study using Scanning Electron Microscopy (SEM) on the Formation of Biofilm in *P. aeruginosa*

To further analyze the potential mechanism that tea polyphenols have on *P. aeruginosa*, scanning electron microscopy (SEM) was performed with control cells and treated cells. SEM was first performed to follow a time course study and observe the detailed process of morphological changes and biofilm formation. SEM was performed after growing bacteria in nutrient rich with 2% sucrose to provide bacteria with ample amounts of nutrients for producing biofilm. Bacteria were grown in 6 well plates with nutrient rich with 2% sucrose and special tissue culture slides purchased from neuVitro for SEM imaging. Bacteria were grown for 6, 12, 24, 48, and 60 hours, respectively, and then processed for SEM imaging to observe the biofilm formation over time. The results shown in Figure 17 indicated that biofilm formation can be observed at 24 hours of incubation and cells began to clump more tightly with one another; at 48 to 60 hours of incubation, increased biofilm formation arises. In the images for 60 hours of incubation, thin films were observed and connected the cells (Fig. 17, E.1/E.2).



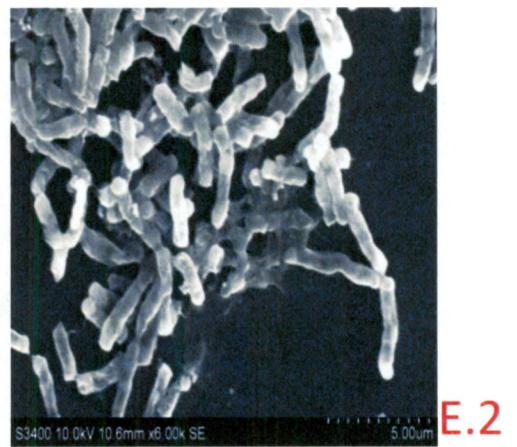
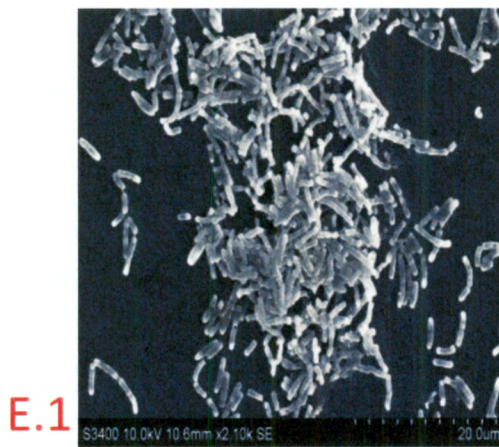
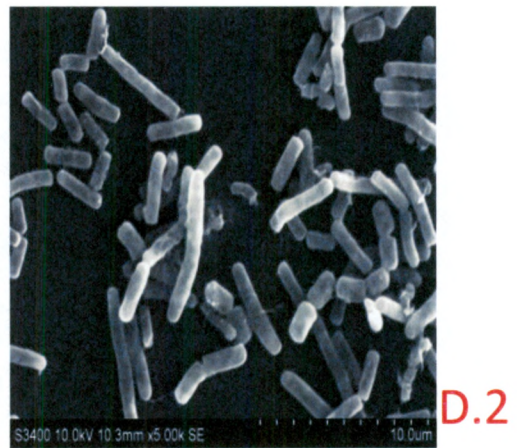
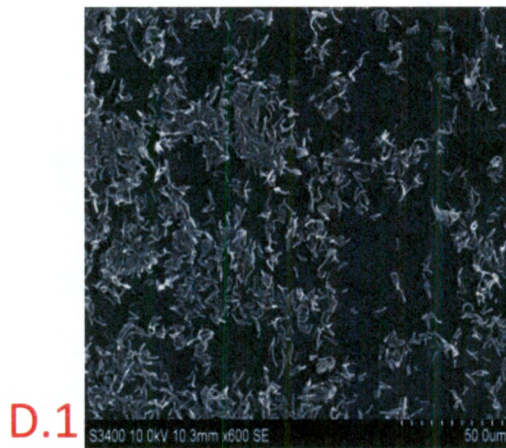


Figure 17: Scanning Electron Microscopy of *Pseudomonas aeruginosa*.

A1/A2: 6 Hours

B1/B2: 12 Hours

C1/C2: 24 Hours

D1/D2: 48 Hours

E1/E2: 60 Hours

Images labeled 1: Low Magnification

Images labeled 2: High Magnification

10. Scanning Electron Microscopy (SEM) Study on Short Term Treatment and Long Term with EGCG-S of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa cells were treated with 50 and 100 $\mu\text{g}/\text{mL}$ of LTP and EGCG-S, respectively, for 24 hours. SEM samples were prepared to view the effects of these lipophilic tea polyphenols on biofilm formation and morphological changes (Figure 18). Results from this experiment demonstrated that with the use of both LTP and EGCG-S at concentrations of 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$, *P. aeruginosa* cells started to become less populated on the slides and exhibited elongated cell structures when compared to the control (0 treatment) experiment. This result correlated very well with the live and dead fluorescence microscopic study and demonstrated that LTP and EGCG-S can inhibit cell growth and biofilm formation. Further long term SEM study, incubating the control and treated culture with 100 $\mu\text{g}/\text{mL}$ EGCG-S for 5 days, was also carried out and the results are shown in Figure 19. Both in low and high magnification, thin biofilm were observed in control samples but not in treated samples. The morphology of the cells also changed after treatment for a long time; the cell lost its integrity and shape.

