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Targeting Prosthetic Joint Infections Using Modified EGCG Derivatives with Antibiotics

Kavneet Chahil

Abstract

Prosthetic joint implantations have revolutionized orthopedic medicine by allowing individuals to regain physical function with minimal side effects. However, they are susceptible to post- surgical complications due to bacterial infections. Three significant bacteria that are responsible for the development of joint infections include *Staphylococcus epidermidis, Staphylococcus aureus,* and *Pseudomonas aeruginosa*, which act by adhering to the prosthesis and form biofilms thus becoming antibiotic-resistant. It has been reported that green tea polyphenols, which are extracted from the leaves of the *Camellia sinensis* plant, have antibacterial, antioxidant, and anti- inflammatory properties. The purified, and modified epigallocatechin-3-gallate-stearate (EGCGS) and palmitoyl-epigallocatechin-3-gallate (P-EGCG) are more efficient and have shown their synergistic effect on antibiotics indicating they could potentially play a positive role in the elimination of infection. In this study, the inhibitory effect of EGCG-S and P-EGCG with or without two antibiotics (Bacitracin or Polymyxin B) on the three most prominent bacteria was investigated. Colony-forming unit (CFU) assays were used to determine the percent of inhibition by the tea polyphenols. Time course studies were performed using CF1 and CF2 formulations to determine the percent of inhibition. Congo Red assays were used to analyze the effect of tea polyphenols on biofilm. Crystal violet quantitative biofilm assays were used to analyze the effect of tea polyphenols on the percent of biofilm inhibition. This study showed that EGCG-S and P-EGCG, when used in combination with Bacitracin or Polymyxin B resulted in the best antibacterial effect and could be used as a treatment against bacteria that cause joint infections. CF1 and CF2 formulations are also promising treatment options.

MONTCLAIR STATE UNIVERSITY

Targeting Prosthetic Joint Infections Using Modified EGCG Derivatives with Antibiotics

by

Kavneet Chahil

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Introduction

We utilize our joints daily to perform activities such as running, walking, climbing stairs among many other functions (Etoundi, Semasinghe, Agrawal, Dobner & Jafari, 2021). A joint is the point of connection where two bones meet and allow for locomotion as well as stability (Voss & Montavon, 2009). Over time, the daily activities that we partake in can cause joint diseases where the cartilage and bones of our joints are torn and damaged as well as ligaments becoming weak (Bullock et al., 2018). Some joint related diseases are osteoarthritis and rheumatoid arthritis (Bullock et al., 2018). Osteoarthritis is a degenerative joint disease that affects 25% of adults (Chen et al., 2017). In osteoarthritis, there is the destruction and progressive loss of cartilage, inflammation of the synovium and the degeneration of the ligaments (Chen et al., 2017). An individual with this disease can have joint instability, chronic pain and stiffness around the joints (Chen et al., 2017). Rheumatoid arthritis is an autoimmune inflammatory disease that starts off by affecting small joints, later progressing to bigger joints (Bullock et al., 2018). In this disease, bone erosion as well as deformities caused by joint damage can result (Bullock et al., 2018). Treatments for osteoarthritis and rheumatoid arthritis are aimed to maximize the function of joints, decrease inflammation, and reduce pain (Bullock et al., 2018).

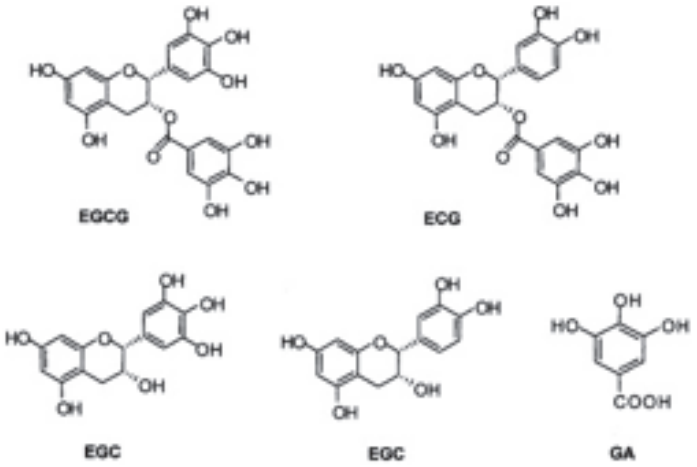
Joint artificial replacement surgery with prosthetic implants has restored mobility in millions of people that have arthritis (Hench, 2005). In 2009, over 905,000 joint replacement surgeries have been performed in the US and osteoarthritis is ranked as the fourth most common cause of hospitalization (Song et al., 2013). A disadvantage of joint replacement surgery is that it can cause bacterial infections, caused by septic or aseptic failure, that are difficult to treat solely with antibiotic treatment (Song et al., 2013; Kasch et al., 2017). Bacterial infections can be

categorized into three categories which include early, delayed, or late onset of symptoms after a joint prosthetic implant. Early infections normally occur within three months from when an individual got a prosthetic joint implant put in. Delayed infections can occur from three months after the joint implant surgery up until two years, while late infections are those that occur more than two years from the surgery (Tande & Patel, 2014).

The main pathogens that cause these bacterial infections are gram-positive bacteria, but gram-negative bacteria can also cause these infections (Rodriguez-Merchan & Liddle, 2018). *Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermidis* are the main three pathogens that cause prosthetic joint infection. These bacteria are opportunistic in the terms that they are able to stick to materials like titanium, polymethylmethacrylate cement and cobalt-chromium, which are used to make prosthetic joint implants and, in the process, form a layer of biofilm to protect itself (Song et al., 2013; Davidson et al., 2019). The formation of biofilm is where bacterial cells are enclosed in a matrix that is primarily made up of nucleic acids, polysaccharides and proteins (Fey & Olson, 2010). Biofilm is formed in four main steps including the adherence of the bacterial cell, growth and accumulation of the bacterial cells, biofilm maturation, and finally detachment (Fey & Olson, 2010). Bacteria use quorum sensing in order to respond to gene expression changes that therefore allow biofilm to form (Trampuz & Zimmerli, 2005). Quorum sensing can also lead to transcriptional changes which produce genes for antibiotic resistance and virulence (Davidson et al., 2019; Nana et al., 2016). Gram-positive bacteria use autoinducing peptides as their quorum sensing system (Rutherford & Bassler, 2012). Gram-negative bacteria use autoinducers, which are extracellular signaling molecules to communicate with one another (Rutherford & Bassler, 2012). Protease and elastase are two

virulence factors that these three bacteria produce and they help in the process of quorum sensing to form biofilm and have the ability to cause tissue damage (Maeda, 1996).

While 1 to 2 percent of prosthetic joint implants lead to bacterial infections, this is cause for concern because it is associated with expensive medical costs, high rates of morbidity and can possibly lead to implant failure (Ribeiro et al., 2012). Treating these bacterial infections usually involves antimicrobial therapy, debridement, or irrigation with antiseptic solution (wash). There are two main problems with these treatments approaches which include that the bacteria can become antibiotic resistant and that the bacteria are able to form biofilm as a defense mechanism and therefore causing the infection to get bigger. Alternative approaches have been researched, one of which is green tea polyphenols. Green tea polyphenols, which are extracted from the leaves of the *Camellia sinensis* plant have anti-microbial, anti-inflammatory, and anti-oxidant properties that can inhibit infectious agents and be helpful in treating infections (Reygaert, 2018). Catechins are major tea polyphenols that are found in green tea (Wu & Brown, 2021), which contribute to green tea's health beneficial properties. There are four main types of catechins including epigallocatechin-3-gallate (EGCG), epicatechin (EC), epigallocatechin (EGC) and epicatechin-3-gallate (ECG) (Cabrera, Artacho & Gimenez, 2006). Epigallocatechin-3-gallate (EGCG) makes up about 59 percent of the catechins (Cabrera, Artacho & Gimenez, 2006).



Structure 1. Molecules of green tea catechins
(Cabrera, Artacho, & Giménez 2006)

Studies have shown that epigallocatechin-3-gallate (EGCG) can inhibit the growth of bacteria (Taylor, Hamilton-Miller & Stapleton, 2005). EGCG has antimicrobial and bactericidal effects on both gram-positive and gram-negative bacteria (Wu & Brown, 2021). EGCG binds to the cell membrane of the bacteria and damages it. This damage to the cell membrane inhibits the ability of the bacteria to bind to host cells as well as inhibiting bacteria to attach to one another to form biofilms, which are important for pathogenesis of bacteria (Reygaert, 2014). EGCG is known to synergistically work together with antibiotics and brings forth an encouraging approach to treat antibiotic resistant strains of bacteria (Reygaert, 2018; Wu & Brown, 2021). Since EGCG is not stable, modified lipophilic palmitoyl-epigallocatechin-3-gallate (P-EGCG) and epigallocatechin-3-gallate stearate (EGCG-S) have been used and reported to have promising antimicrobial activity (Liu, Kang & Yan, 2021). Recently, several reports suggested that EGCG-S and P-EGCG can enhance the activities of different antibiotics (Yussof et al., 2019). In addition, these tea polyphenols have also displayed antibiofilm activities in many Gram-positive and Gram-negative bacteria (Shinde et al., 2021). The process of making optimal

P-EGCG containing formulations are underway. Two most advanced formulations CF1, a gel form and CF2, in a solution form are established (Chu et al., 2020). The effect of P-EGCG, EGCG-S and the new formulations (CF1 and CF2) containing P-EGCG will be used to evaluate their effect on the joint infecting bacteria and further develop organic antimicrobial agents to combat the major microorganisms and prevent joint infection.

Objectives of this Study

The objectives of this study are:

1. Characterization of the three bacteria: *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis*.
2. Study the synergistic effect of EGCG-S and P-EGCG with antibiotics/wash on *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis*.
3. Evaluate CF1 and CF2 formulations as antibacterial agents.
4. Examine the possible synergistic effects that EGCG-S and P-EGCG with antibiotics/wash have on biofilm formation.
    I. Congo Red Qualitative Biofilm Assay
    II. Crystal Violet Quantitative Biofilm Assay

Materials and Methods

1. Bacterial Cultures

Three bacteria were used: *Pseudomonas aeruginosa (P. aeruginosa)*, *Staphylococcus aureus (S. aureus)*, and *Staphylococcus epidermidis (S. epidermidis)*. The bacterial cultures were maintained on nutrient agar (NA) plates and/or in nutrient broth. The nutrient agar plates were stored in the refrigerator at 4℃. For every experiment, fresh overnight cultures were made from the stock plates. The overnight cultures were made by taking a small amount of the bacteria from the stock plates using a inoculating loop or cotton swab and placing the bacteria into a falcon tube containing nutrient broth (NB). The falcon tubes containing the bacteria and nutrient broth were labeled with the name of the bacteria, the date and the initials of the person performing the experiment. They were left to incubate overnight at 250 rpm and 37℃. The next day, the overnight culture was removed from the incubator.

2. Media Preparation

The media used for the experiments performed in this study were Nutrient Broth and Nutrient Agar. The nutrient broth was prepared as follow: 8 grams of media was mixed with 1 Liter of distilled water. The nutrient agar was prepared as follows: 23 grams of the media from Difco was mixed with 1 Liter of distilled water in a 2 Liter flask. After the media was mixed and dissolved, it was autoclaved at 121℃ for 30 minutes and set to the liquid cycle setting. When the nutrient broth and nutrient agar were done being autoclaved, the nutrient broth was cooled at room temperature, followed by being stored in the -4℃ cold room. The nutrient agar, after being autoclaved was cooled down at room temperature and poured intro petri dishes aseptically.

3. Gram Stain

Gram staining was performed to make sure that the bacterial culture is pure and not contaminated. It was also used to determine the morphological characteristics of the bacteria. A sterile cotton swab was used to take some bacteria from the stock plate and then smeared onto a sterile microscope slide. The microscope slide containing the bacterium sample was dried and heat fixed. Crystal violet was applied to the slide for 20 seconds and then rinsed off with deionized water for 2 seconds. Gram's iodine was added to the slide for 1 minute, and then washed off with Gram's decolorizer for 10 seconds. The decolorizer was rinsed off with deionized water for 2 seconds. Safranin was used to counter-stain the slide and applied for 1 minute. After 1 minute, the safranin was rinsed off the slide with deionized water. A cover slip was then put on the slide and the slide was dried with bibulous paper. Using a light microscope, the bacteria sample was then observed at 1000x total magnification (Lee et al., 2015).

4. Preparation of Treatments

a. Tea Polyphenols

EGCG-S and P-EGCG were purchased from Camellix LLC, Augusta, GA. A stock of 10X 250 μg/mL was prepared. The EGCG-S and P-EGCG compounds were dissolved in 200 proof ethanol. The stocks were then stored in a -20℃ freezer. Below is the calculation of the stock and the dilution:

250 μg/mL= 0.00025 grams of compound + 1 mL of EtOH/H_2O

b. Antibiotics

The powders for Polymyxin B and Bacitracin, which were the two antibiotics used in this study were purchased from Sigma Aldrich. Polymyxin B and Bacitracin were made at 100X stock concentrations at 1.98 mg/mL and 22.5 mg/mL, respectively. Polymyxin B was made at 10X using 100 μL from the 100X stock of 1.98 mg/mL Polymyxin B and diluting it with 900 μL of 200 proof Ethanol. Bacitracin was made at 10X using 100 μL from the 100X stock of 22.5 mg/mL and diluting it with 900 μL of 200 proof Ethanol. The antibiotics were stored in the -20℃ freezer.

c. Wash

The wash that was used was prepared by mixing 10 mL of 0.9% saline with 1.57 mL of 0.25% sodium hypochlorite and 0.3 mL povidone iodine (Dynarex).

d. Combination Treatments

Combination Treatments were prepared as followed:

EGCG-S+Wash: 100 μL of 250 μg/mL EGCG-S + 900 μL of Wash

EGCG-S+ Polymyxin B: 100 μL of 250 μg/mL EGCG-S + 10 μL of 100X Polymyxin B

EGCG-S+ Bacitracin: 100 μL of 250 μg/mL EGCG-S + 10 μL of 100X Bacitracin

Wash+Polymyxin B: 990 μL of Wash + 10 μL of 100X Polymyxin B

Wash+Bacitracin: 990 μL of Wash + 10 μL of 100X Bacitracin

Polymyxin B+Bacitracin: 10 μL of 100X Polymyxin B + 10 μL of 100X Bacitracin + 980 μL Saline

EGCG-S+ Wash+Polymyxin B: 100 μL of 250 μg/mL EGCG-S + 890 μL of Wash + 10 μL of 100X Polymyxin B

EGCG-S+ Wash+Bacitracin: 100 μL of 250 μg/mL EGCG-S + 890 μL of Wash + 10 μL of 100X Bacitracin

EGCG-S+ Polymyxin B+Bacitracin: 100 μL of 250 μg/mL EGCG-S + 10 μL of 100X Polymyxin B + 10 μL of 100X Bacitracin

Wash+Polymyxin B+Bacitracin: 980 μL of Wash + 10 μL of 100X Polymyxin B+ 10 μL of 100X Bacitracin

EGCG-S+ Wash+Polymyxin B+Bacitracin: 100 μL of 250 μg/mL EGCG-S + 880 μL of Wash + 10 μL of 100X Polymyxin B + 10 μL of 100X Bacitracin

P-EGCG+ Wash: 100 μL of 250 μg/mL P-EGCG + 900 μL of Wash

P-EGCG+ Polymyxin B: 100 μL of 250 μg/mL P-EGCG + 10 μL of 100X Polymyxin B

P-EGCG+ Bacitracin: 100 μL of 250 μg/mL P-EGCG + 10 μL of 100X Bacitracin

P-EGCG+ Wash+Polymyxin B: 100 μL of 250 μg/mL P-EGCG + 890 μL of Wash + 10 μL of 100X Polymyxin B

P-EGCG+ Wash+Bacitracin: 100 μL of 250 μg/mL P-EGCG + 890 μL of Wash + 10 μL of 100X Bacitracin

P-EGCG +Polymyxin B+Bacitracin: 100 μL of 250 μg/mL P-EGCG + 10 μL of 100X Polymyxin B + 10 μL of 100X Bacitracin

P-EGCG+ Wash+Polymyxin B+Bacitracin: 100 μL of 250 μg/mL P-EGCG + 880 μL of Wash + 10 μL of 100X Polymyxin B + 10 μL of 100X Bacitracin

After the combination treatments were prepared, they were used for Colony-forming unit (CFU) Assays, which were treated for 30 seconds to 5 minutes. Overnight cultures of the bacteria were prepared. 10 μL of overnight culture was put into an eppendorf tube for each

treatment. To the eppendorf tube, 90 μL of treatment was added and the eppendorf tube was vortexed for 10-15 seconds. The eppendorf tubes were then treated for 30 seconds and 5 minutes. After the treatment time of either 30 seconds or 5 minutes, 25μL was taken out of the eppendorf tube and plated out on nutrient agar X plates. The nutrient agar X plates were then incubated overnight and then next day, the colonies on the X plates were counted. The percent of inhibition was calculated as follows:

% Inhibition= ((Control CFU-Treated CFU)/Control CFU)) X 100

Log Reduction was also calculated as follows:

Log Reduction= log(Control CFU/Treated CFU)

5. Congo Red Qualitative Biofilm Assay Experiment

To study the effects of antibiotics and tea polyphenols on biofilm formation, congo red assays were performed. Overnight cultures of the bacteria were made. Treatment of Biofilm formation: 5 μL of the respective overnight culture was mixed with 45 μL of treatment in micro-centrifuge tubes and treated for 5 minutes. After 5 minutes, 25 μL was plated in its respective well. The plate was placed in the incubator at 37℃ for two days, followed by analysis.