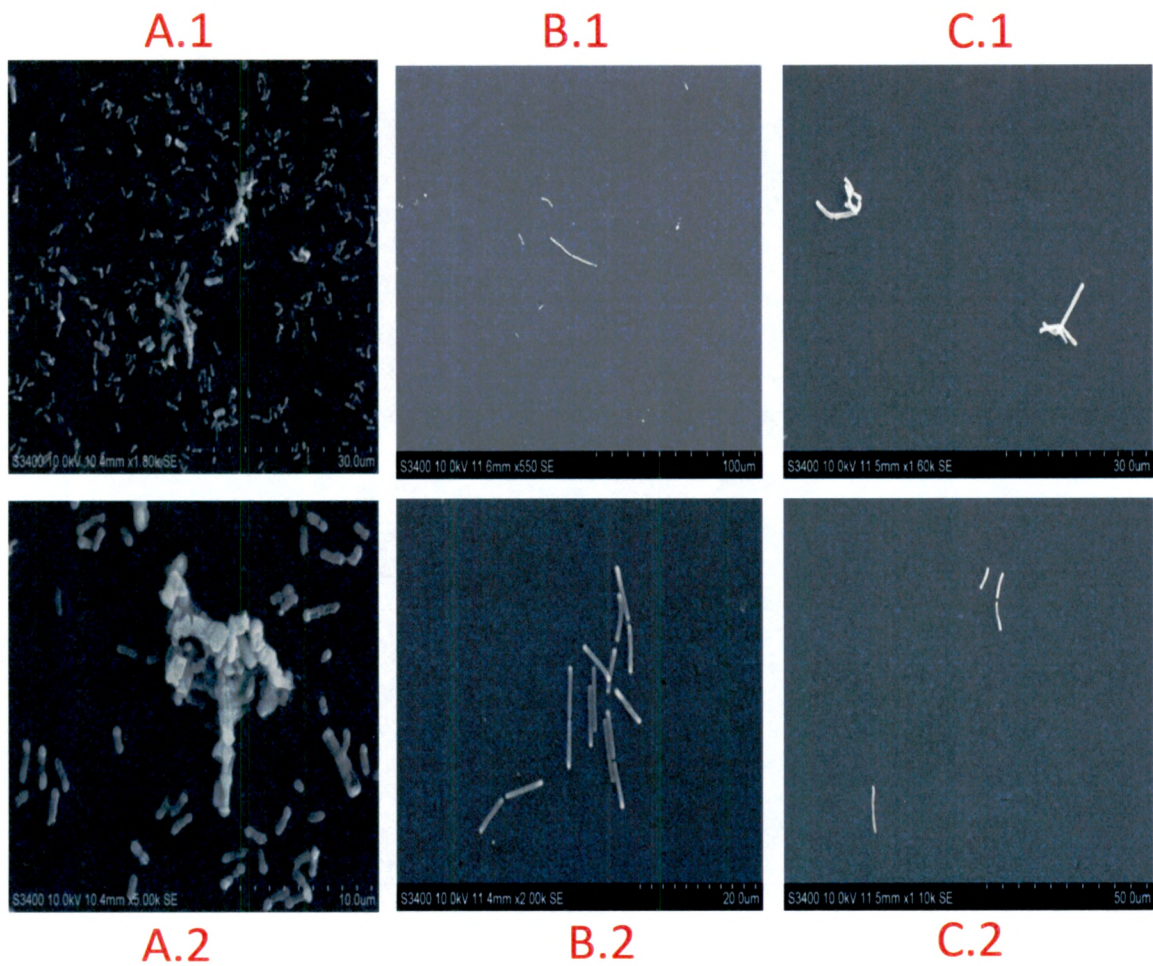


Figure 18: Scanning Electron Microscopy of *Pseudomonas aeruginosa* LTP and EGCG-S treated bacteria cells after 24 hours.

- A1: Control 24 Hours Low Magnification
- A2: Control 24 Hours High Magnification
- B1: 50 µg/mL LTP
- B2: 100 µg/mL LTP
- C1: 50 µg/mL EGCG-S
- C2: 100 µg/mL EGCG-S

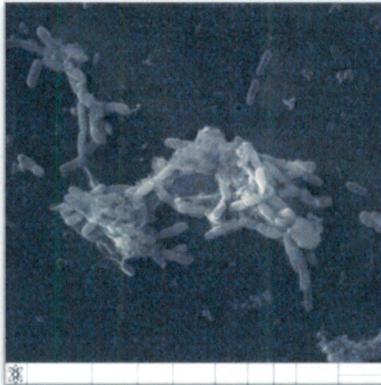


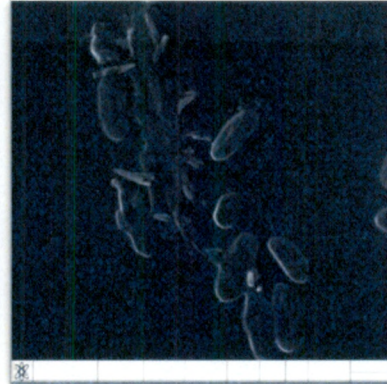
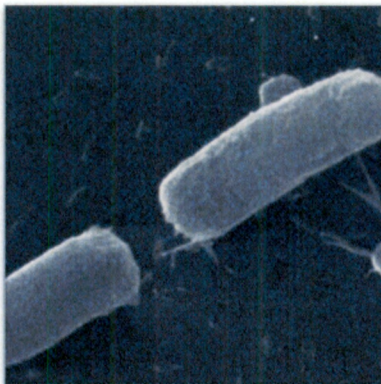

<p>Lower power</p> <p>Control 5 Days</p>			<p>Lower power</p> <p>100 µg/mL EGCG-S</p> <p>5 Days</p>
<p>Higher power</p> <p>Control 5 Days</p>			<p>Higher power</p> <p>100 µg/mL EGCG-S</p> <p>5 Days</p>
<p>Morphology</p> <p>Control 5 Days</p>			<p>Morphology</p> <p>100 µg/mL EGCG-S</p> <p>5 Days</p>

Table 19: Scanning Electron Microscopy of *Pseudomonas aeruginosa* Long Term Treatment for 5 days with 100 ug/mL EGCG-S

B. Study the Effect of Tea Polyphenols on Endospore Germination

1. Endospore Enrichment and Purification

To culture and grow endospores, proper methods and protocols must be used to ensure that a high percentage of endospores were obtained in the culture and stay at the endospore state. To grow endospores typically a specific agar or broth is required and a set amount of days in a nutrient deprived state is also required. This protocol was set up using reported methods to evaluate different procedures to determine which would produce the highest amount of endospores [45]. Originally methods were used to simply grow vegetative cells of endospore forming bacteria within an incubator for an extended period of time (10 days to two weeks) to hopefully generate a high yield of endospores. However, this protocol did not facilitate the growth of endospores and provided a low yielding amount of endospores. Different reported methods were used to enrich endospores formation were analyzed. A modified nutrient agar which simply had MgSO₄ and KH₂PO₄ added was used to enrich endospores and further increase the yield of endospores after 10 days of incubation at 37°C. The microorganisms used in the study were *Bacillus cereus*, *Bacillus megaterium*, and *Bacillus subtilis*. A differential staining method, Schaeffer-Fulton stain, was performed both before and after the growth to view both vegetative cells (in pink) and endospores (in green). The results show vegetative cells of *Bacillus cereus* before the process of enrichment of spore formation. Figures 21 to 23 indicated that almost all the vegetative cells have converted into endospores in all three bacterial species. *B. cereus* produced approximately 99% endospores after 10 days of incubation (Figure 21), *B. subtilis* cultures also showed a very high yield of

endospores of above 95 % (Figure 23). *B. megaterium* showed fewer endospore formation, but still a significant amount of endospores were observed (Figure 22). The results suggested that this method produced around 90%-99% endospore growth when enriching the endospores with modified nutrient agar in these microorganisms.

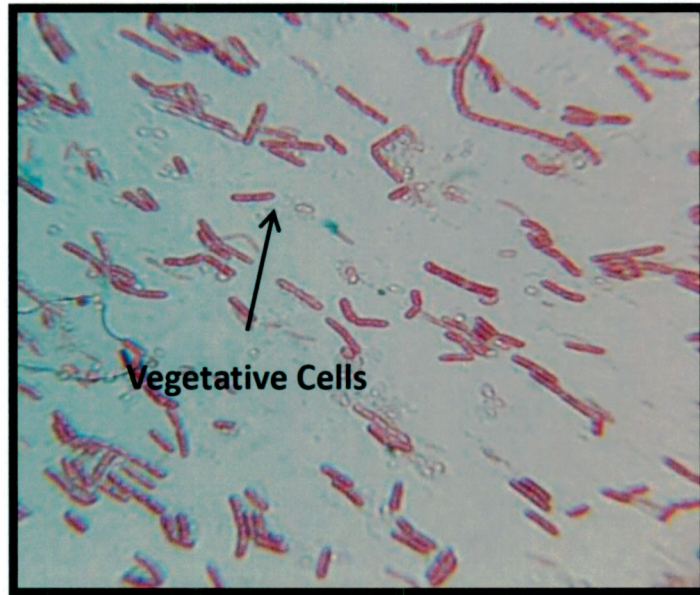


Figure 20: Schaeffer-Fulton stain prior to growing *Bacillus cereus* on modified nutrient agar for 10 days at 37°C in an incubator (1000x Oil)

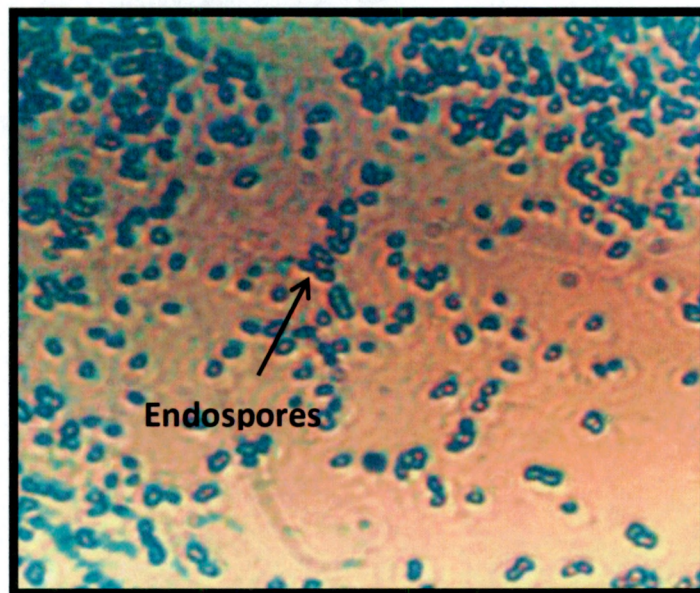


Figure 21: Schaeffer-Fulton stain after growing *Bacillus cereus* on modified nutrient agar for 10 days at 37°C in an incubator. (1000x Oil)

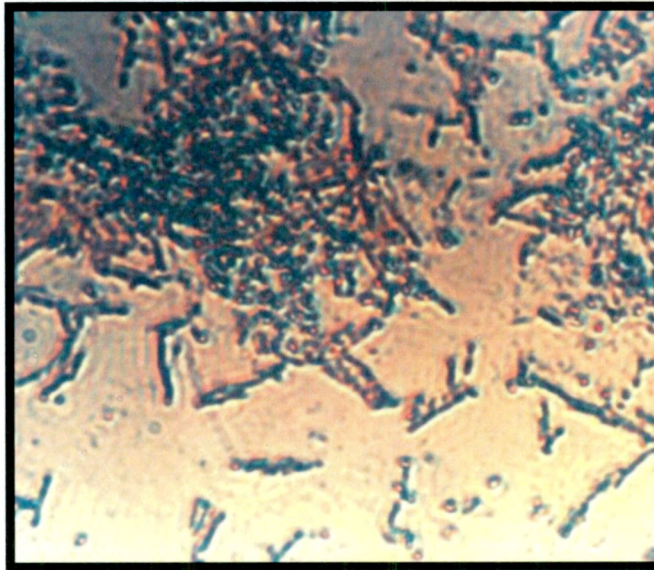


Figure 22: Schaeffer-Fulton stain after growing *Bacillus megaterium* on modified nutrient agar for 10 days at 37°C in an incubator. (1000x Oil)

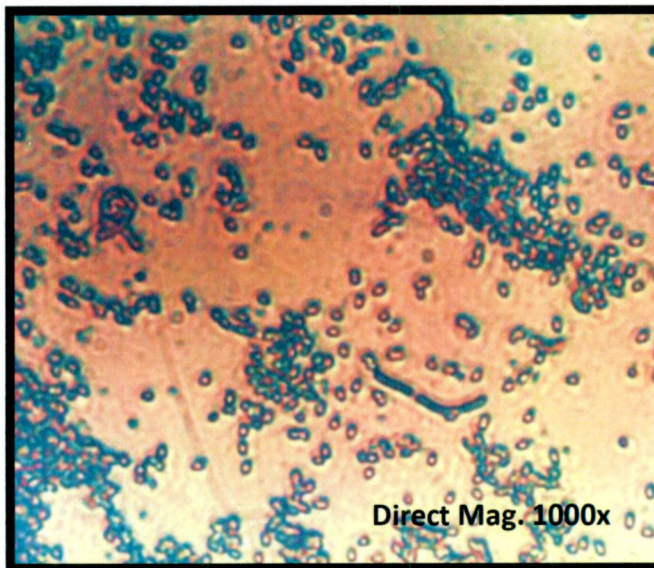


Figure 23: Schaeffer-Fulton stain after growing *Bacillus subtilis* on modified nutrient agar for 10 days at 37°C in an incubator. (1000x Oil)

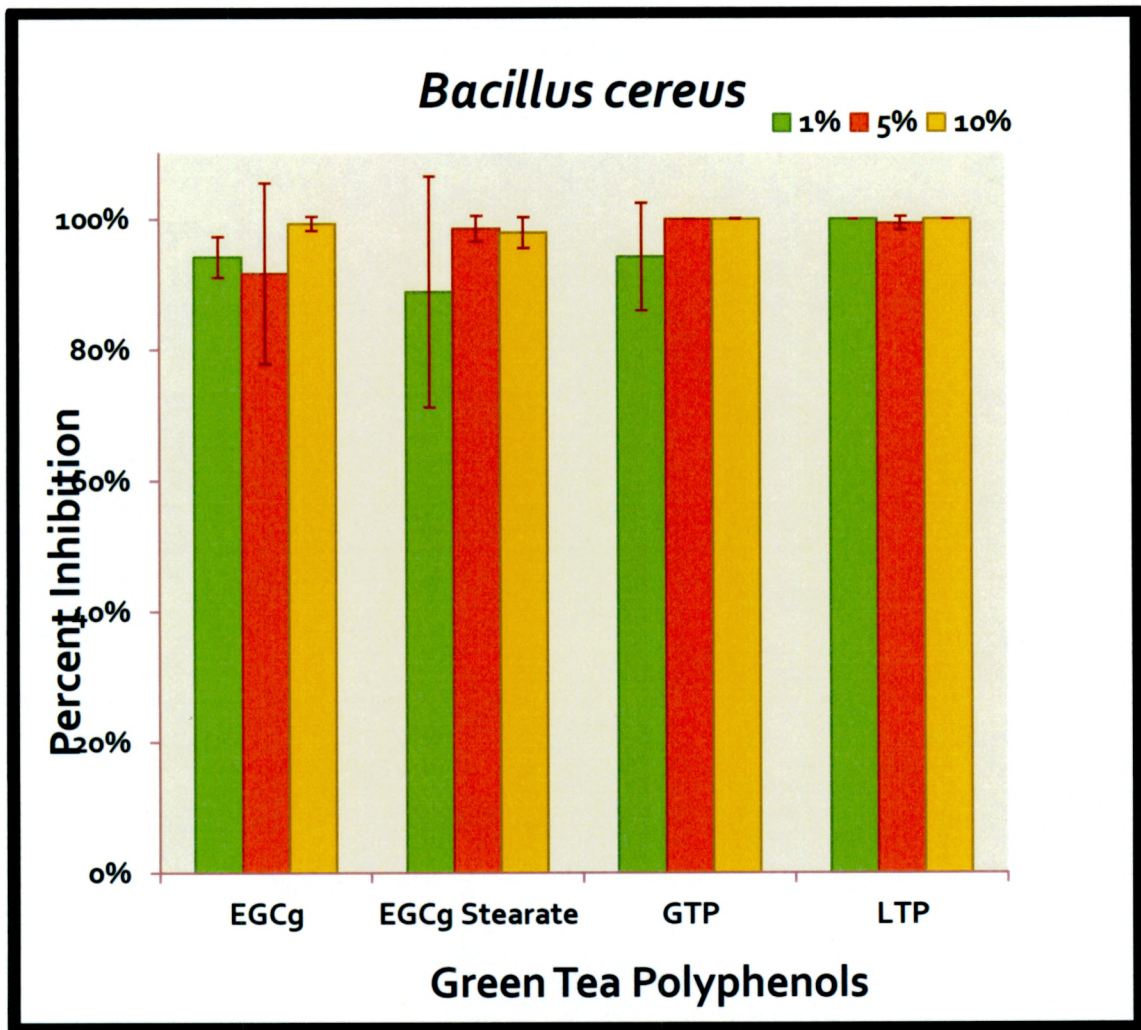
2. Endospore Purification and Treatment with Tea Polyphenols