TSB/NB	EGCG-S (250 ug/mL)	P-EGCG (250 ug/mL)	Wash	Polymyxin B (1.98 mg/mL)	Bacitracin (22.5 mg/mL)
EGCG-S+ Wash	EGCG-S+ Polymyxin B	EGCG-S+ Bacitracin	Wash+ Polymyxin B	Wash+ Bacitracin	Polymyxin B+ Bacitracin
EGCG-S+ Wash+ Polymyxin B	EGCG-S+ Wash+ Bacitracin	EGCG-S+ Polymyxin B+ Bacitracin	Wash+ Polymyxin B+ Bacitracin	EGCG-S+ Wash+ Polymyxin B+ Bacitracin	P-EGCG+ Wash
Blank	P-EGCG+ Polymyxin B	P-EGCG+ Bacitracin	P-EGCG+ Wash+ Polymyxin B	P-EGCG+ Wash+ Bacitracin	P-EGCG+ Polymyxin B+ Bacitracin
P-EGCG+ Wash+ Polymyxin B+ Bacitracin	Bleach				

Diagram 1: Plate set up of Congo Red assay

6. Crystal Violet Quantitative Biofilm Assay Experiment

To study the effect of tea polyphenols, antibiotics and CF1/CF2 formulations on biofilm formation, crystal violet assays were performed. Overnight cultures of the bacteria were made. The samples were prepared in micro-centrifuge tubes according to the following template:

Treatment: EGCG-S (250 μg/mL) 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment	**Treatment: EGCG-S (250 μg/mL) +Polymyxin B (10X)** 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment
Treatment: Bacitracin (10X) 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment	**Treatment: EGCG-S (250 μg/mL) +Polymyxin B (10X) +Bacitracin (10X)** 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment
Treatment: Polymyxin B (10X) 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment	**Treatment: CF1** 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment
Treatment: EGCG-S (250 μg/mL) + Bacitracin (10X) 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment	**Treatment: CF2** 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment
Treatment: Bleach 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment	**Treatment: P-EGCG (250 μg/mL)** 600 μL of TSB + 300 μL of Overnight Culture + 100 μL of Treatment

Diagram 2: Set up of Crystal Violet Experimental Plate

***P. aeruginosa* Control:** 700 μL TSB +300 μL Culture	***S. aureus* Control:** 700 μL TSB +300 μL Culture	***S. epidermidis* Control:** 700 μL TSB +300 μL Culture	**Negative Control: EGCG-S (250 μg/mL)** 900 μL TSB +100 μL EGCG-S	**Negative Control: Bleach** 900 μL TSB +100 μL Bleach	**Negative Control: CF1** 900 μL TSB + 100 μL CF1
Negative Control: CF2 900 μL TSB + 100 μL CF2	**Negative Control: Erythromycin** 900 μL TSB + 100 μL Erythromycin	**Negative Control: Tetracycline** 900 μL TSB + 100 μL Tetracycline	**Negative Control: Bacitracin (10X)** 900 μL TSB + 100 μL Bacitracin	**Negative Control: Polymyxin B (10X)** 900 μL TSB + 100 μL Polymyxin B	**Negative Control: EGCG-S+ Erythromycin** 900 μL TSB + 100 μL Treatment
Negative Control: EGCG-S+ Tetracycline 900 μL TSB + 100 μL EGCG-S+ Tetracycline	**Negative Control: EGCG-S+ Bacitracin** 900 μL TSB + 100 μL EGCG-S+ Bacitracin	**Negative Control: EGCG-S+ Polymyxin B** 900 μL TSB + 100 μL EGCG-S+ Polymyxin B	**Negative Control: EGCG-S+ Polymyxin B+ Bacitracin** 900 μL TSB + 100 μL EGCG-S+ Polymyxin B + Bacitracin	**Negative Control: Polymyxin B + Bacitracin** 900 μL TSB + 100 μL Polymyxin B + Bacitracin	**Negative Control: Blank. Only TSB** 1000 μL TSB

Diagram 3: Set up of Crystal Violet Control/Negative Control Plate

The plates were labeled with date and placed into the incubator at 37℃ for 4 days (Nowak et al., 2015). On the fourth day, the plates were aspirated, washed with 1X PBS, and aspirated (Nowak et al., 2015). The wells were stained with 0.1% crystal violet solution and left to sit for 30 minutes (Nowak et al., 2015). After 30 minutes, the wells were aspirated, then washed with 1X PBS, aspirated followed by the plates being inverted overnight to dry (Nowak et al., 2015). Once dry, sterile cotton swabs were used to remove excess dye on the sides of wells using 30% acetic acid (Nowak et al., 2015). Then, 1 mL of 30% acetic acid was added to each well and the plates were swirled 3-4 times to lift the attached cells and/or the biofilm (Nowak et al., 2015). The absorbance was performed at 595nm with 30% acetic acid being used as a blank (Nowak et al., 2015). The percent of biofilm inhibition was calculated as follows:

% of Biofilm Inhibition= ((control OD_{595}-treated OD_{595})/control OD_{595}) X 100

Results

This study consisted of the use of three microorganisms: Gram-negative Bacteria: *Pseudomonas aeruginosa (P. aeruginosa)*; and Gram-positive Bacteria: *Staphylococcus aureus (S. aureus)* and *Staphylococcus epidermidis (S. epidermidis)*. These three bacteria are known to be the main bacteria involved in the pathogenesis of prosthetic joint infections. Various experiments were performed to establish the potential uses of EGCG-S and P-EGCG tea polyphenols with antibiotics. Prosthetic joint implants are associated with a 1-2 percent risk of acquiring a bacterial infection (Ribeiro et al., 2012). Sometimes the bacterial infection is also the reason for the removal of the prosthetic joint. Current treatment methods to treat bacterial infections include antibiotic therapy with Polymyxin B or Bacitracin, debridement, or irrigation with wash. There are two main problems with this current treatment. First, bacteria are able to become resistant to the antibiotics. This can happen when bacteria produce efflux pumps that are able to decrease the concentration of the antibiotic inside the bacterial cell, therefore causing the antibiotic to become less efficient and not able to reach its target (Lin et al., 2015). Bacteria are also able to produce antibiotic resistance enzymes, which can change the conformation of antibiotics and make them inactive (Lin et al., 2015). The second problem with the current treatment method is that bacteria can form biofilm as a defense mechanism, which protects the bacteria from the antibiotic therapy (Hollmann et al., 2014). In this study, different approaches were used to evaluate and profile the effect that EGCG-S and P-EGCG have on the different steps of the current treatments to establish the most critical step (s) where the tea polyphenols can combat this problem. In addition, the newly formulated P-EGCG containing formulations CF1 and CF2 were also used to evaluate their effect on these three bacteria.

1. Characterization of the three bacteria: *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis*.

A. ***Pseudomonas aeruginosa***

A gram stain is a technique in which bacteria can be classified into two groups, either being Gram-positive or Gram-negative. It is important to distinguish which bacteria are Gram-positive and which bacteria are Gram-negative because different bacteria need to be treated in different ways depending on their characteristics. The gram stain of *P. aeruginosa* is shown in Figure 1, which shows that *P. aeruginosa* is an aerobic, rod-shaped bacterium. In Figure 1, the pink stained cells in the gram stain indicate that *P. aeruginosa* is a gram-negative bacterium.

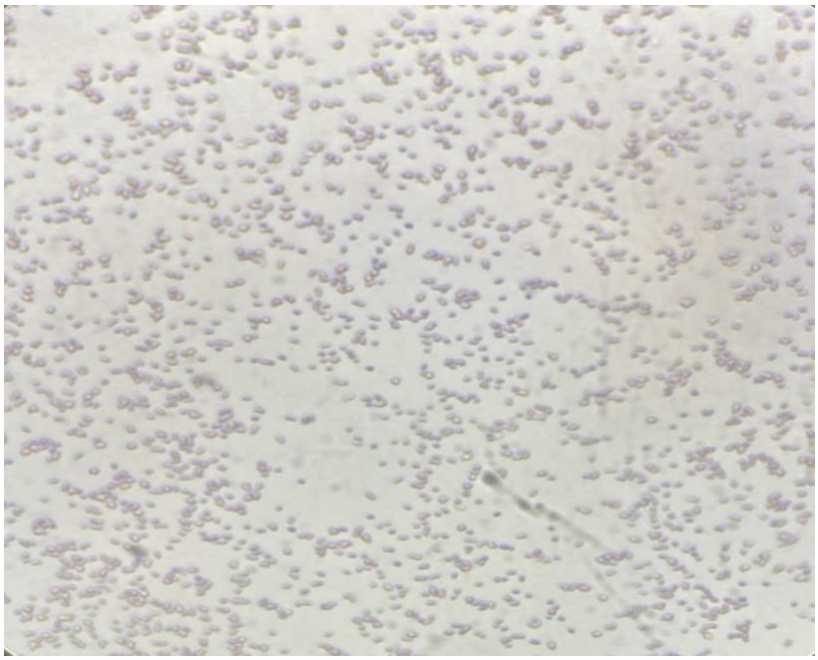


Figure 1: Gram Stain of *P. aeruginosa* under the light microscope at 1000x under oil immersion. *P. aeruginosa* displaying gram-negative characteristics.

B. *Staphylococcus aureus*

Figure 2 shows a gram stain of *Staphylococcus epidermidis*. *Staphylococcus epidermidis* is a gram-positive bacterium that is purple in color. It is an aerobic, cocci shaped bacterium in regard to its morphology.

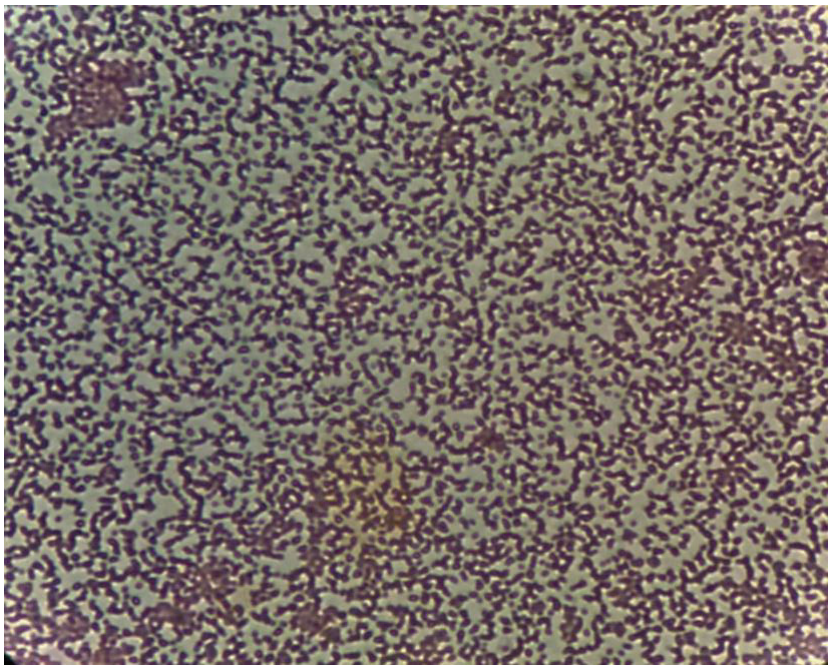


Figure 2: Gram stain of *S. aureus* under the light microscope at 1000X under oil immersion. *S. aureus* displaying gram-positive characteristics.

C. ***Staphylococcus epidermidis***

Figure 3 shows a gram stain of *Staphylococcus epidermidis*. *Staphylococcus epidermidis* is a gram-positive bacterium that is purple in color. It is an aerobic, cocci shaped bacterium in regard to its morphology.

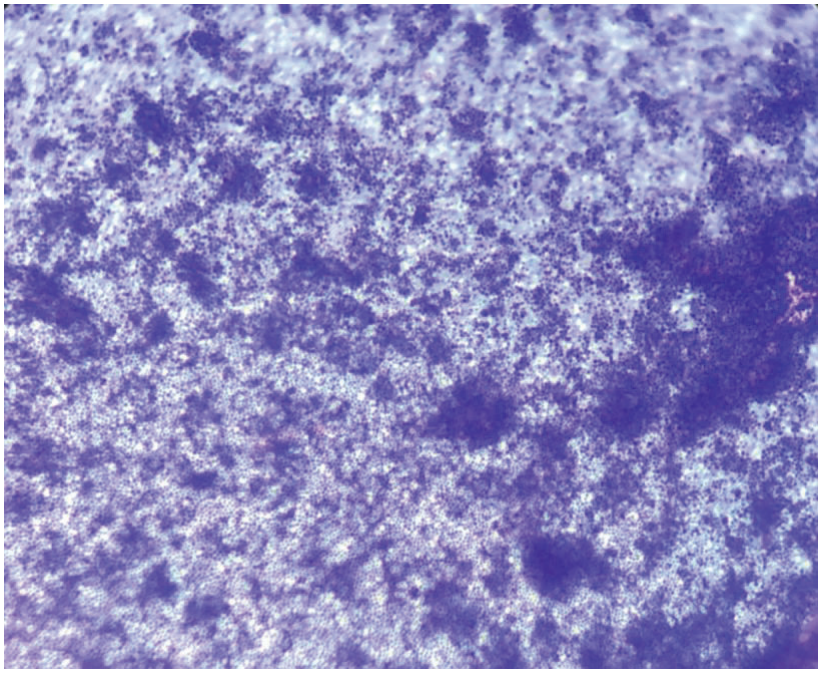


Figure 3: Gram stain of *S. epidermidis* under the light microscope at 1000X under oil immersion. *S. epidermidis* displaying gram-positive characteristics.

Summary: The *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis* bacterial cultures are pure and homogeneous. DNA extraction and sequencing was performed to confirm the identity of the bacteria (data not shown).

2. Study the synergistic effect of EGCG-S and P-EGCG with antibiotics/wash on *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis*.

   This was performed to profile all of the possible treatment combinations and evaluate which treatments are the best combinations in terms of inhibition and efficacy.

A. *Pseudomonas aeruginosa*

An overnight culture for *P. aeruginosa* was prepared to see how various treatments affect the inhibition of *P. aeruginosa*. Figure 4 shows that when *P. aeruginosa* is treated with EGCG-S and antibiotics at the same time, the percent of inhibition is 100 percent, regardless of if one antibiotic is used or both antibiotics.

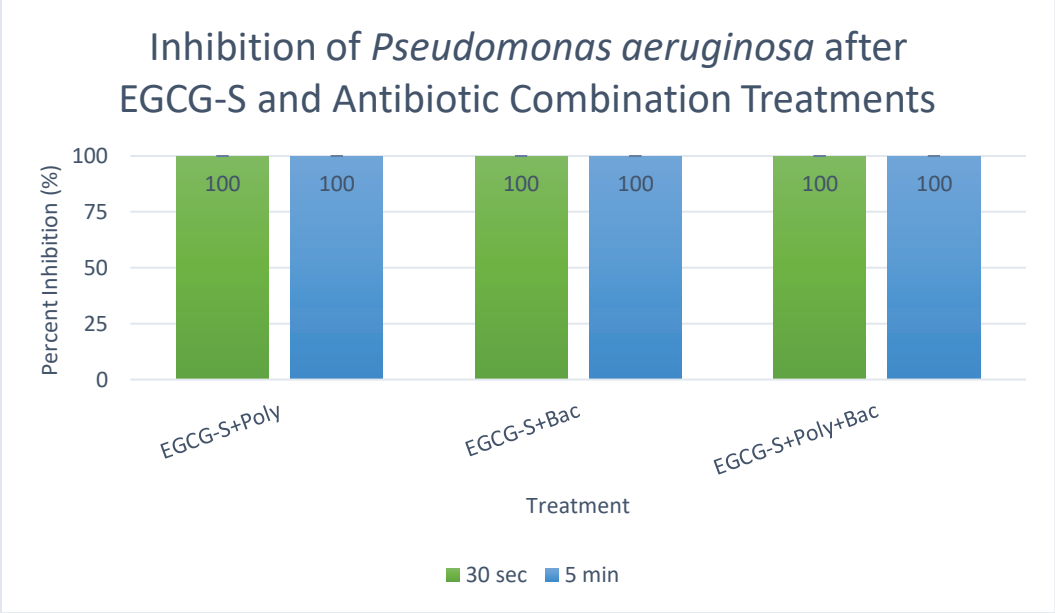


Figure 4: Inhibition of *Pseudomonas aeruginosa* after EGCG-S and Antibiotic Combination treatments.

Figure 5 shows a bar representing the inhibition of *P. aeruginosa* growth after various treatments, which included the tea polyphenol P-EGCG, Polymyxin B and Bacitracin. Regardless of if *P. aeruginosa* was treated with P-EGCG and one antibiotic or both antibiotics, all of the treatments resulted in a percent inhibition of 100 percent.