The procedure for separating endospores that grew on modified nutrient agar from vegetative cells that grew within the same plate consisted of developing a purification protocol that ensured limited vegetative cells would be present prior to endospore germination and treatment. After the 10 day enriched growth period and purity checking, cells were put through a purification procedure detailed within the Materials and Methods, section 14. At the end of the purification procedure, endospores were then treated with tea polyphenols to study the effect of tea polyphenols on endospore germination. This consisted of treating *B. cereus*, *B. megaterium*, and *B. subtilis* endospores with LTP, GTP, EGCG, and EGCG-S, respectively, for 2 hours. Concentrations of 1%, 5%, and 10% of each tea polyphenol were used to treat the three mentioned microorganisms and colony forming unit (CFU) was obtained from serial dilutions for each treatment. Three replicas were carried out for each microorganism and each condition. The results are shown in Figures 24-25. The results indicate that when all tea polyphenols, GTP, LTP, EGCG, and EGCG-S were used at concentrations of 10%, this concentration demonstrated the highest endospore germination inhibition when compared to the control and other conditions. Each tea polyphenol that was used at 10% treatment demonstrated an average inhibition of endospore germination between 97%-100%; EGCG-S with the lowest inhibition at 97.14% on *B. megaterium* and both GTP and LTP with the highest at 100% on *B. cereus* and *B. subtilis*. When each tea polyphenol was used at 5% for treatment, they demonstrated an average inhibition of endospore germination between 91%-100%; EGCG resulted in the lowest at 91.68% on *B. cereus* and EGCG-S resulted in the lowest on *B. megaterium* at 91.91%. Both GTP and LTP had

the highest at 100% on *B. cereus* and *B. subtilis*. Finally, when each tea polyphenol was used at 1% for treatment, each demonstrated an average inhibition of endospore germination ranging from 88% to 100%. These results indicated that each of the tea polyphenols at the concentration of 1%, 5% and 10% have an inhibitory effect on endospore germination from endospore forming bacteria. Overall, all the tea polyphenols studied showed a significant endospore inhibition as noted by CFU.

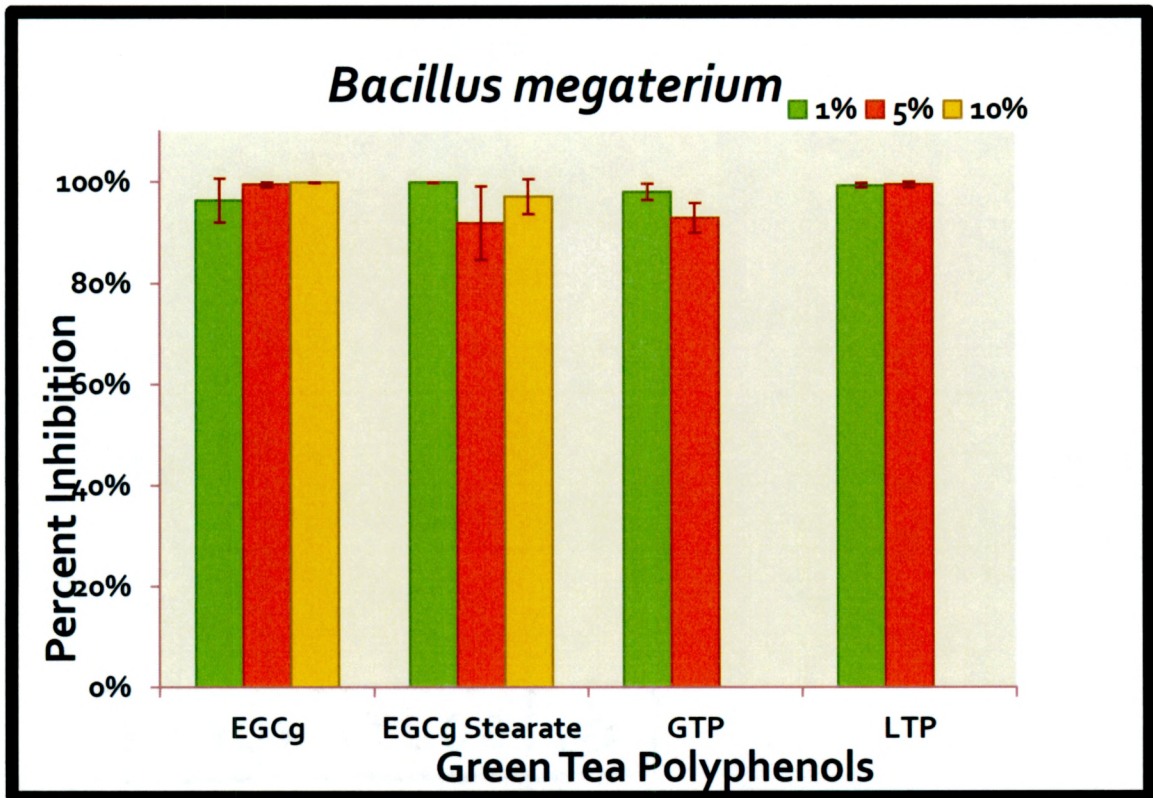
After observing that all treatments were able to inhibit endospore germination, the next step was to determine the minimum inhibitory concentration (MIC) and minimum time to inhibit spores germination with different tea polyphenols. Results from previous experiments, which focused on using a wide range of concentrations for each polyphenol at 1%-10%, and a 2 hour treatment time have shown a significant inhibition for spore germination. Thus, the same experiment and protocol used within the Endospore Purification and Treatment experiment was performed with the concentration set at 1% and 5%. Additionally, the tea polyphenols LTP and EGCG-S were selected for further study since they demonstrated the highest stability and lipophilic properties. The microorganisms selected for the study were also narrowed down to only *B. cereus* since it showed the highest susceptibility to the tea polyphenols. Instead of utilizing the previous 2 hour treatment time, this time kill study focused on minimizing the time to 5, 10, 15, and 30 minutes of treatment in hopes of lowering treatment time. Three replicas were performed and the results are shown in Figure 26, indicating that when using 1% LTP there was an average percentage inhibition of 88.98%-96.53% when treating endospores from 5 minutes to 30 minutes. When using LTP at 5% there was an average percentage inhibition of 92.08%-100% when treating endospores from 5 minutes to 30 minutes.