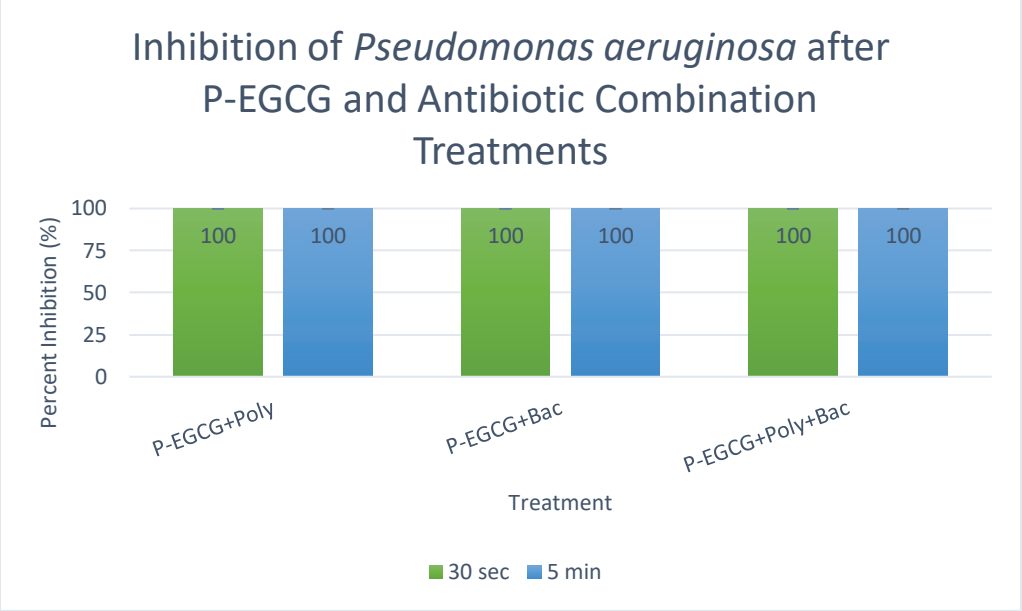


Figure 5: Inhibition of *Pseudomonas aeruginosa* after P-EGCG and Antibiotic Combination treatments.

When EGCG-S, antibiotics Polymyxin B and Bacitracin as well as wash are used together to treat *P. aeruginosa,* there is 92.690 percent of inhibition as shown in Figure 6. The treatment EGCG-S plus wash and Polymyxin B resulted in the lowest percent of inhibition at 82.915 percent compared to the other three treatments that had higher inhibition percentages. The percent inhibition increased slightly by 6 percent when both Polymyxin B and Bacitracin were added to the EGCG-S+Wash combination treatment.

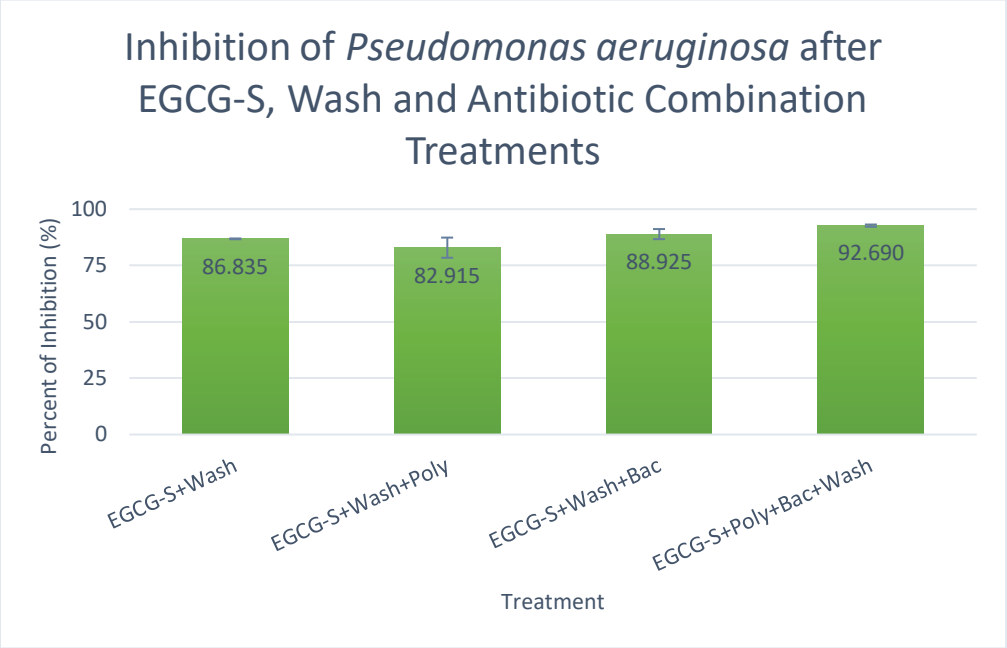


Figure 6: Inhibition of *Pseudomonas aeruginosa* after EGCG-S, Wash and Antibiotic Combination Treatments.

Figure 7 shows the inhibition of *P. aeruginosa* after it is treated with P-EGCG, Polymyxin B, Bacitracin, and wash in different combinations. For all the treatments shown in Figure 11, the inhibition percentage for *P. aeruginosa* ranges from 82.370 percent to 85.790 percent, with the P-EGCG, wash, and Bacitracin combination treatment displaying the highest percent of inhibition.

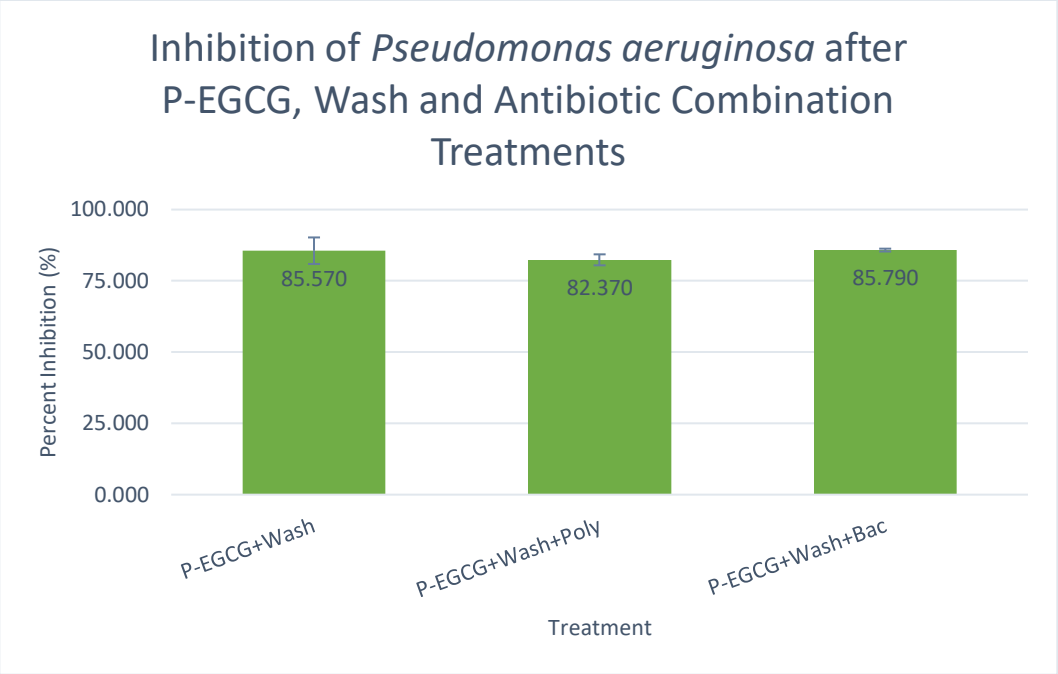


Figure 7: Inhibition of *Pseudomonas aeruginosa* after P-EGCG, Wash and Antibiotic Combination Treatments.

B. *Staphylococcus aureus*

An overnight culture of *S. aureus* was prepared to see how different treatments affect the inhibition of *S. aureus.* When *S. aureus* is treated with EGCG-S and antibiotics together, as shown in Figure 8, the results are the same as seen for *P. aeruginosa.* It does not matter if *S. aureus* is treated with EGCG-S and one antibiotic or with two antibiotics, the percent of inhibition is constant at 100 percent.

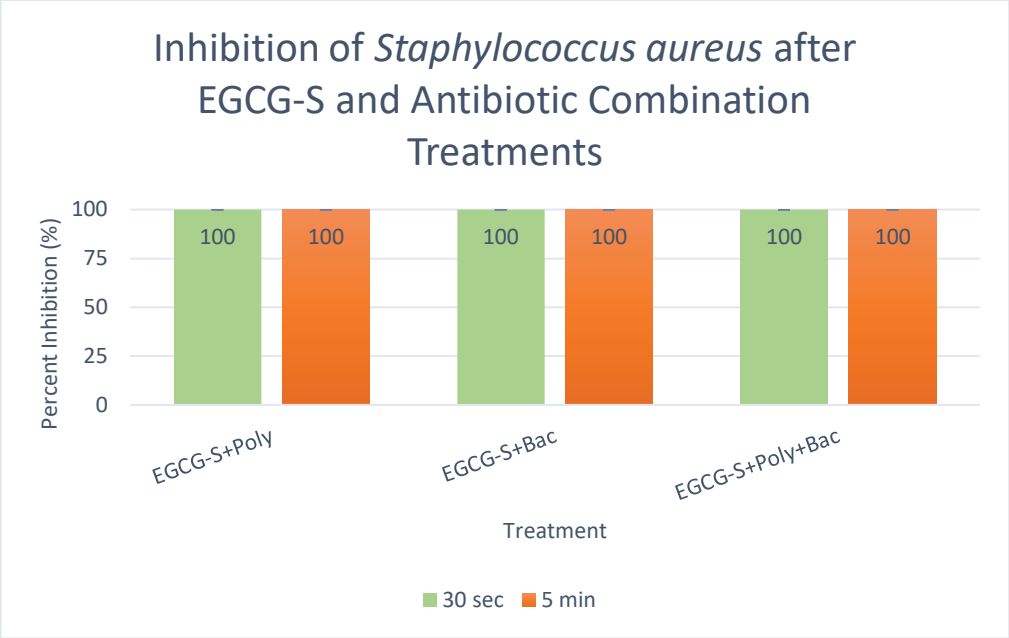


Figure 8: Inhibition of *Staphylococcus aureus* after EGCG-S and Antibiotic Combination Treatments.

Figure 9 shows that when *S. aureus* is treated with P-EGCG with a single antibiotic, either being Polymyxin B or Bacitracin, or with both antibiotics, the inhibition of *S. aureus* is similar around 100 percent.

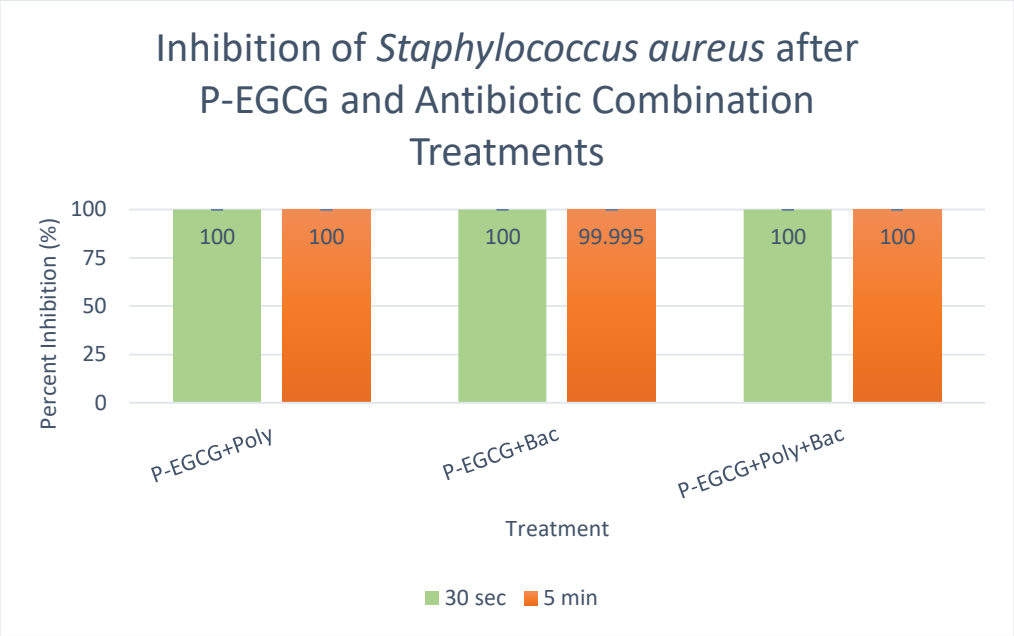


Figure 9: Inhibition of *Staphylococcus aureus* after P-EGCG and Antibiotic Combination Treatments.

Regardless of whether *S. aureus* is treated with EGCG-S+Wash alone or with the two antibiotics together, the percent of inhibition was very similar. When *S. aureus* is treated with EGCG-S, Polymyxin B, Bacitracin and wash all combined together, the bacterium is inhibited by 96.665 percent compared to the 96.775 percent when *S. aureus* is just treated with EGCG-S and Wash as shown in Figure 10. The same results were observed for P-EGCG. Figure 11 shows that when *S. aureus* is treated with P-EGCG, Wash and both antibiotics in different combinations, the percentage of inhibition ranges from 91.025 percent to 94.325 percent. The data shows that the addition of antibiotics to EGCG-S+ Wash or P-EGCG+ Wash does not really impact the percent of inhibition towards *S. aureus*.

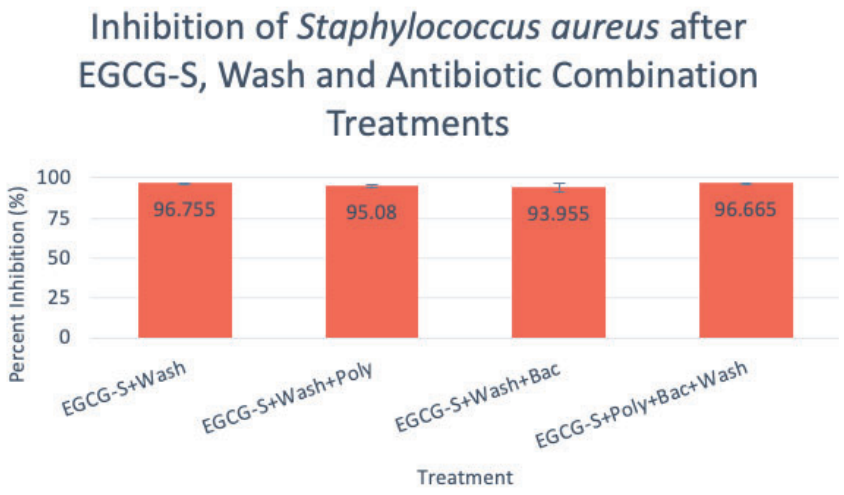


Figure 10: Inhibition of *Staphylococcus aureus* after EGCG-S, Wash and Antibiotic Combination Treatments.

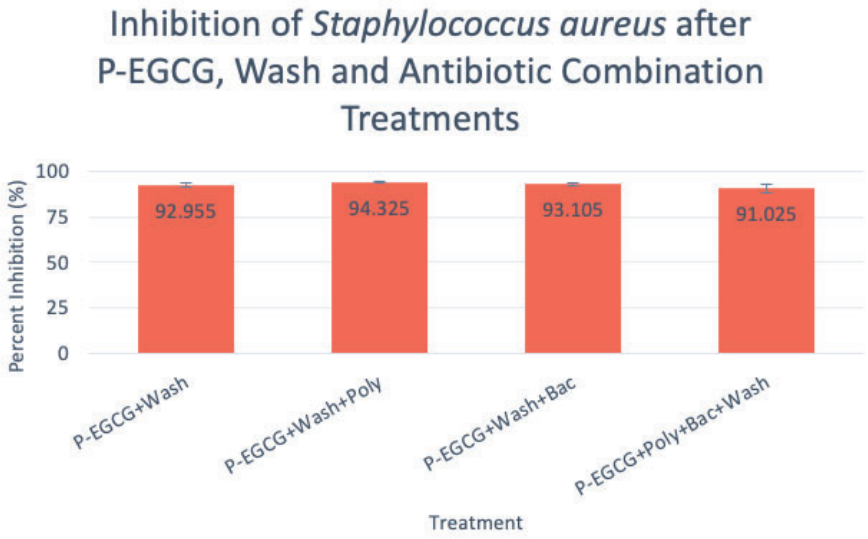


Figure 11: Inhibition of *Staphylococcus aureus* after P-EGCG, Wash and Antibiotic Combination Treatments.

C. ***Staphylococcus epidermidis***

An overnight culture of *S. epidermidis* was prepared. Figure 12 shows that when *S. epidermidis* is treated with EGCG-S and antibiotics at the same time for both 30 seconds and 5 minutes, there is 100 percent inhibition, regardless of one antibiotic being used or two. When P-EGCG, Polymyxin B and Bacitracin were used together to treat *S. aureus,* it resulted in a percent inhibition of 99.865 percent regarding the growth of *S. epidermidis* after 30 seconds of treatment. The percent inhibition increased to 100 percent after 5 minutes of treatment.

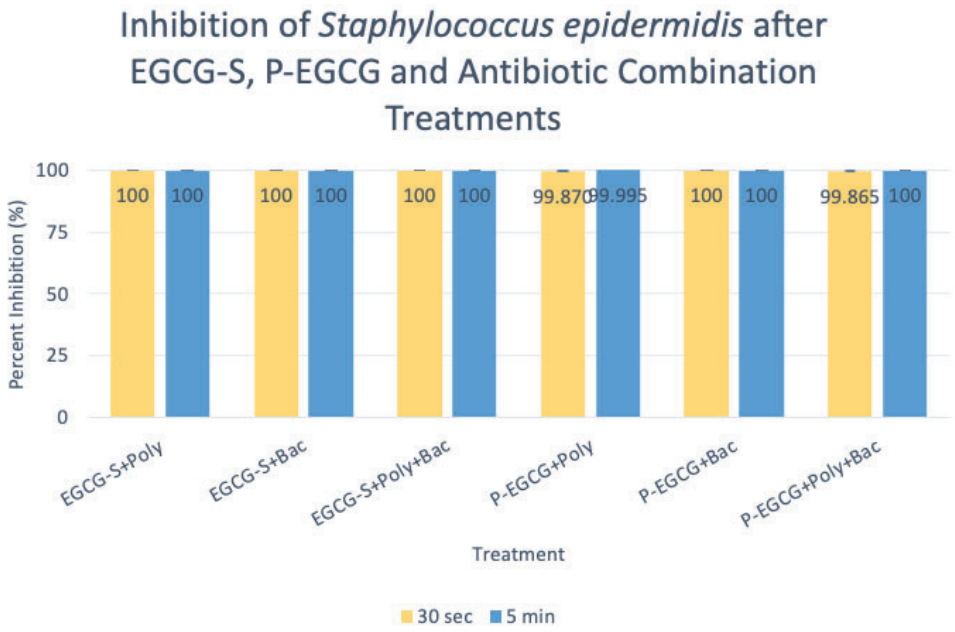


Figure 12: Inhibition of *Staphylococcus epidermidis* after EGCG-S, P-EGCG and Antibiotic Combination Treatments.

When EGCG-S, wash, Polymyxin B and Bacitracin were used in different combinations as treatments against *S. epidermidis,* they resulted in percent inhibitions ranging from 64.81 percent to 95.66 percent as shown in Figure 13. The highest percentage of inhibition, which

was 95.66 percent was from the EGCG-S and Wash combination treatment. When P-EGCG, wash and either of the two antibiotics were combined together, the results were similar for the percent inhibition, which ranged from 89.53 percent to 93.11 percent as shown in Figure 14.

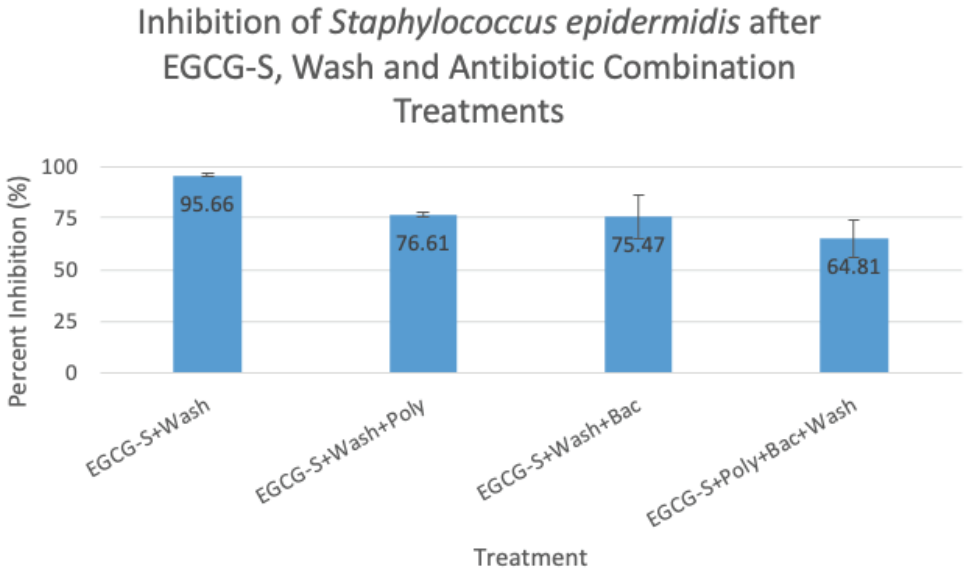


Figure 13: Inhibition of *Staphylococcus epidermidis* after EGCG-S, Wash and Antibiotic Combination Treatments.

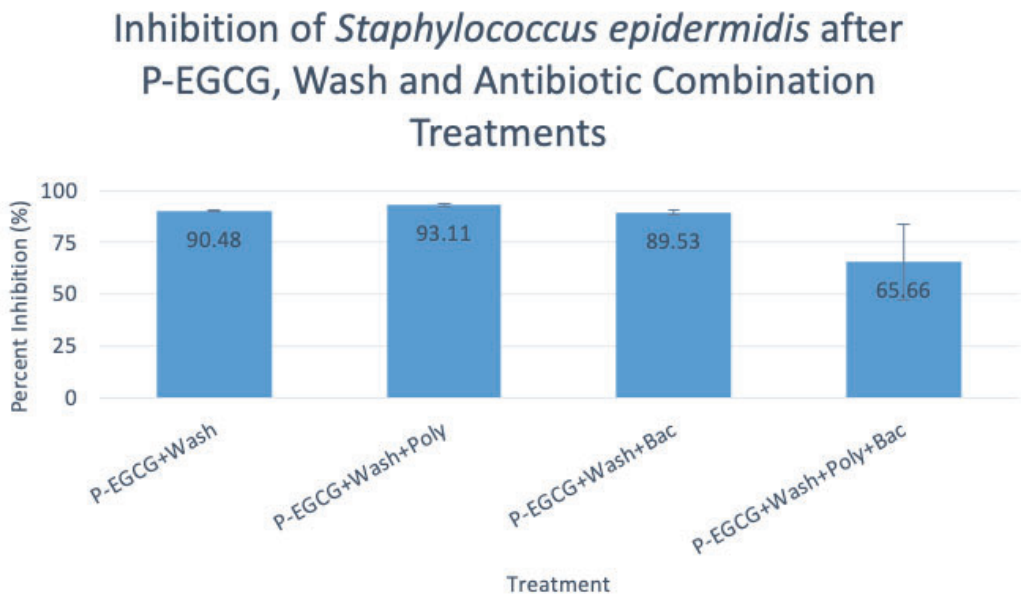


Figure 14: Inhibition of *Staphylococcus epidermidis* after P-EGCG, Wash and Antibiotic Combination Treatments.

D. Combination of Bacteria: *Pseudomonas aeruginosa*+ *Staphylococcus aureus* + *Staphylococcus epidermidis*

After an overnight bacterial culture of *P. aeruginosa, S. aureus,* and *S. epidermidis* was combined together was prepared, different treatments were applied to see how they affect the inhibition regarding the growth of the combination bacteria. Figure 15 shows that when the EGCG-S or P-EGCG tea polyphenol is used in different combinations with wash and antibiotics, the range of inhibition across all of the treatments is 50.490 percent to 65.975 percent. The highest percent of inhibition was when P-EGCG, wash and Polymyxin B were used together as a treatment and resulted in a 65.975 percent inhibition. EGCG-S with wash as a treatment worked slightly less at inhibiting the growth of the combination bacteria

compared to the EGCG-S+ Wash+ Polymyxin B or Bacitracin combination treatment. Overall, it was shown that it is harder to treat a combination of microorganisms.

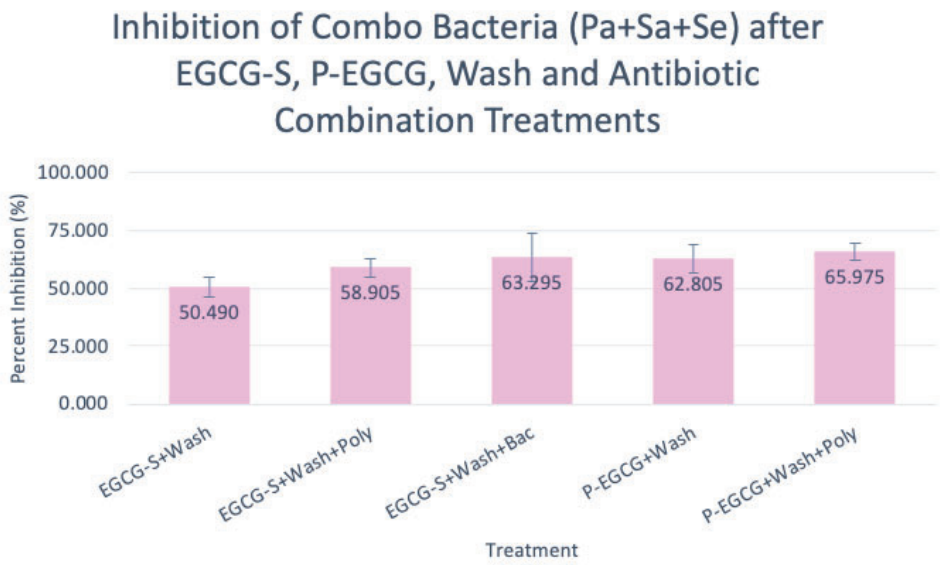


Figure 15: Inhibition of Combination Bacteria (*P. aeruginosa* + *S. aureus*+ *S. epidermidis)* after EGCG-S, P-EGCG, Wash and Antibiotic Combination Treatments.

Summary:

When EGCG-S or P-EGCG is combined with either Polymyxin B or Bacitracin, the percent inhibition is near 100 percent against each individual bacterium and the combination bacteria, shown in Figure 17 (outlined in red). Out of all the wash combination treatments, the EGCG-S+ Polymyxin B+ Bacitracin+ Wash treatment worked the best against *Pseudomonas aeruginosa* with a percent inhibition of 92.690 percent. EGCG-S+ Wash worked well for *Staphylococcus aureus* and *Staphylococcus epidermidis*, 96.755 percent and 95.66 percent inhibition respectively. The combination treatments worked to some extent for the combination bacteria. EGCG-S or P-EGCG combined with single antibiotics got the best

results in all three bacteria, the percent of inhibition is about 100 percent. When the wash step was added into the combination treatment, the percent of inhibition was reduced. The chemical components in the wash may react with the antibiotics or tea polyphenols.

Percentage of Inhibition (%)						
	EGCG-S+ Poly	EGCG-S+ Bac	EGCG-S+ Poly+ Bac	P-EGCG+ Poly	P-EGCG+ Bac	P-EGCG+ Poly+ Bac
PA	100%	100%	100%	100%	100%	100%
SA	100%	100%	100%	100%	99.995%	100%
SE	100%	100%	100%	99.933%	100%	99.933%

Percentage of Inhibition (%)								
	EGCG-S +Wash	EGCG-S+ Wash+ Poly	EGCG-S+ Wash+ Bac	EGCG-S+ Poly+ Bac+ Wash	P-EGCG +Wash	P-EGCG +Wash +Poly	P-EGCG +Wash +Bac	P-EGCG +Poly+ Bac+ Wash
PA	86.835%	82.915%	88.925%	92.690%	85.570%	82.370%	85.790%	-
SA	96.755%	95.08%	93.955%	96.665%	92.955%	94.325%	93.105%	91.025
SE	95.66%	76.61%	75.47%	64.81%	90.48%	93.11%	89.53%	65.66%
Combo	50.49%	58.905%	63.295%	-	62.805%	65.975%	-	-

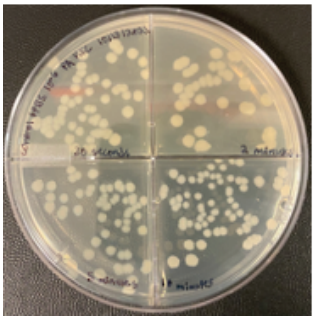
Figure 16: Summary of a combination profiling study using EGCG-S, P-EGCG, Wash, Polymyxin B and Bacitracin. The table displays how the various treatments affect the percentage of inhibition. PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*; SE: *Staphylococcus epidermidis*; Combo: *Pseudomonas aeruginosa*+ *Staphylococcus aureus*+ *Staphylococcus epidermidis*.

3. Evaluate CF1 and CF2 formulation as antibacterial agents.

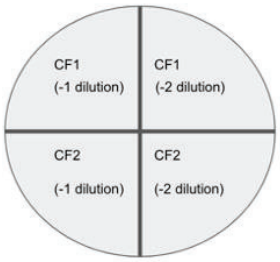
This study was performed to see how P-EGCG containing formulations affect the inhibition of *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis* and if they can inhibit their growth. A Colony Forming Unit (CFU) Assay was performed using X plates for a timed study for 30 seconds, 5 minutes and 10 minutes.

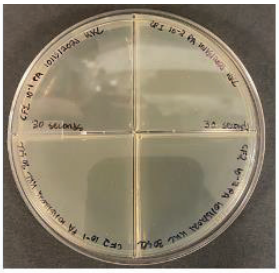
A. *Pseudomonas aeruginosa*

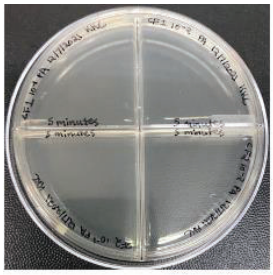
Figure 17 shows a *Pseudomonas aeruginosa* control plate as well as nutrient agar quad plates treated with the CF1 and CF2 formulations at different dilutions for 30 seconds, 5 minutes and 10 minutes. It was shown in Figure 18, that CF1 and CF2 formulations at 10^{-1} and 10^{-2} dilutions inhibited *Pseudomonas aeruginosa* growth up to 99.99945 percent and 99.99971 percent, respectively in as little as 30 seconds of treatment. After being treated for 5 minutes with either the CF1 or CF2 formulations, the growth of *Pseudomonas aeruginosa* was inhibited by 100 percent.

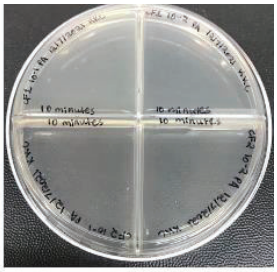


P. aeruginosa Control	
Treatment Time	Count
30 sec	56×10^6
5 min	58×10^6
10 min	68×10^6









30 seconds 5 minutes 10 minutes

Figure 17: *Pseudomonas aeruginosa* CF1 and CF2 Time Course Study. **Top row:** *Pseudomonas aeruginosa* control for 30 seconds, 5 minutes and 10 minutes. **Bottom row:** *Pseudomonas aeruginosa* treated with CF1 and CF2 formulations at 10^{-1} and 10^{-2} dilutions.

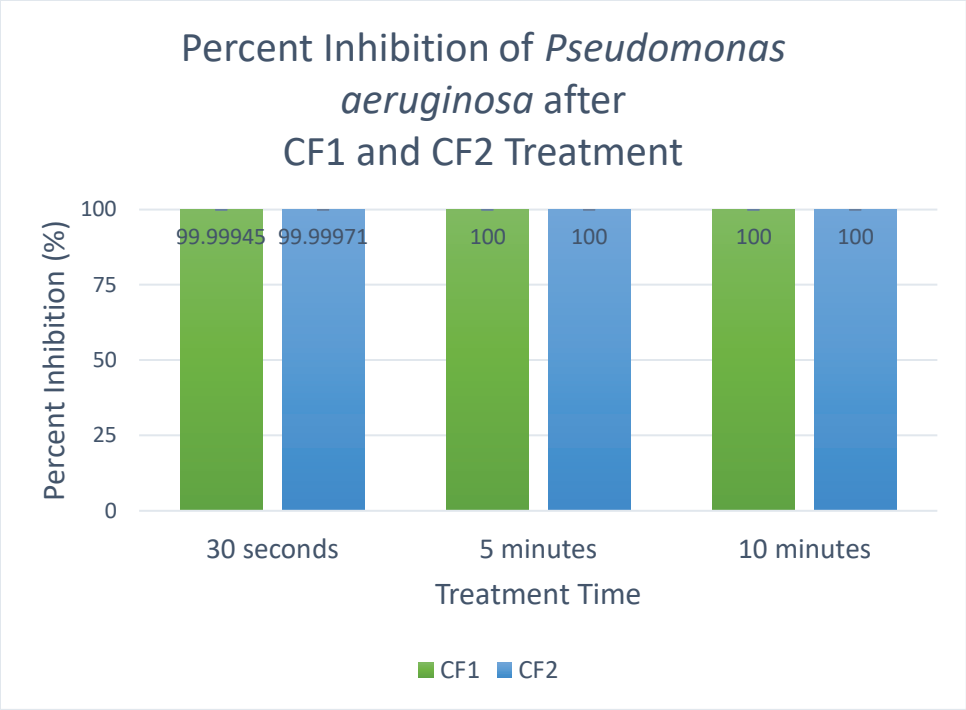
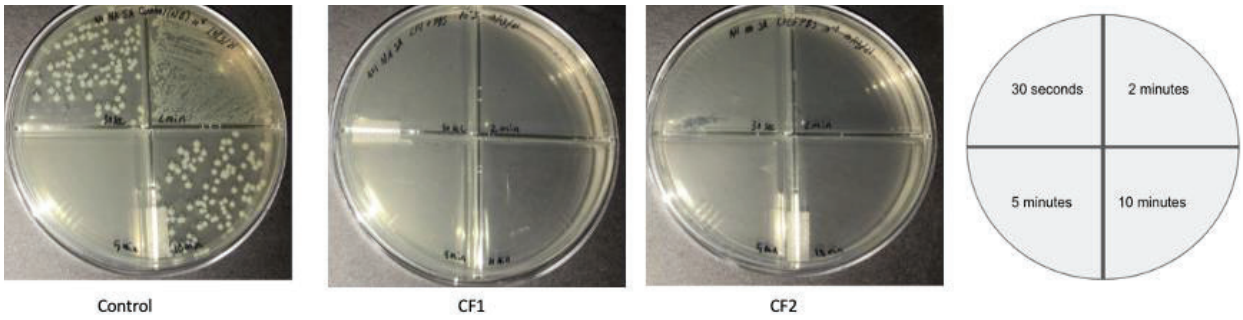


Figure 18: Bar graph displaying the percent inhibition of *Pseudomonas aeruginosa* after P-EGCG containing CF1 and CF2 Formulation Treatments.