Similarly, when using EGCG-S at 1% there was an average percentage inhibition of 80.98%-99.73% when treating endospores from 5 minutes to 30 minutes. When using EGCG-S at 5% there was an average percentage inhibition of 85.98%-100% when treating endospores from 5 minutes to 30 minutes. In summary, results show that even when *B. cereus* endospores were treated for 5 minutes with 1% and 5% of both LTP and EGCG-S, there was a significant inhibition of endospore germination from *B. cereus*. Additionally, when treatment time increased up to 15 minutes both LTP and EGCG-S reached their maximum inhibitory concentration. This indicates that treatment time can be minimized from the previous 2 hour treatment to 15 minutes for both LTP and EGCG-S. Different concentrations (1% and 5%) of LTP and EGCG-S were used to treat the spores for 15 minutes and the results shown in Table 1 indicated that both 1% to 5% of these tea polyphenols can achieve near 100 % inhibition of endospore germination.



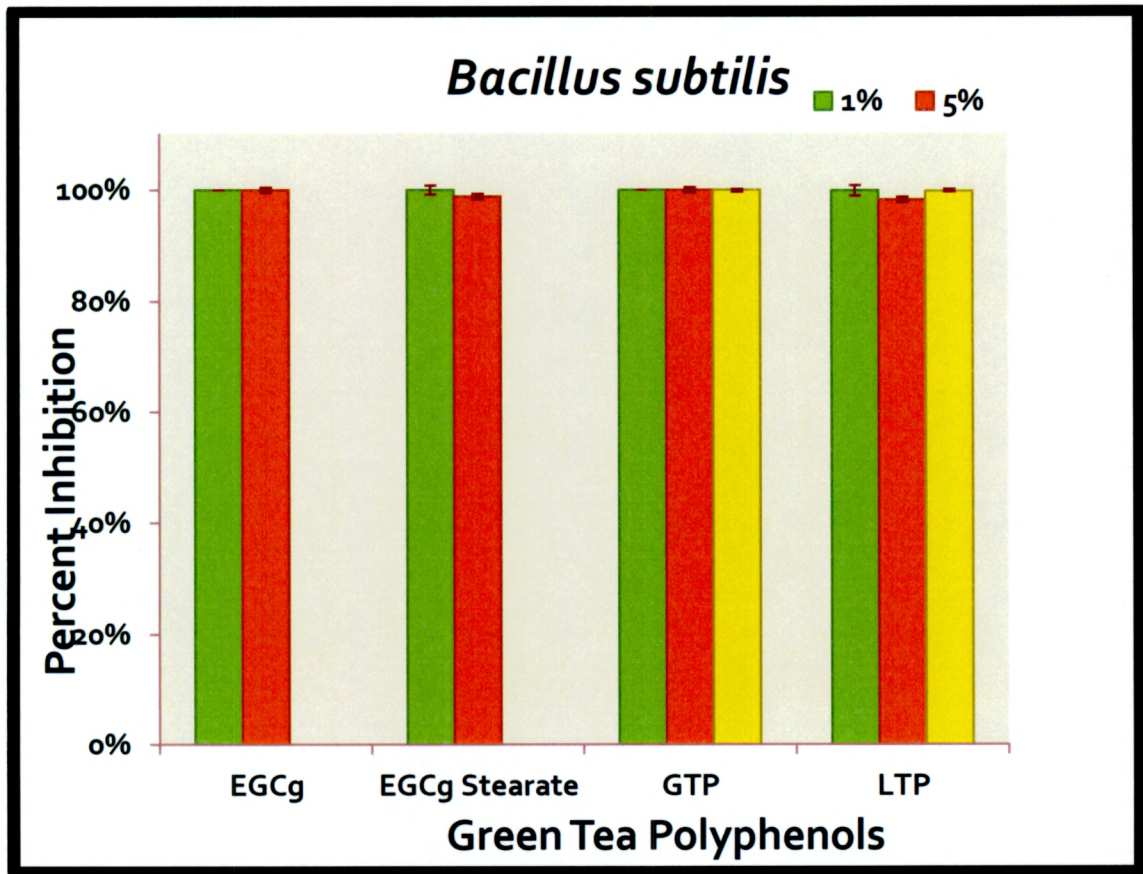
Average Percent Inhibition of <i>B. cereus</i>				
<u>Concentration</u>	<u>EGCg</u>	<u>EGCg-Stearate</u>	<u>GTP</u>	<u>LTP</u>
1%	94.18%	88.81%	94.21%	100%
5%	91.68%	98.49%	100%	99.28%
10%	99%	98%	100%	100%

Figure 24: Percentage of Inhibition of *Bacillus cereus* Endospore Germination using GTP, LTP, EGCG, EGCG-S at 1%, 5%, and 10%.



<u>Average Percent Inhibition of <i>B. megaterium</i></u>				
<u>Concentration</u>	<u>EGCg</u>	<u>EGCg-Stearate</u>	<u>GTP</u>	<u>LTP</u>
1%	96.39%	99.84%	98.02%	99.39%
5%	99.48%	91.91%	96.54%	99.46%
10%	99.85%	97.14%	--	--

Figure 25: Percentage of Inhibition of *Bacillus megaterium* endospore germination using GTP, LTP, EGCG, EGCG-S at 1%, 5%, and 10%.



<u>Average Percent Inhibition of <i>B. subtilis</i></u>				
<u>Concentration</u>	<u>EGCg</u>	<u>EGCg-Stearate</u>	<u>GTP</u>	<u>LTP</u>
1%	99.95%	99.93%	99.96%	99.98%
5%	100%	98.77%	100%	98.17%
10%	--	--	100%	99.68%

Figure 26: Percentage of Inhibition of *Bacillus subtilis* Endospore Germination using GTP, LTP, EGCG EGCG-S, at 1%, 5%, and 10%

3. Transmission Electron Microscopy (TEM) Study on Endospore Germination

After finding that the tea polyphenols work on inhibiting endospore germination, the possible mode of action was studied. To determine how exactly the tea polyphenols were working to inhibit endospore germination, transmission electron microscopy (TEM) was performed on control, LTP (5%) and EGCG-S (5%) treated *B. cereus* endospores. The results are shown in Figures 27 to 30. The standard cells and endospores are shown in Figure 27 and in the control samples, both vegetative cells and endospore forming cells are shown in Figure 28. Images in Figure 29 revealed that LTP was working in a mechanism that was leading to destruction of the spore coat of the endospore and agglutination of spores. Images in Figure 30 clearly demonstrated that the spores' coats were completely destroyed in most of the endospores. The results suggested that one mechanism of these polyphenols is to work on the spore coat and lead to the prevention of the endospores germination.