B. ***Staphylococcus aureus***

Figure 19 shows a *S. aureus* control plate and two nutrient agar plates that were treated with the CF1 and CF2 formulations at the following times: 30 seconds, 5 minutes, and 10 minutes. It was shown in Figure 20, that both the CF1 and CF2 formulations inhibited *S. aureus* growth 100 percent within 30 seconds of treatment.



S. aureus Control	
Treatment Time	Count
30 sec	111×10^6
5 min	73×10^4
10 min	60×10^4

Figure 19: *Staphylococcus aureus* CF1 and CF2 Time Course Study. Left to Right: *Staphylococcus aureus* control for 30 seconds, 5 minutes and 10 minutes. *Staphylococcus aureus* treated with CF1 and CF2 formulations at 10^{-1} and 10^{-2} dilutions.

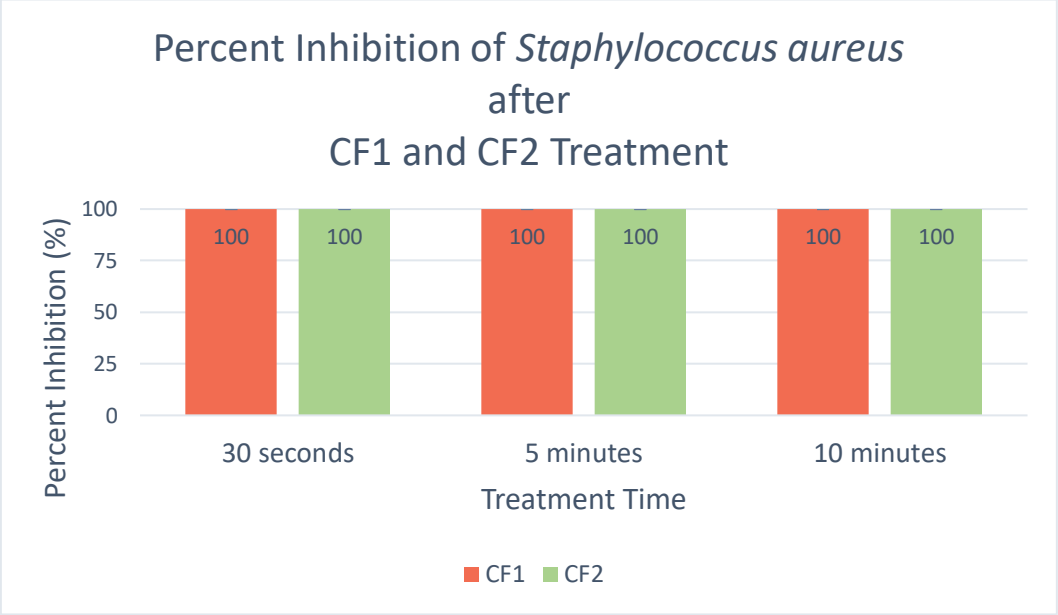
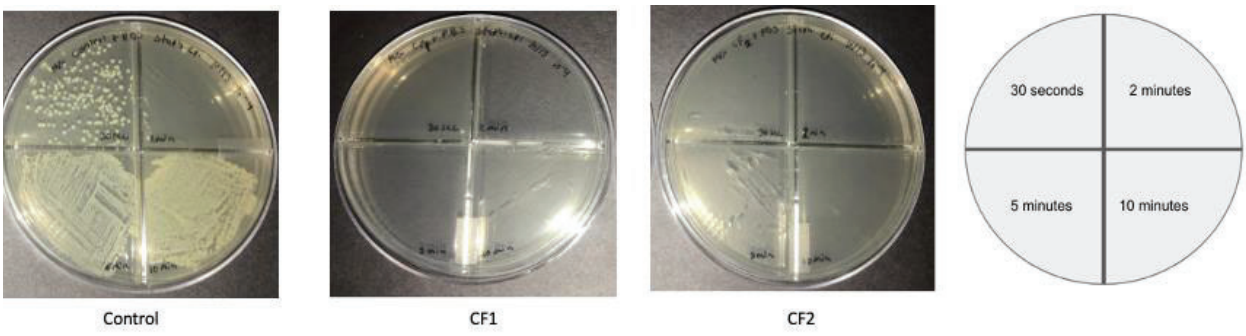


Figure 20: Inhibition of *Staphylococcus aureus* after P-EGCG containing CF1 and CF2 Formulation Treatments.

C. *Staphylococcus epidermidis*

Figure 21 shows a *S. epidermidis* control plate as well as nutrient agar plates, which were treated with the CF1 and CF2 formulations at different dilutions for 30 seconds, 5 minutes and 10 minutes. It was shown in Figure 22 that the CF1 and CF2 formulations at 10^{-1} and 10^{-2} dilutions inhibited *S. epidermidis* growth up to 100 percent in as little as 30 seconds of treatment time.



S. epidermidis Control	
Treatment Time	Count
30 sec	30 x 10^6
5 min	132 x 10^6
10 min	76 x 10^6

Figure 21: *Staphylococcus epidermidis* CF1 and CF2 Time Course Study. The figure shows the *Staphylococcus epidermidis* control plate and the two agar plates that were treated with the CF1 and CF2 formulations at 10^{-1} and 10^{-2} dilutions.

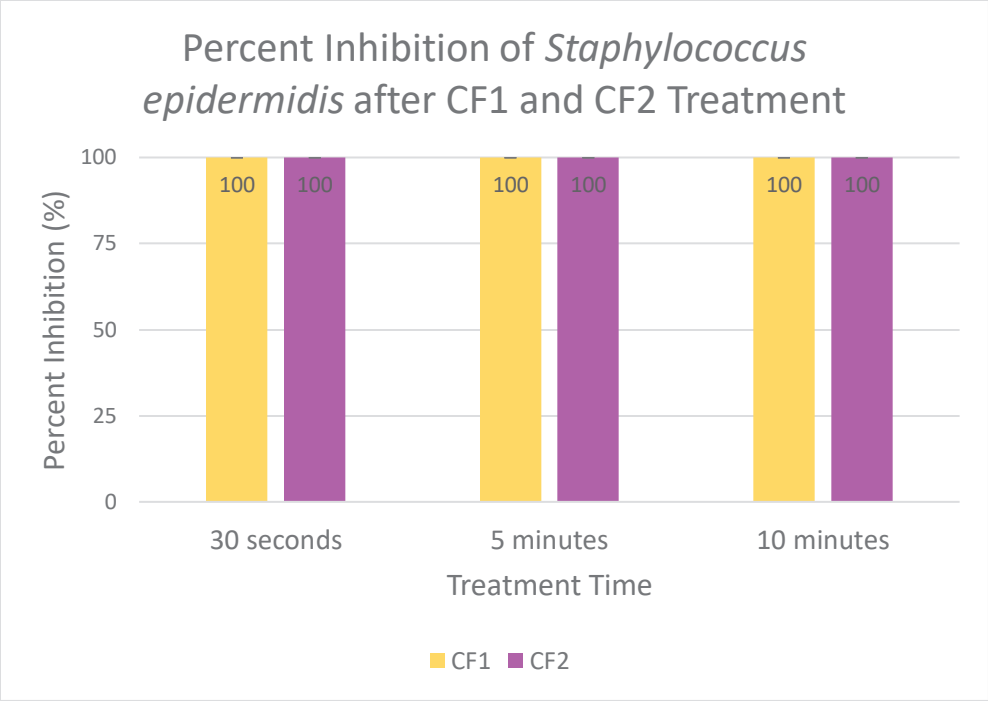


Figure 22: Inhibition of *Staphylococcus epidermidis* after P-EGCG containing CF1 and CF2 formulation treatments.

Summary:

Both of the P-EGCG containing formulations, CF1 and CF2 work in inhibiting bacterial growth of *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis.* For *P. aeruginosa*, 99.99945 percent of bacterial growth is inhibited in 30 seconds after treatment with the CF1 formulation and this treatment is associated with a log reduction value of 5.7085. The P-EGCG containing CF2 formulation showed similar results, with a 99.99971 percent of inhibition regarding *P. aeruginosa* bacterial growth and a respective log reduction of 5.859. The CF1 and CF2 formulations can inhibit the bacterial growth of *S. aureus* and *S. epidermidis* to 100 percent in as little as 30 seconds with a log reduction values higher than 5.859. These results indicate that these P-EGCG

containing CF1 and CF2 formulations really work with a potent antimicrobial activity and have the potential to be commercialized.

	Treatment	Mean Percent Inhibition (%)	Mean Log Reduction
	CF1 (30 sec)	99.99945%	5.7085
	CF2 (30 sec)	99.99971%	5.859
	CF1 (5 min)	100%	-
	CF2 (5 min)	100%	-
	CF1 (10 min)	100%	-
P. aeruginosa	CF2 (10 min)	100%	-
	CF1 (30 sec)	100%	-
	CF2 (30 sec)	100%	-
	CF1 (5 min)	100%	-
	CF2 (5 min)	100%	-
	CF1 (10 min)	100%	-
S. aureus	CF2 (10 min)	100%	-
	CF1 (30 sec)	100%	-
	CF2 (30 sec)	100%	-
	CF1 (5 min)	100%	-
	CF2 (5 min)	100%	-
	CF1 (10 min)	100%	-
S. epidermidis	CF2 (10 min)	100%	-

Figure 23: Summary of *P. aeruginosa, S. aureus,* and *S. epidermidis* bacterial growth inhibition after P-EGCG containing CF1 and CF2 formulation treatments.

4. Examine the possible synergistic effects that EGCG-S and P-EGCG with antibiotics have on biofilm formation.

This study was performed to see which treatments using EGCG-S and P-EGCG are the most effective in preventing the bacteria from forming biofilm. If we know which treatment prevents biofilm, then we can utilize that information with the goal of overcoming the antibiotic resistance problem that *P. aeruginosa, S. aureus,* and *S.*

epidermidis are associated with. Two different combination profiling studies were done with various treatments, including a Congo Red Qualitative Biofilm Assay and a Crystal Violet Quantitative Biofilm Assay.

I. Congo Red Qualitative Biofilm Assay to Analyze the Effect of Tea Polyphenols and Antibiotics/wash on Biofilm Formation

A. ***Pseudomonas aeruginosa***

In this study, EGCG-S and P-EGCG tea polyphenols as well as the antibiotics, Polymyxin B and Bacitracin were used to analyze the inhibition of biofilm formation. Figure 24 shows a template of how the Congo red plates were set up as well as which treatments prevented biofilm to form (shown in green). The wells on the plate were treated with the respective treatments for 5 minutes and are shown in Figure 25. After the plate was incubated, it was qualitatively analyzed. The results shown in Figure 25 indicate that tea polyphenols EGCG-S and P-EGCG have a considerable synergistic effect with antibiotics on *P. aeruginosa* than with either of the antibiotics alone. Wells 5 and 6, which are treatments Polymyxin B and Bacitracin individually, respectively are positive for biofilm formation as shown in Figure 25. When *Pseudomonas aeruginosa* was treated with the combination of EGCG-S, Polymyxin B and Bacitracin together, there was no formation of biofilm as shown in well 15. Wells 19 and 20, which are treatments P-EGCG plus Polymyxin B and P-EGCG plus

Bacitracin, respectively were negative for biofilm formation. 24 hours after *Pseudomonas aeruginosa* was treated with the combination of EGCG-S, wash, Polymyxin B and Bacitracin, shown by well 17 there was biofilm that formed, shown in the top two plates of Figure 25. Though, after 48 hours the EGCG-S, wash, Polymyxin B and Bacitracin combination treatment started decreasing the amount of biofilm that had formed. This is shown in the bottom two plates of Figure 25, well 17. EGCG-S, P-EGCG and Wash in combination with single antibiotics (either Polymyxin B or Bacitracin) or with both antibiotics inhibited biofilm formation in *P. aeruginosa*. This was represented by the wells remaining red in color. *P. aeruginosa* is biofilm resistant to Polymyxin B.

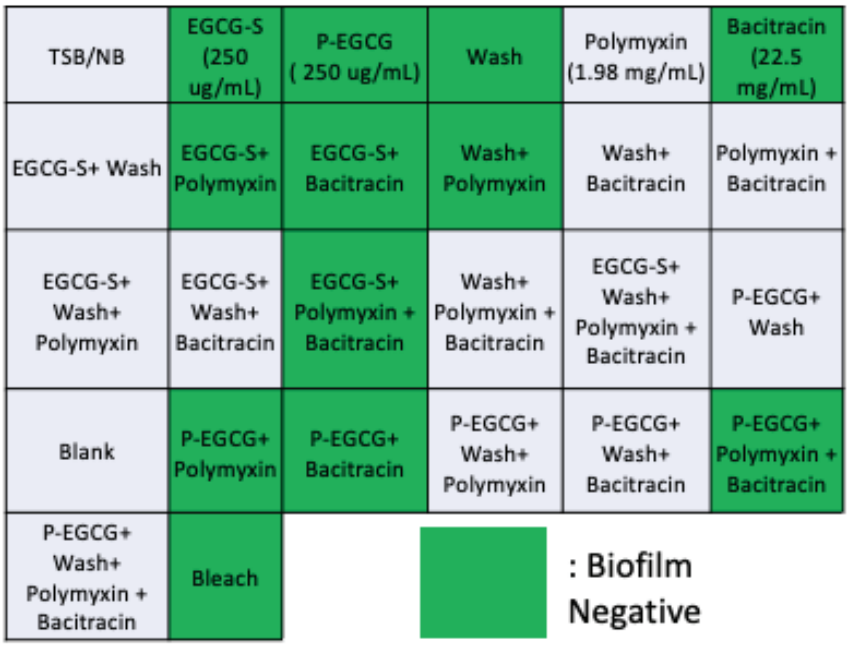


Figure 24: *Pseudomonas aeruginosa* Congo Red Assay plate template. The green represents the wells which are biofilm negative and do not allow *Pseudomonas aeruginosa* to form biofilm with those respective treatments.