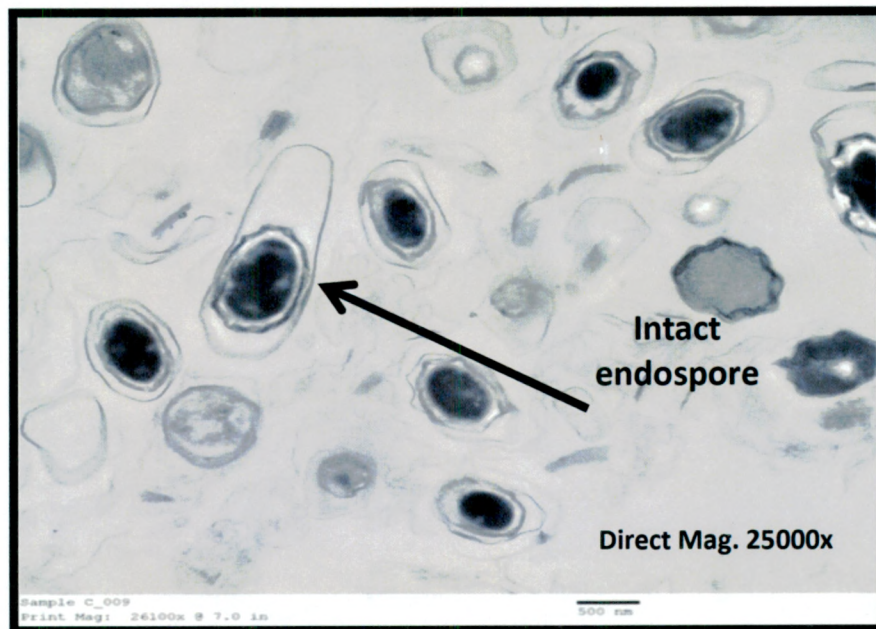
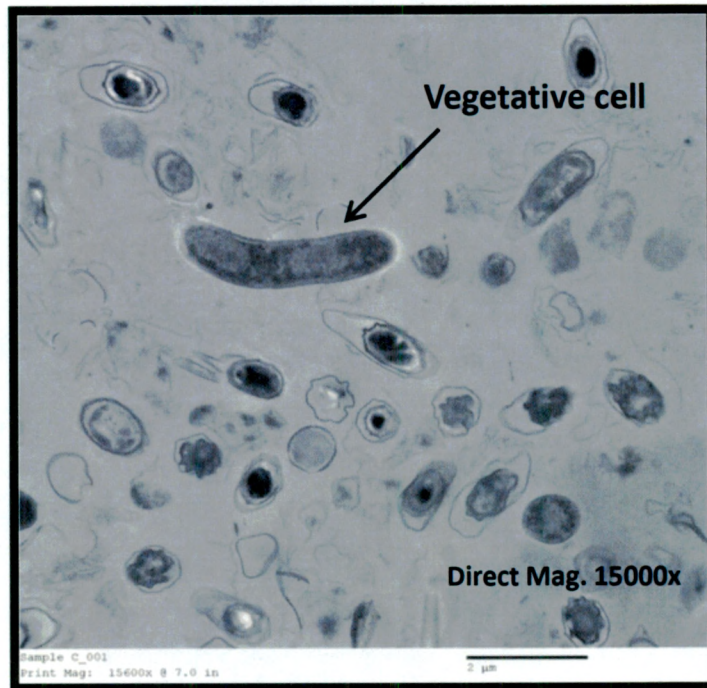


Figure 27: Top. Transmission Electron Microscopy image of Control sample prior to enrich growth for *B. cereus*. Image displays intact vegetative cell (15000x).

Figure 28: Bottom. Intact endospore cell (25000x)

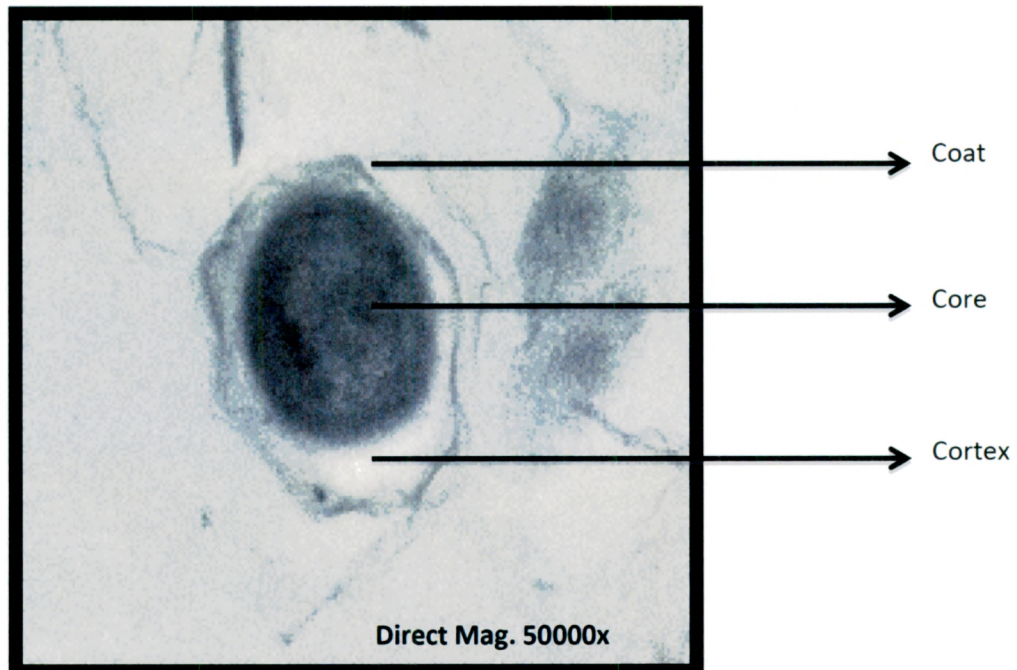


Figure 29: Transmission Electron Microscopy image of Control sample for *B. cereus*.

Image displays intact endospore cell (50000x)

Direct Mag. 80000x

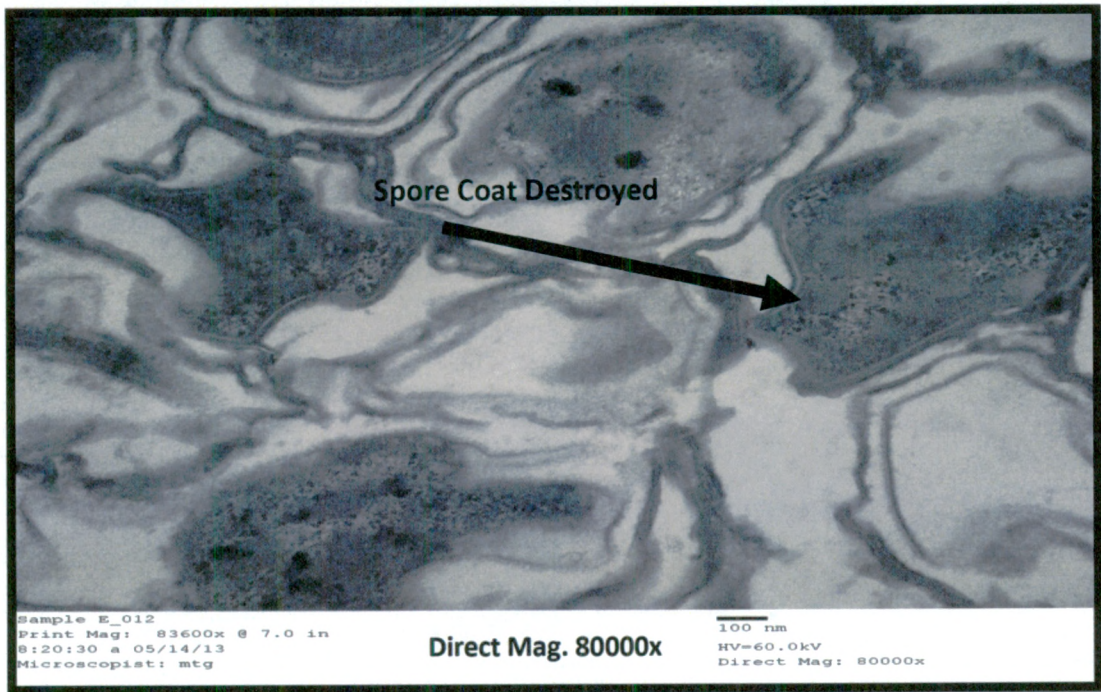
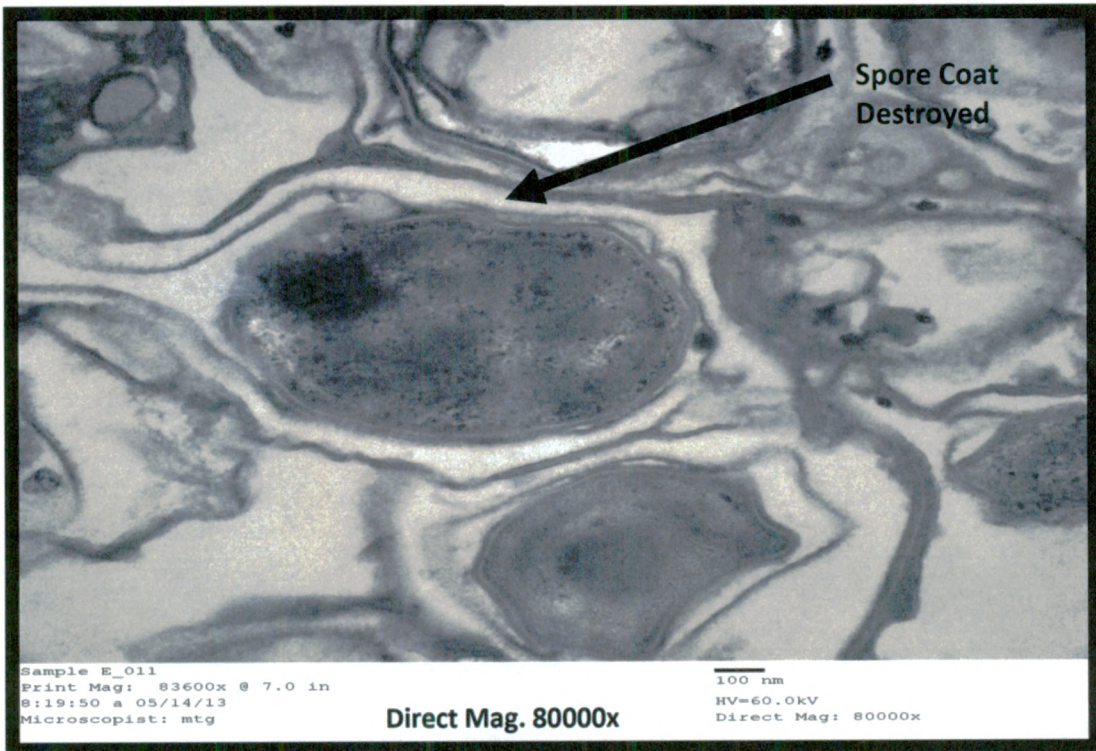
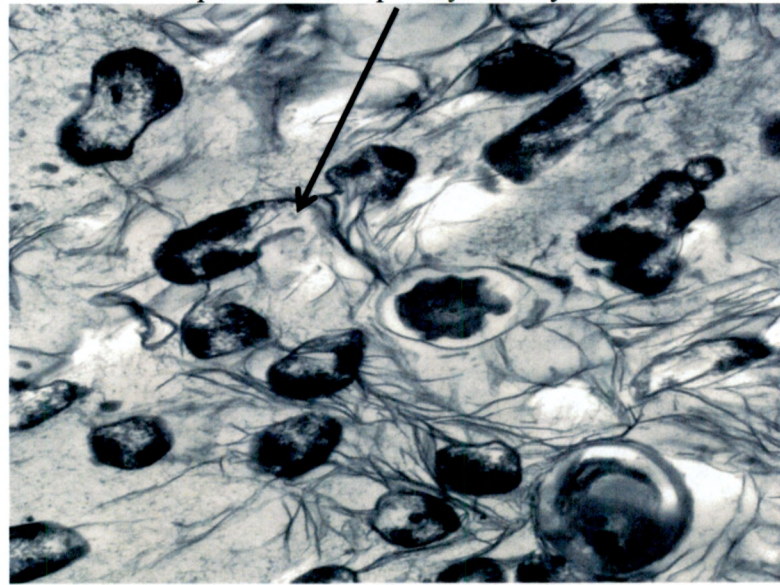


Figure 30: TEM image of LTP treated sample growth for *B. cereus* displays destroyed spore coat (top) and spores agglutination of endospore (80000x)