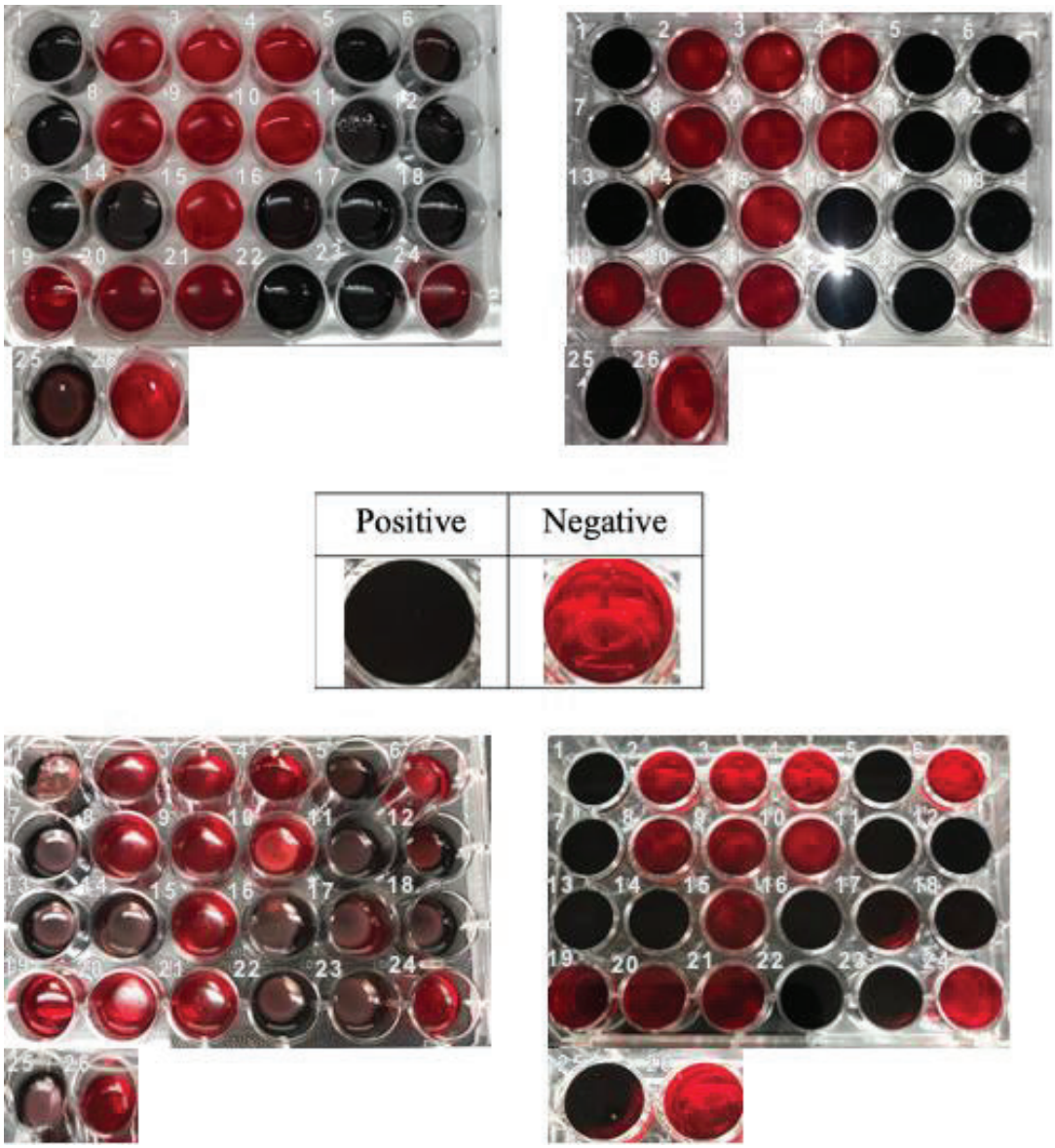


Figure 25: *Pseudomonas aeruginosa* Congo Red Qualitative Biofilm Assay. *Pseudomonas aeruginosa* was treated with various treatments for 5 minutes, as listed in Figure 24. **Top two plates:** Congo Red plate 24 hours after treatment. Left: front of the plate. Right: back of the plate. **Bottom two plates**: Congo Red plate 48 hours after treatment. Left: front of the plate. Right: back of the plate.

B. ***Staphylococcus aureus***

Figure 26 shows a template displaying how the Congo Red Plates for the *Staphylococcus aureus* bacterium were set up. Figure 26 also shows which treatments inhibited biofilm formation, that was shown in green. Each of the wells were treated for 5 minutes with their respective treatments and then incubated overnight. The results shown in Figure 27 show that the EGCG-S and P-EGCG tea polyphenols have a synergistic effect when combined with the antibiotics, Polymyxin B and Bacitracin. Well 5 which is just treated with Polymyxin B alone results in the formation of biofilm as shown in Figure 27 represented by the color of the well changing to black. *S. aureus* is biofilm resistant to Polymyxin B. Though, when *S. aureus* was treated with EGCG-S and Polymyxin B combined, it was negative for biofilm shown in Well 8. Wells 15 and 24 which was the treatment of EGCG-S or P-EGCG treated together with both antibiotic inhibited biofilm formation. Well 16, which was treated with EGCG-S, wash and both antibiotics together, showed biofilm that formed 24 hours after incubation, but by 48 hours the biofilm that had formed was started to decrease as shown in Figure 27.

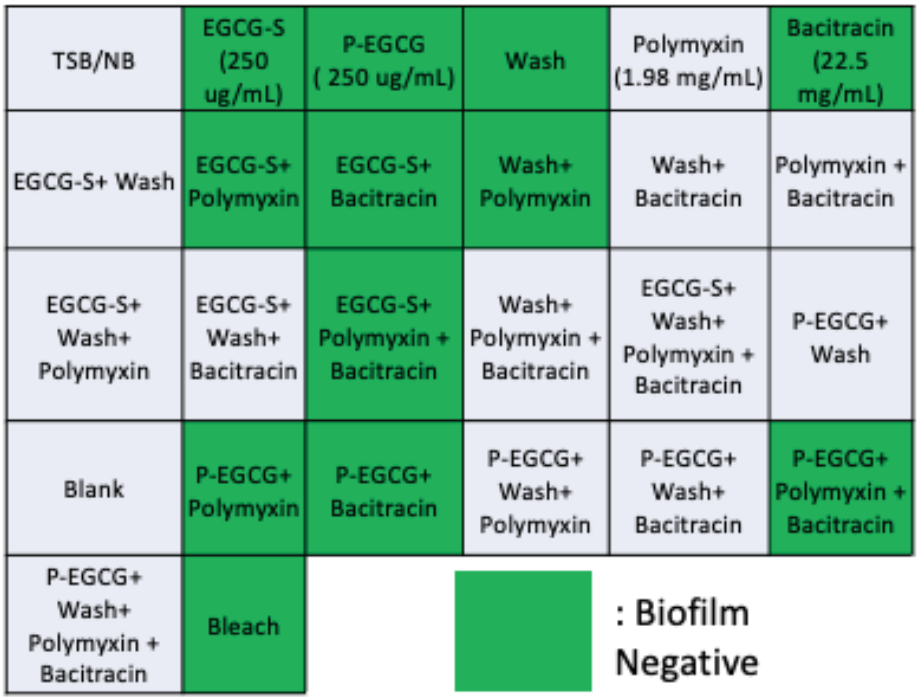


Figure 26: *Staphylococcus aureus* Congo Red Assay plate template. The green represents the wells which are biofilm negative and do not allow *Staphylococcus aureus* to form biofilm with those respective treatments.

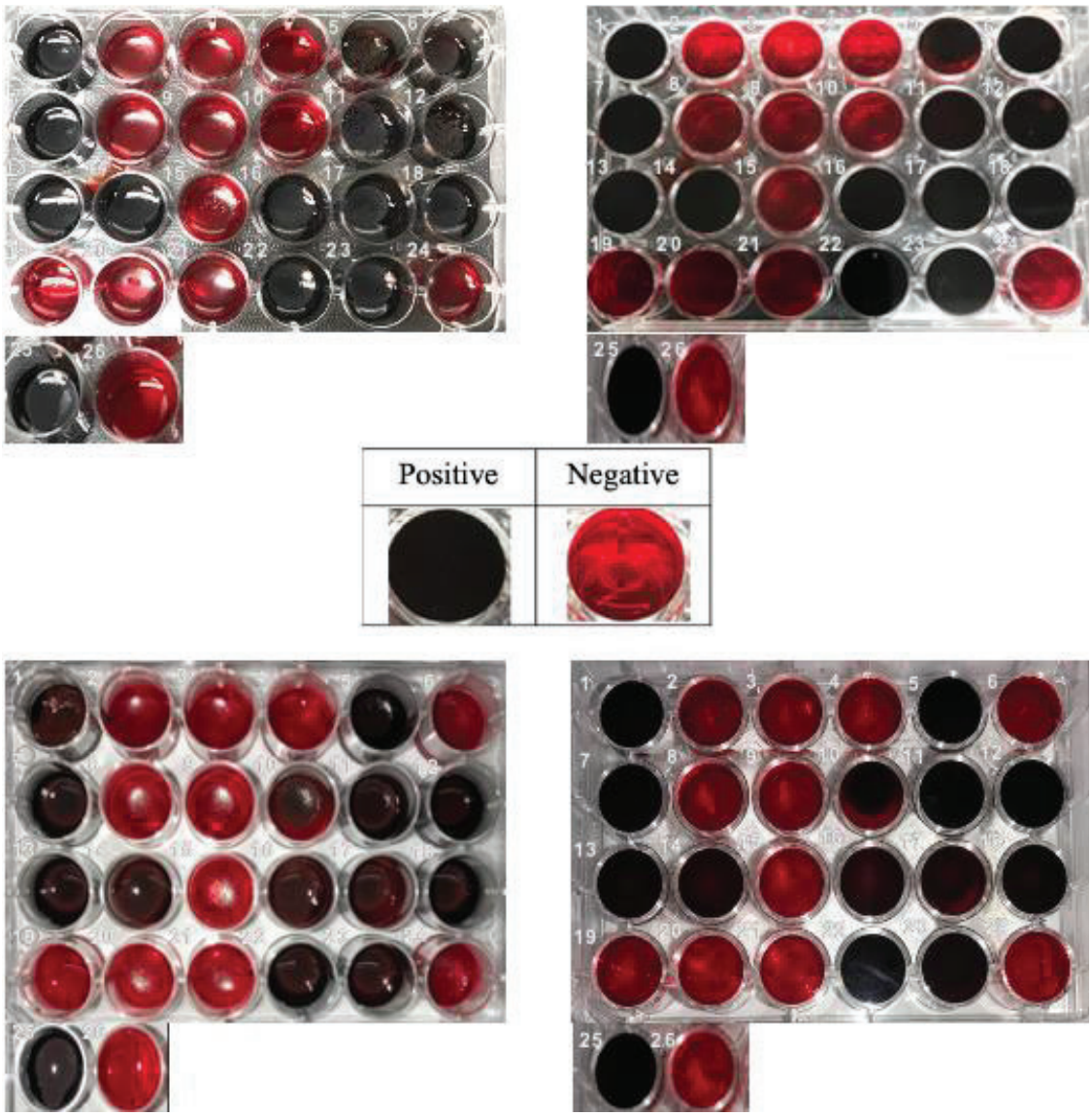


Figure 27: *Staphylococcus aureus* Congo Red Qualitative Biofilm Assay. *Staphylococcus aureus* was treated with various treatments for 5 minutes, as listed in Figure 26. **Two top plates:** Congo Red plate 24 hours after treatment. Left: front of the plate. Right: back of the plate. **Bottom two plates:** Congo Red plate 48 hours after treatment. Left: front of the plate. Right: back of the plate.

C. ***Staphylococcus epidermidis***

Figure 28 shows a template of how the Congo red plate was set up and it also shows which treatments prevented the formation of biofilm, shown in green. The wells on the plate were treated for 5 minutes with their respective treatments and then incubated overnight and analyzed, shown in Figure 29. Wells 5 and 6, which were treated with Polymyxin B and Bacitracin alone, respectively were positive for biofilm formation, shown by the black color in Figure 29. *S. epidermidis* is resistant to both Polymyxin B and Bacitracin. Though, when EGCG-S was used with either Polymyxin B or Bacitracin together, wells 8 and 9 respectively, did not result in the formation of biofilm. This data suggests that the tea polyphenols EGCG-S and P-EGCG have a synergistic effect with the antibiotics Polymyxin B and Bacitracin on *S. epidermidis* and enhance antimicrobial activity.

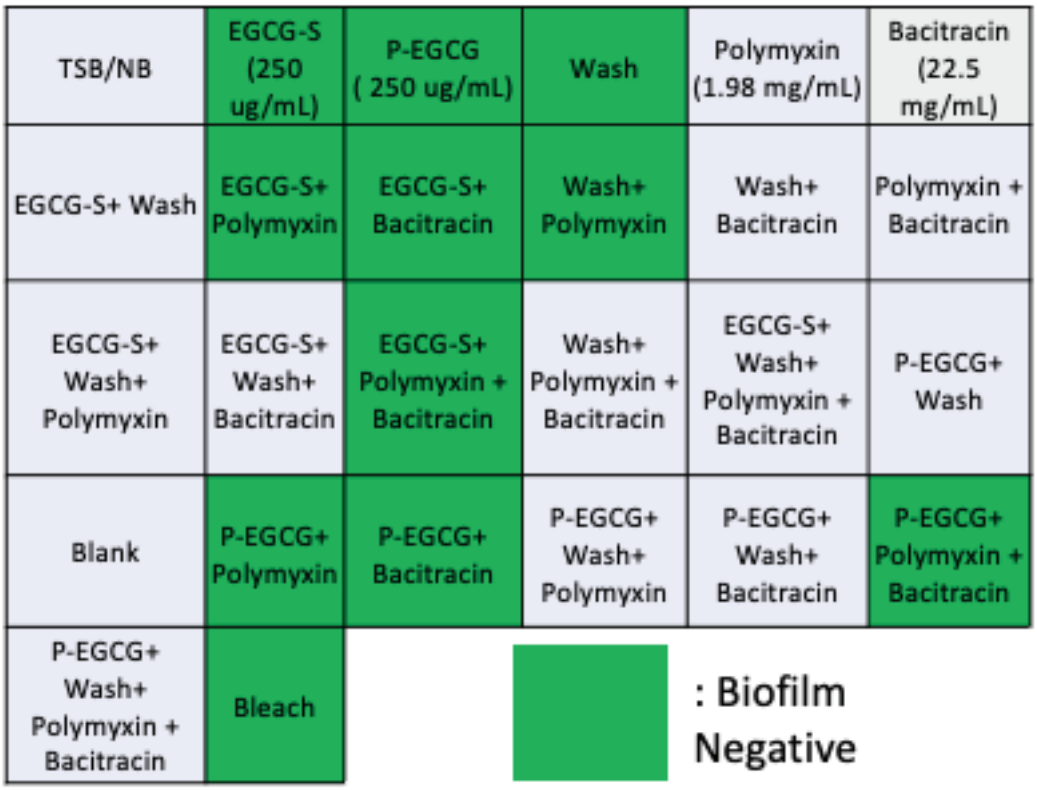


Figure 28: *Staphylococcus epidermidis* Congo Red Assay plate template. The green represents the which treatments are biofilm negative and do not allow *Staphylococcus epidermidis* to form biofilm.

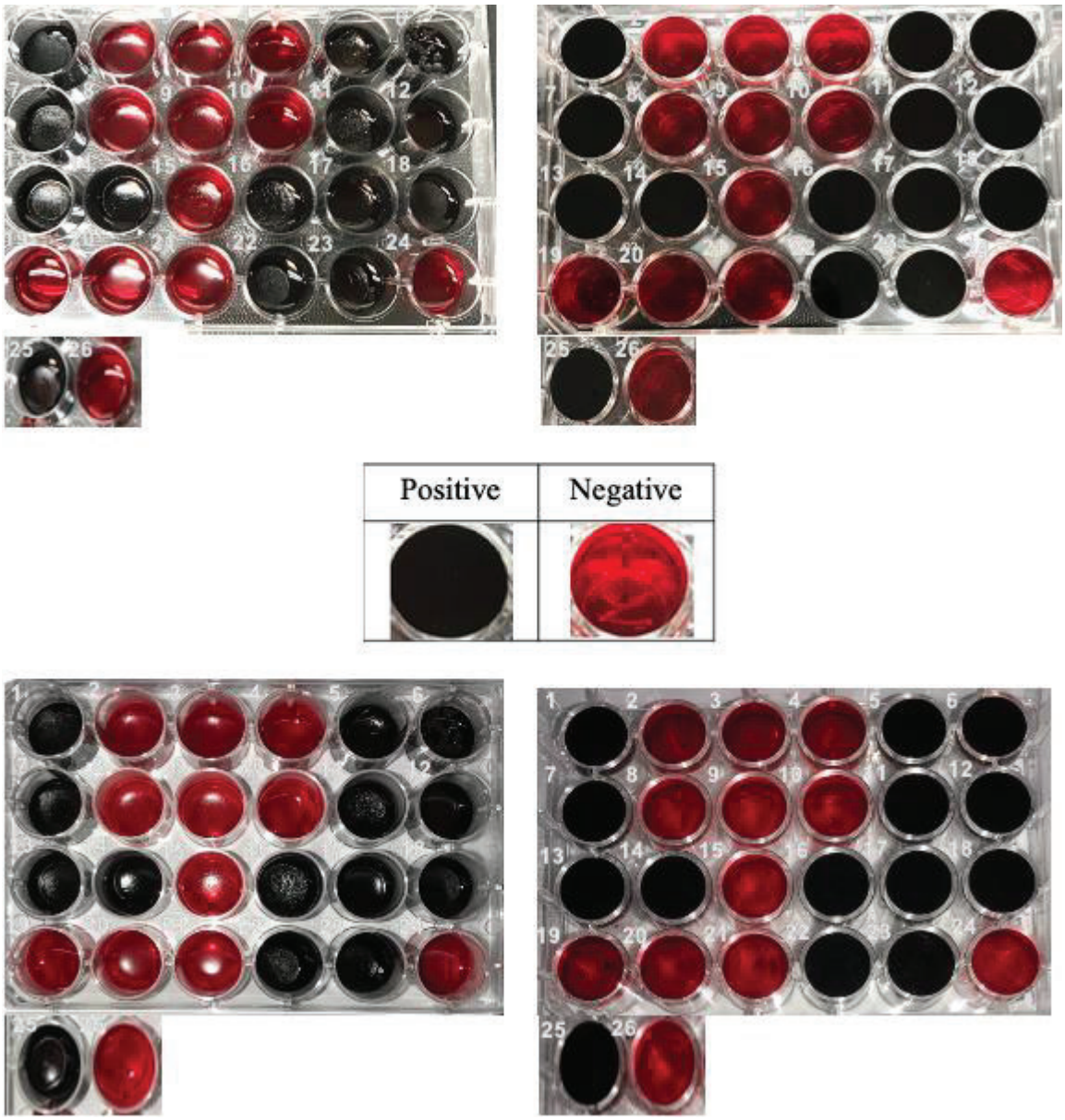


Figure 29: *Staphylococcus epidermidis* Congo Red Qualitative Biofilm Assay. *Staphylococcus epidermidis* was treated with various treatment for 5 minutes, as shown in Figure 28. **Top two plates:** Congo Red plate 24 hours after treatment. Left: front of the plate. Right: back of the plate. **Bottom two plates:** Congo Red plate 48 hours after treatment. Left: front of the plate. Right: back of the plate.