Spore coat completely destroyed

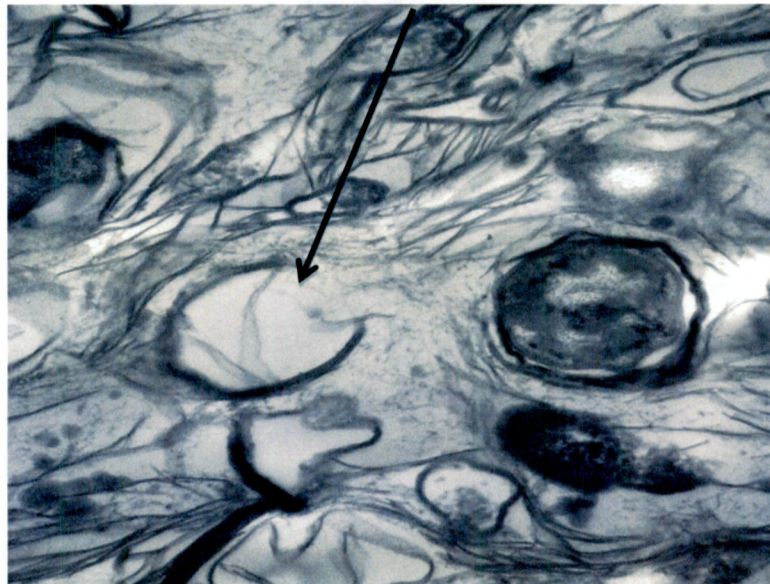


Sample EG_022
Print Mag: 31300x @ 7.0 in
9:30:51 a 05/14/13
Microscopist: mtg

500 nm
HV=60.0kV
Direct Mag: 30000x

Direct Mag. 52000x

Spore coat completely destroyed



Sample EG_007
Print Mag: 62700x @ 7.0 in
8:36:10 a 05/14/13
Microscopist: mtg

500 nm
HV=60.0kV
Direct Mag: 60000x

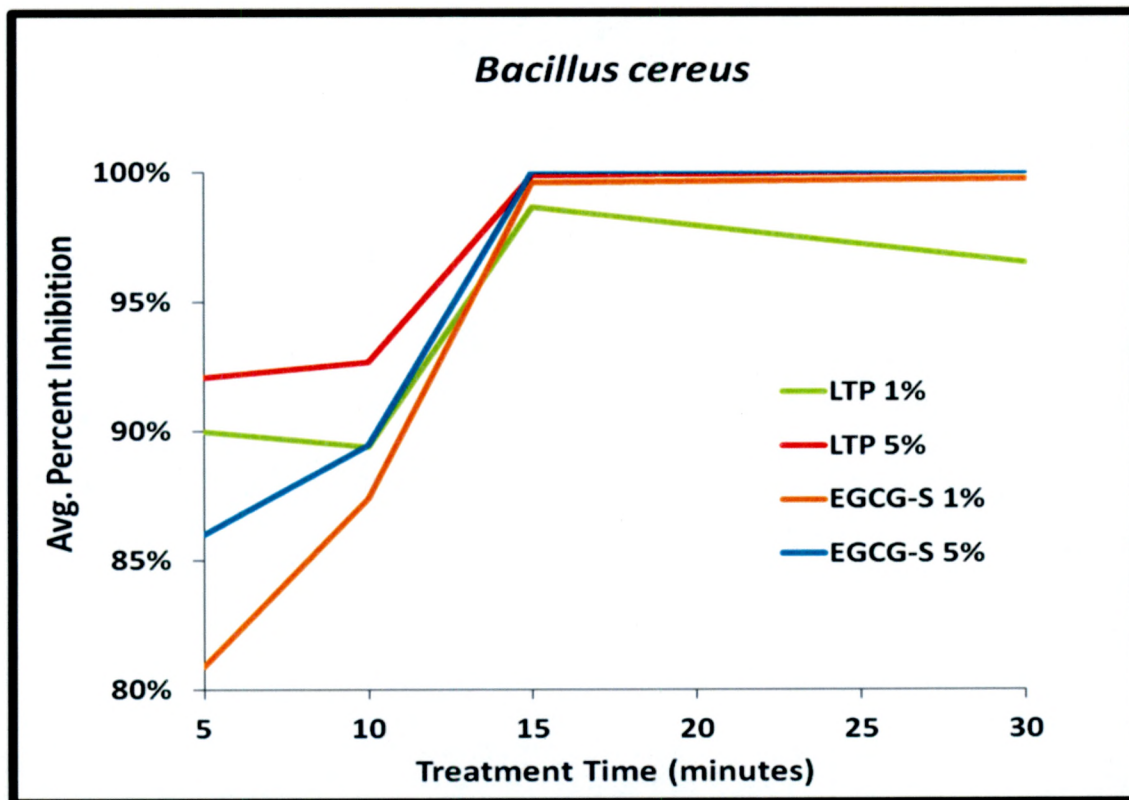
Direct Mag. 52000x

Figure 31: Transmission Electron Microscopy image of EGCG-S treated sample growth for *B. cereus*. Image displays destroyed spore coat (52000x)

4. Endospore Time-Kill Study

After viewing that all treatments were able to inhibit endospore germination, the next step was to determine the minimum inhibitory concentration and minimum time to inhibit spores germination with different tea polyphenols. Results from previous experiments which focused on using a wide range of concentrations for each polyphenol, 1%-10%, and a 2 hour treatment time have shown a significant inhibition for spore germination. Thus, the same experiment and protocol used within the Endospore Purification and Treatment experiment was performed with the concentration range set between 1% and 5% because 10% mostly demonstrated similar effects at 1% and 5% with certain tea polyphenols. Additionally, the tea polyphenols LTP and EGCG-Stearate were selected for further study since they demonstrated the highest inhibitory percentages from previous studies and for their stability and lipophilic properties. The microorganisms selected for the study were also narrowed down to only *B. cereus* because it showed the highest susceptibility to the tea polyphenols. Instead of utilizing the previous 2 hour treatment time, this time kill study focused on minimizing time down to 5, 10, 15, and 30 minutes of treatment in hopes of lowering treatment time. Results shown in Figure 35 indicated that when using LTP at 1% there was an average percentage inhibition of 88.98%-96.53% when treating endospores from 5 minutes to 30 minutes. When using LTP at 5% there was an average percentage inhibition of 92.08%-100% when treating endospores from 5 minutes to 30 minutes. Similarly, when using EGCG-S at 1% there was an average percentage inhibition of 80.98% - 99.73% when treating endospores from 5 minutes to 30 minutes. When using EGCG-S at 5%, there was an average percentage inhibition of 85.98%-100% when treating endospores from 5

minutes to 30 minutes. In summary, results show that even when *B. cereus* endospores were treated for 5 minutes with 1% and 5% of both LTP and EGCG-S there was a significant inhibition of endospore germination from *B. cereus*. Additionally, when treatment time increased up to 15 minutes both LTP and EGCG-S reached their maximum inhibitory concentration. This indicates that treatment time can be minimized from the previous 2 hour treatment to 15 minutes and both LTP and EGCG-S demonstrate the same effects. This minimizes both time and the amount of polyphenols used. The results suggested that 15 minutes and 5% LTP and EGCG-S completely inhibit the endospore.



Treatment Type	EGCG-S 1%		EGCG-S 5%	
	Avg. % Inhibition	Standard Deviation	Avg. % Inhibition	Standard Deviation
5	80.89%	± 1.8985	85.98%	± 2.0529
10	87.39%	± 3.5487	89.44%	± 2.1353
15	99.60%	± 0.4000	100.00%	± 0.0000
30	99.73%	± 0.4619	100.00%	± 0.0000

Figure 32: Percentage of inhibition of *Bacillus cereus* Endospore Germination using EGCG-S at 1% and 5% for 5, 10, 15 and 30 minutes.

Average Percent of Inhibition at a 15-minute Treatment Time.	
LTP 1%	98.7%
LTP 5%	99.9%
EGCG-S 1%	99.6%
EGCG-S 5%	100.0%

Table 6: Average Percentage of Inhibition of *Bacillus cereus* Endospore Germination using LTP and EGCG-S at 1% and 5% for 15 minutes.

Conclusion

After performing numerous experiments with green tea polyphenols, it can be concluded that the green tea polyphenols are able to work to inhibit biofilm formation from both bacteria, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. This has been seen when using each of the green tea polyphenols: GTP, LTP, EGCG, and EGCG-S. Concentrations of 50µg/mL to 200µg/mL all demonstrated inhibitory effects on the production of biofilm. In addition to working to inhibit biofilm formation, these green tea polyphenols demonstrated the ability to damage the cell membrane of bacteria cells and cause agglutination of biofilm forming cells which seems to be the mechanism of how they inhibit biofilm formation. The MIC for each green tea polyphenol varied, but was typically between the concentrations of 50µg/mL to 100µg/mL. As demonstrated by the SEM images, the green tea polyphenols not only morphologically change but they also do not demonstrate any biofilm formation.

These tea polyphenols can potentially be used to lower the amount of endospores present in environmental samples such as food, catheters, stents, etc. As seen by the images, tea polyphenols appear to be affecting endospore's spore coat in a way that leads to a disruption of the endospore state of these cells. Potential mechanisms could deal with the structural component of endospore formation and how the tea polyphenols could be effecting the transition from vegetative cells to endospore cells. In addition to a disruption in how endospores germinate the tea polyphenols seem to be forcing endospore cells to agglutinate with one another and inhibit endospore germination. As seen by the time kill study, inhibiting endospore germination through the application of tea polyphenols seem to require only a treatment time of 15 minutes as compared to the

original 2 hours of treatment applied in previous experiments. These experiments show that the tea polyphenols only require a small period of time and a lower concentration of tea polyphenols to effectively inhibit endospore germination. Although, these seem as plausible mechanisms, further experiments should be carried out to study the potential usage for tea polyphenols as a food preservative to prevent food spoilage and contamination due to endospore forming bacteria.

These research findings demonstrate the broad and applicable spectrum of effects these green tea polyphenols have on both biofilm forming bacterial cells and endospores. It can be concluded that GTP, LTP, EGCG, and EGCG-S work very well as antimicrobial nature derived agents. The purified green tea polyphenols, EGCG and EGCG-S both seem to have a stronger inhibitory effect against biofilms and endospores. The potential applicable use of these polyphenols are not limited to use in medical settings, food industries, and industrials. They can also potentially become integrated into drugs for pharmaceutical production.

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