D. Combination of Bacteria: *Pseudomonas aeruginosa*+ *Staphylococcus aureus*+ *Staphylococcus epidermidis*

In this Congo Red study with all three of the bacteria combined, EGCG-S and P-EGCG tea polyphenols were used to see their effect on the formation of biofilm. Figure 30 shows a template of how the Congo Red experimental plate was set up and it also shows which treatments were negative for biofilm and inhibition its formation, and those treatments are shown in green in Figure 30. The wells on the plate were treated with their respective treatments for 5 minutes and are shown in Figure 31. After the plate was incubated, it was qualitatively analyzed. The results shown in Figure 31 indicate that the EGCG-S and P-EGCG tea polyphenols have a considerable synergistic effect with the antibiotics, Polymyxin B and Bacitracin on the combination of the three bacteria together (*P. aeruginosa* + *S. aureus* + *S. epidermidis)*. Wells 5 and 6, which were treated with Polymyxin B and Bacitracin respectively were positive for biofilm formation and this is represented in Figure 31 with that corresponding well turning black. Though, it was found that when the tea polyphenol EGCG-S is used in combination with either Polymyxin B or Bacitracin (wells 14 and 15, respectively), the treatment inhibited the formation of biofilm and the wells remained red as shown in Figure 31. Wash, Polymyxin B and Bacitracin by themselves do not work as a treatment since they allow biofilm to form.

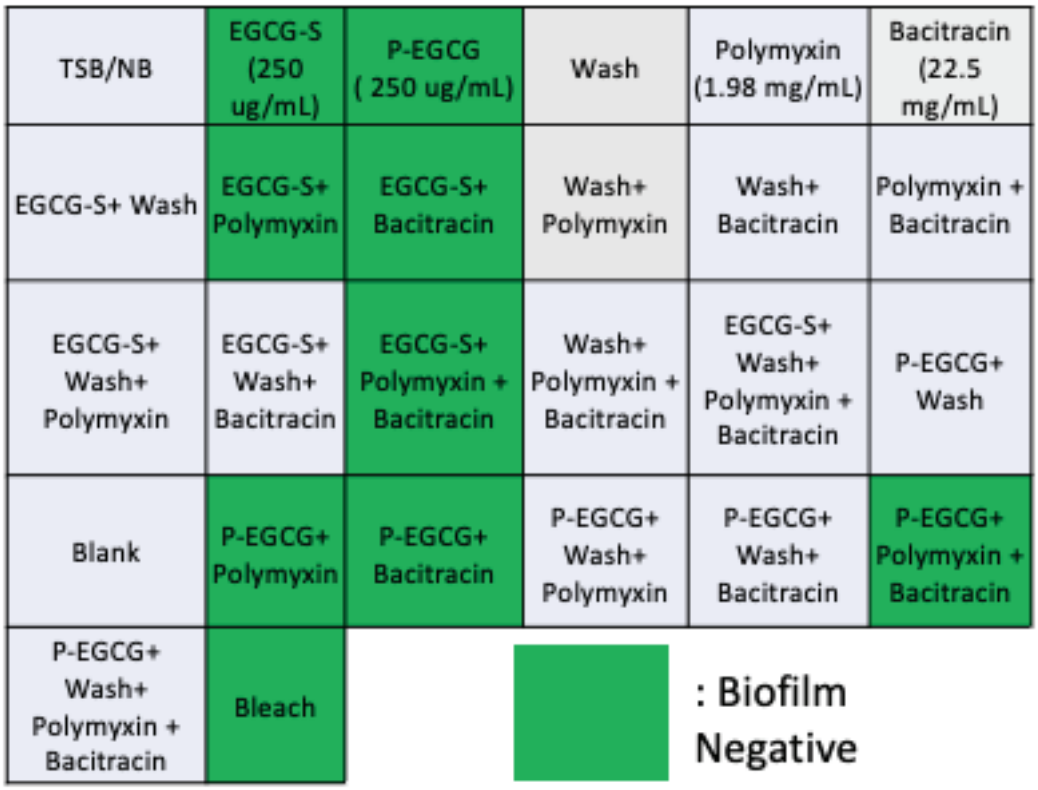


Figure 30: Combination of all three of the bacteria (*Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermidis)* Congo Red Assay plate template. The green represents the well which are biofilm negative and do not allow the combination bacteria to form biofilm with those respective treatments.

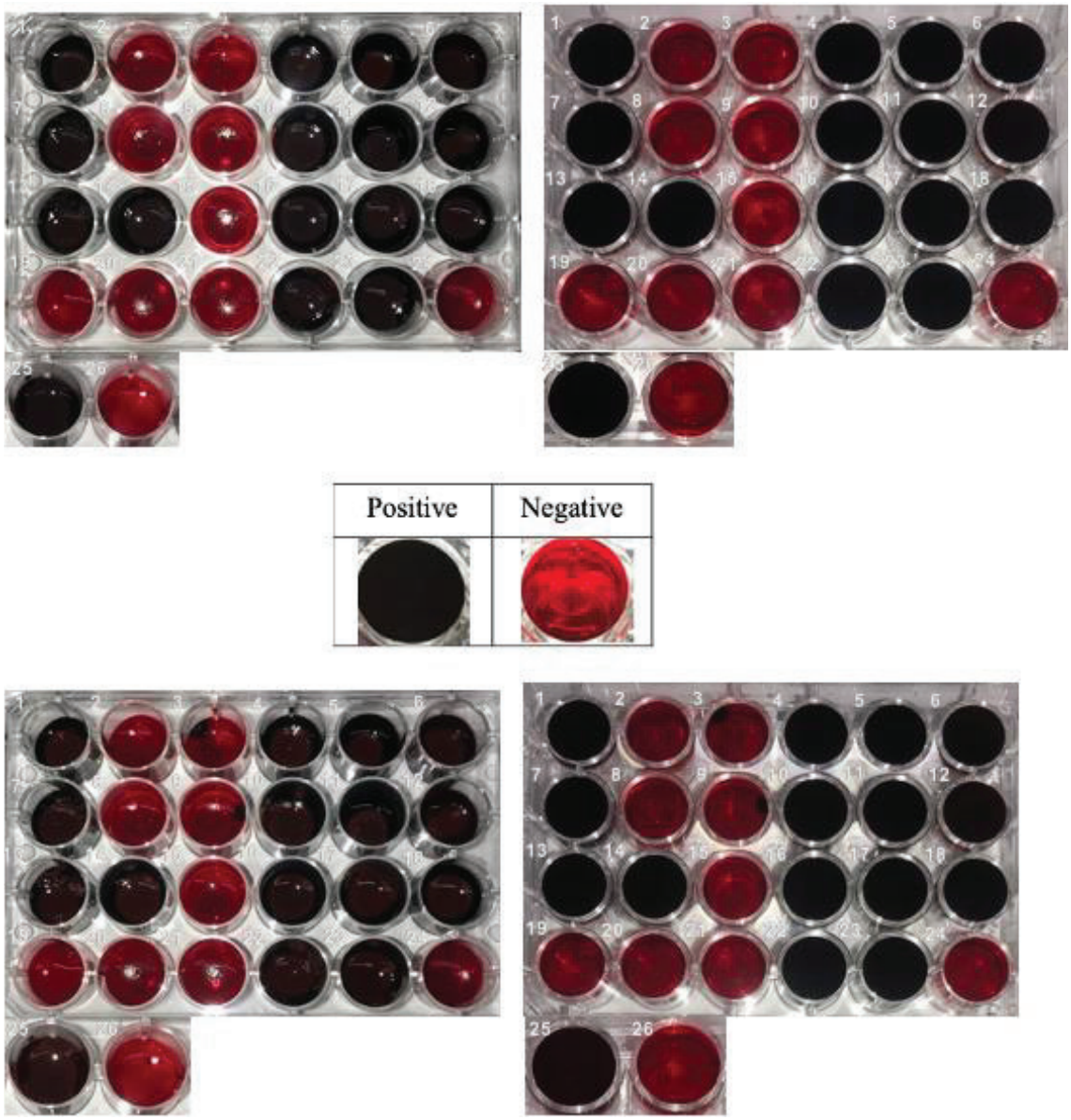


Figure 31: Combination Bacteria (*P. aeruginosa, S. aureus and S. epidermidis)* Congo Red Qualitative Biofilm Assay. The combination bacteria was treated with various treatments for 5 minutes, which are listed in Figure 30. **Top two plates:** Congo Red plate 24 hours after treatment. Left: front of the plate. Right: back of the plate. **Bottom two plates:** Congo Red plated 48 hours after treatment. Left: front of the plate. Right: back of the plate.

Summary:

The single treatments EGCG-S, P-EGCG and Wash inhibited the formation of biofilm for *P. aeruginosa, S. aureus,* and *S. epidermidis*. The profiling patten is identical for *P. aeruginosa, S. aureus* (Figure 24, and Figure 26. The patten is slightly different for S. *epidermidis* (Figure 28). Treatment with Bacitracin alone only inhibited biofilm formation against *P. aeruginosa* and *S.* aureus but not for *S. epidermidis*. The antibiotic Polymyxin B by itself did not work on any of the individual bacteria. All of the different combinations of EGCG-S and P-EGCG combined with Polymyxin B and Bacitracin inhibited biofilm formation of *P. aeruginosa, S. aureus,* and *S. epidermidis*. The combination bacteria showed similar results (Figure 20) as the individual bacteria, except that the Wash treatment did not inhibit biofilm formation against it. The reason that the Wash treatment did not work on the combination bacteria could be because some kind of interaction or competition could be occurring among these three organisms.

Individual Bacteria	Combo Bacteria (PA+SA+SE)
EGCG-S	+
P-EGCG	+
Wash	-
Bacitracin (only PA, SA)	-
Polymyxin B (doesn't work)	-
EGCG-S+ Polymyxin B	+
EGCG-S+ Bacitracin	+
EGCG-S+ Polymyxin B+ Bacitracin	+
P-EGCG+ Polymyxin B	+
P-EGCG+ Bacitracin	+
P-EGCG+ Polymyxin B+ Bacitracin	+
Bleach (control)	+

Figure 32: Summary of Congo Red Qualitative Biofilm Assay. Summary of how different treatments against individual bacteria and combination bacteria affect biofilm formation. PA: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*; SE: *Staphylococcus epidermidis*; Combo: Combination Bacteria (*Pseudomonas aeruginosa*+ *Staphylococcus aureus*+ *Staphylococcus epidermidis*). The (-) represents that biofilm did not form with the respective treatment. The (+) represents that biofilm formed regardless of the respective treatment.

II. Crystal Violet Quantitative Assay to Analyze the Effect of Tea Polyphenols on Biofilm Inhibition

A Congo Red qualitative assay tells us if different treatments are able to inhibit the growth of biofilm. In order to see what percentage of biofilm is inhibited, a Crystal Violet quantitative assay was carried out.

A. ***Pseudomonas aeruginosa***

In this study, EGCG-S and P-EGCG tea polyphenols as well as the antibiotics, Polymyxin B and Bacitracin were used to analyze the effect that they have on biofilm inhibition. Figure 33 shows the experimental plate on the top and the control well at the bottom. The way the wells are numerically labeled in Figure 33 corresponds to the treatment numbers that are shown in Figure 34. Figure 35 shows the what the negative control plate looked like after the crystal violet quantitative assay and its corresponding template. Figure 36 shows how the formation of biofilm was inhibited regarding *P. aeruginosa* cell cultures after being treated for 96 hours. The percentage of biofilm inhibition across all the treatments ranged from 64.70 percent to 99.89 percent. When *P. aeruginosa* cell cultures were treated with EGCG-S, Polymyxin B and Bacitracin together, that resulted in an inhibition of biofilm formation percentage of 99.32 percent. This treatment represented the second highest inhibition percentage, with the first highest inhibition percentage represented by bleach at 99.89 percent. When EGCG-S was used with Polymyxin B together, the percent of inhibition for *P.*

aeruginosa increased approximately 16 percent compared to using Polymyxin B alone as a treatment (98.10 percent versus 82.95 percent).

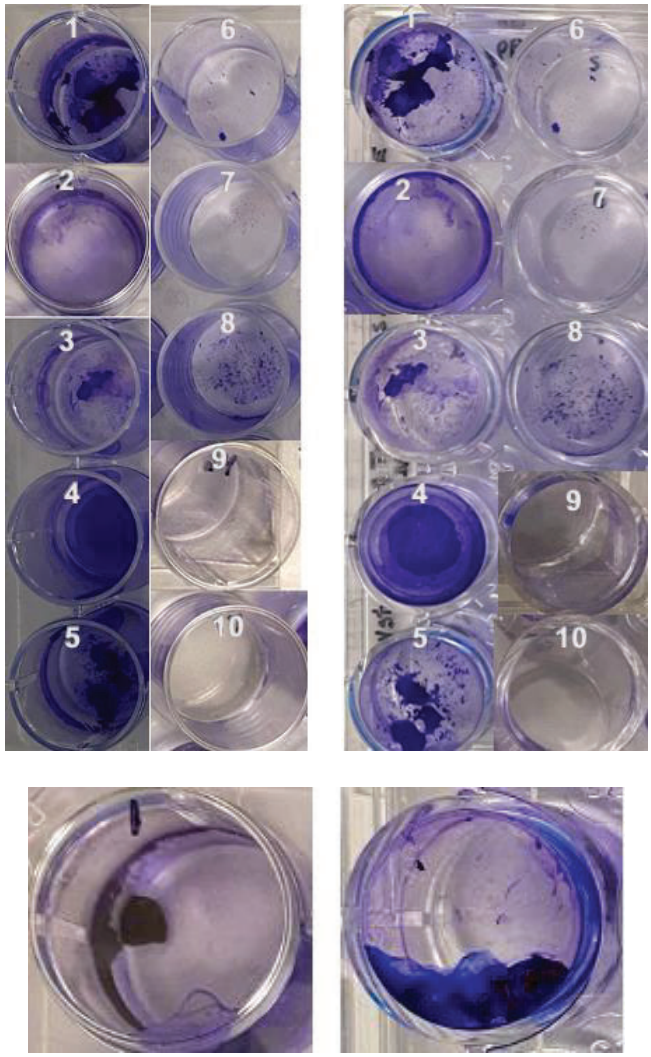
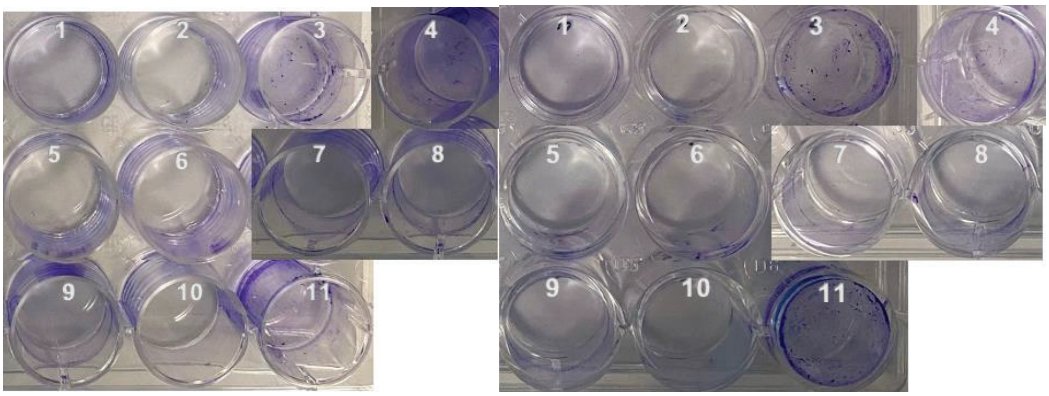


Figure 33: *Pseudomonas aeruginosa* Crystal Violet Quantitative Assay Experimental Plate and Control. **Top:** Shows the experimental plate. Left- front of the plate. Right- back of the plate. **Bottom:** The front and back of the control well. The well numbers displayed are associated to the treatments shown in Figure 34.

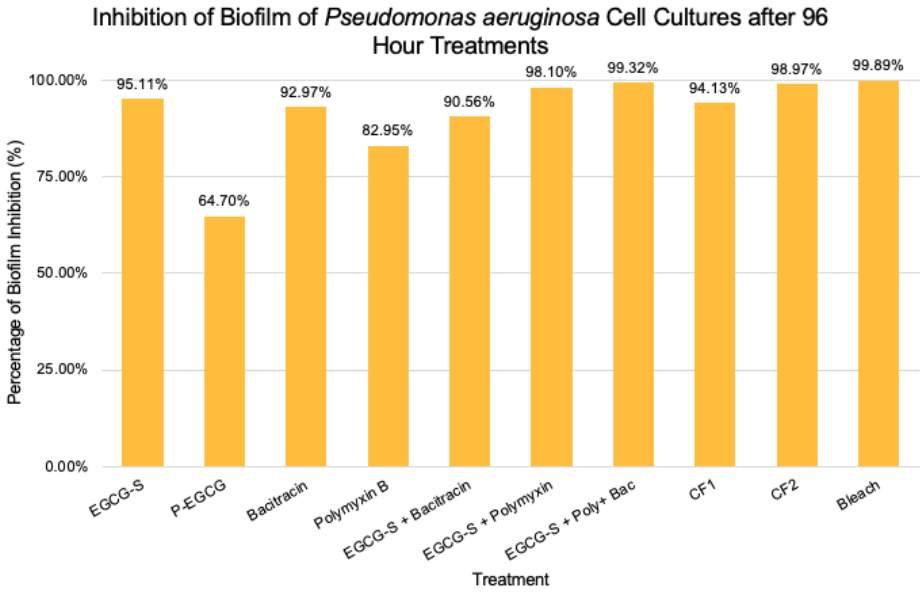
Well 1: EGCG-S	**Well 6: EGCG-S+ Polymyxin B**
Well 2: P-EGCG	**Well 7: EGCG-S+ Polymyxin B+ Bacitracin**
Well 3: Bacitracin	**Well 8: CF1**
Well 4: Polymyxin B	**Well 9: CF2**
Well 5: EGCG-S+ Bacitracin	**Well 10: Bleach**

Figure 34: Template of Crystal Violet assay treatment set up.



Well 1:EGCG-S	**Well 2: Bleach**	**Well 3: CF1**	**Well 4: CF2**
Well 5: Bacitracin	**Well 6: Polymyxin B**	**Well 7: EGCG-S+ Bacitracin**	**Well 8: EGCG-S+ Polymyxin B**
Well 9: EGCG-S+ Bacitracin+ Polymyxin B	**Well 10: Bacitracin+ Polymyxin B**	**Well 11: Blank. Only TSB**	

Figure 35: Negative Control Plate for the *Pseudomonas aeruginosa* Crystal Violet Assay. **Top:** Front and back of the negative control plate, from left to right respectively. **Bottom:** Template of the negative control plate.

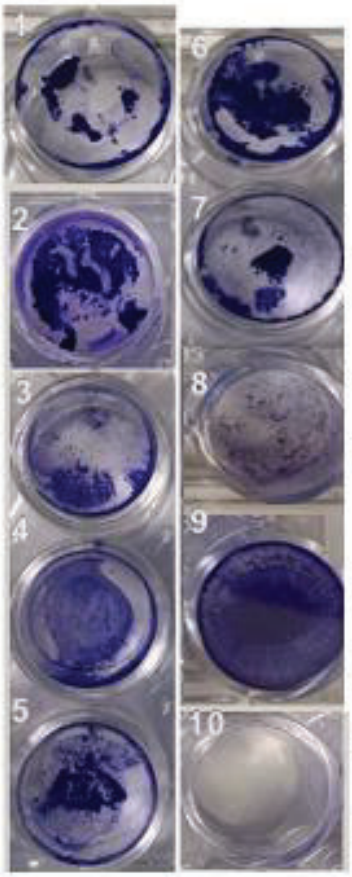


Pseudomonas aeruginosa		
Well Number	**Treatment**	**% of Biofilm Inhibition**
1	EGCG-S (2.5 mg/mL)	95.11%
2	P-EGCG (2.5 mg/mL)	64.70%
3	Bacitracin (10x)	92.97%
4	Polymyxin B (10x)	82.95%
5	EGCG-S + Bacitracin	90.56%
6	EGCG-S + Polymyxin B	98.10%
7	EGCG-S + Polymyxin B+ Bacitracin	99.32%
8	CF1	94.13%
9	CF2	98.97%
10	Bleach	99.89%

Figure 36: Inhibition of *Pseudomonas aeruginosa* Cell Cultures after 96 Hour Treatments. The treatments used involved the EGCG-S and P-EGCG tea polyphenols as well as Polymyxin B, Bacitracin, CF1 and CF2 formulations and bleach.

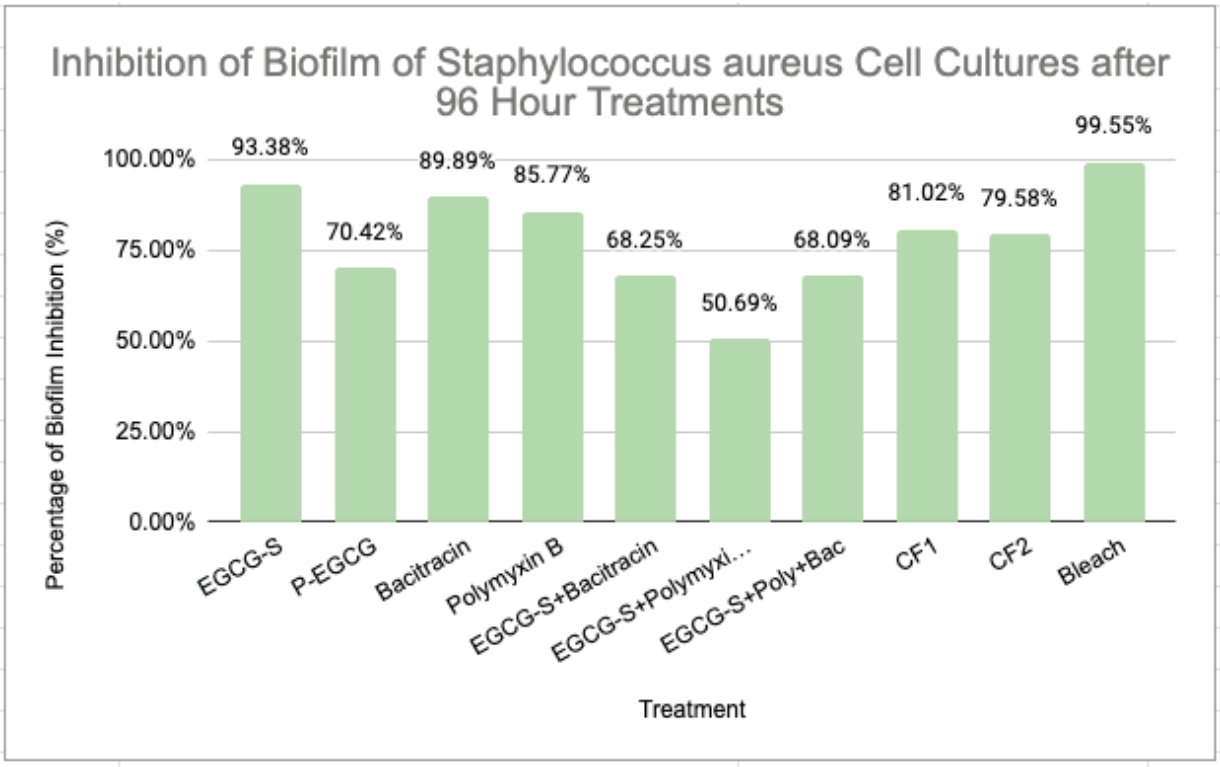
B. *Staphylococcus aureus*

Figure 37 shows the experimental plate and the control well. Figure 38 also shows which treatments were applied to which well. Figure 38 shows how the formation of biofilm was inhibited regarding *S. aureus* cell cultures after being treated for 96 hours. The percentage of biofilm inhibition across all of the treatments ranged from 50.69 percent to 99.55 percent. It was shown that EGCG-S works better than P-EGCG at inhibiting biofilm formation. When *S. aureus* was treated with EGCG-S, Polymyxin B and Bacitracin together, it resulted in an inhibition of biofilm formation percentage of 68.09 percent. When Bacitracin and Polymyxin B alone were used to treat *S. aureus*, the results showed 89.89 percent and 85.77 percent for inhibiting *S. aureus* biofilm formation.



Well 1: EGCG-S	**Well 6: EGCG-S+ Polymyxin B**
Well 2: P-EGCG	**Well 7: EGCG-S+ Polymyxin B+ Bacitracin**
Well 3: Bacitracin	**Well 8: CF1**
Well 4: Polymyxin B	**Well 9: CF2**
Well 5: EGCG-S+ Bacitracin	**Well 10: Bleach**

Figure 37: *Staphylococcus aureus* Crystal Violet Quantitative Assay Experimental Plate and Control. **Top:** Shows the experimental plate. **Bottom:** Shows the control well. The numbers displayed on the top of wells are associated to the treatments shown to the right in the figure.

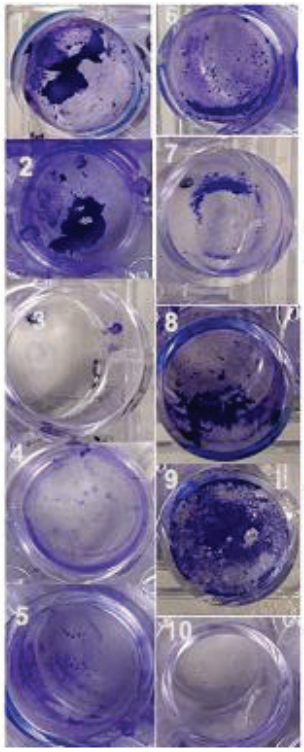


Staphylococcus aureus		
Well Number	**Treatment**	**% of Biofilm Inhibition**
1	EGCG-S	93.38%
2	P-EGCG	70.42%
3	Bacitracin	89.89%
4	Polymyxin B	85.77%
5	EGCG-S + Bacitracin	68.25%
6	EGCG-S + Polymyxin B	50.69%
7	EGCG-S + Bac+Poly	68.09%
8	CF1	81.02%
9	CF2	79.58%
10	Bleach	99.55%

Figure 38: Inhibition of *Staphylococcus aureus* Cell Cultures after 96 Hour treatments. The treatments used involved EGCG-S and P-EGCG tea polyphenols as well as Polymyxin B, Bacitracin, CF1 and CF2 formulations and bleach.

C. ***Staphylococcus epidermidis***

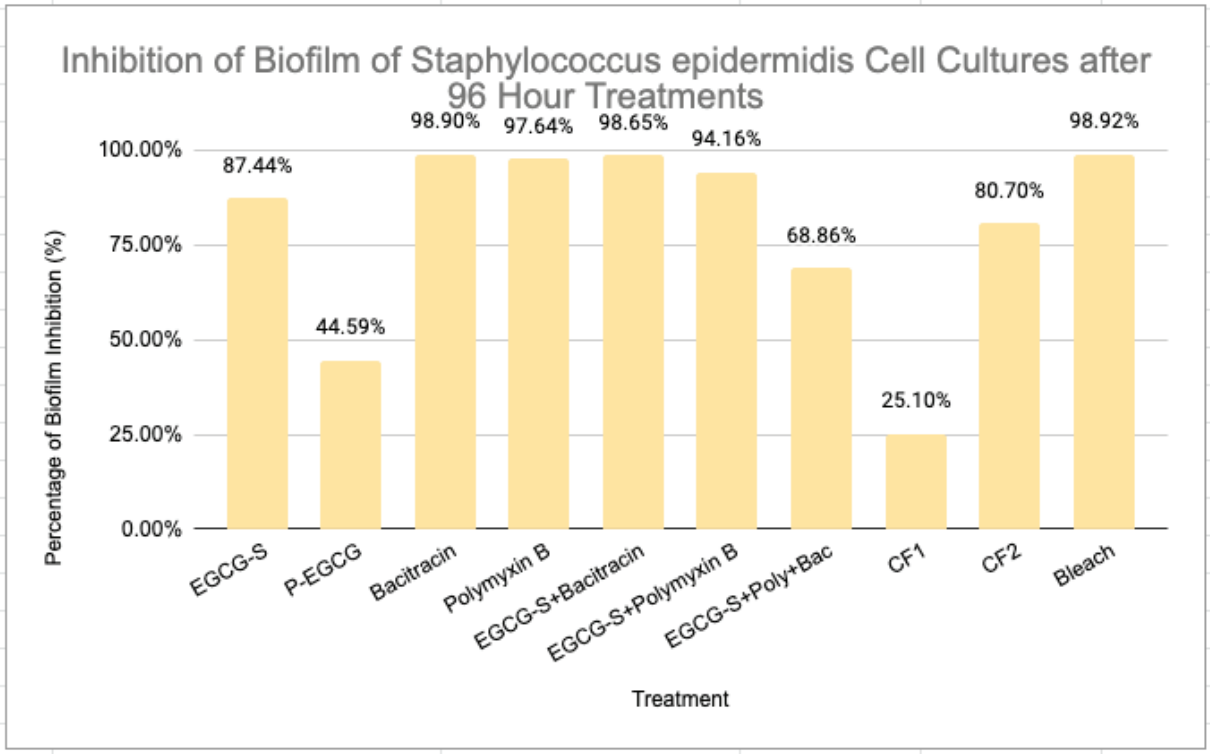
EGCG-S and P-EGCG tea polyphenols were used with antibiotics, Polymyxin B and Bacitracin to analyze the effect that they have on biofilm inhibition. Figure 39 shows the experimental plate on the top and the control well at the bottom. The percentage of biofilm inhibition ranged from 25.10 percent to 98.92 percent as shown in Figure 40. EGCG-S was shown to work better than P-EGCG at inhibiting *S. aureus* biofilm formation. When *S. epidermidis* cell cultures were treated with EGCG-S and Bacitracin together, that resulted in an inhibition of 98.65 percent. This treatment represented the third highest inhibition percentage, with the first two being bleach and Bacitracin. The use of EGCG-S with Polymyxin B increases biofilm inhibition by approximately 11 percent. EGCG-S with single antibiotics (Polymyxin B: 94.16 percent, Bacitracin: 98.65 percent) as treatments work better than EGCG-S with both antibiotics combined together which resulted in a percent inhibition of 68.86 percent regarding biofilm formation.



Well 1: EGCG-S	**Well 6: EGCG-S+ Polymyxin B**
Well 2: P-EGCG	**Well 7: EGCG-S+ Polymyxin B+ Bacitracin**
Well 3: Bacitracin	**Well 8: CF1**
Well 4: Polymyxin B	**Well 9: CF2**
Well 5: EGCG-S+ Bacitracin	**Well 10: Bleach**

Control

Figure 39: *Staphylococcus epidermidis* Crystal Violet Quantitative Assay Experimental Plate and Control. **Top:** Shows the experimental plate. **Bottom:** Shows the control well. The numbers displayed on the top of the wells are associated to the treatments shown to the right in the figure.

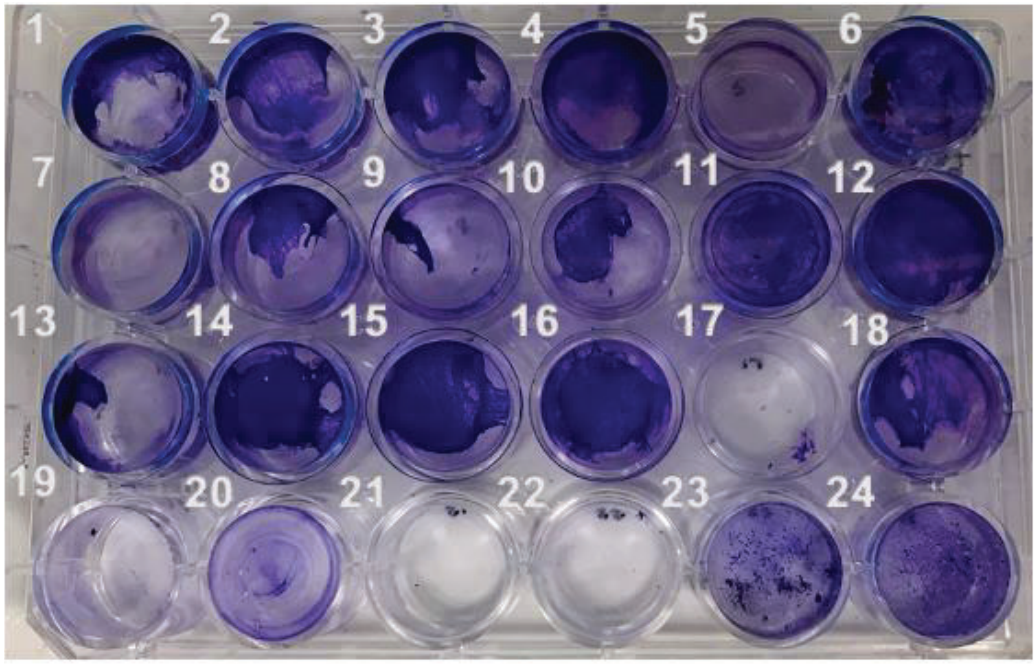


Well Number	Treatment	% of Biofilm Inhibtion
1	EGCG-S	87.44%
2	P-EGCG	44.59%
3	Bacitracin	98.90%
4	Polymyxin B	97.64%
5	EGCG-S+Bacitracin	98.65%
6	EGCG-S+Polymyxin B	94.16%
7	EGCG-S+Poly+Bac	68.86%
8	CF1	25.10%
9	CF2	80.70%
10	Bleach	98.92%

Figure 40: Inhibition of *Staphylococcus epidermidis* Cell Cultures after 96 Hour Treatments. The treatments used involved the EGCG-S and P-EGCG tea polyphenols as well as Polymyxin B, Bacitracin, CF1 and CF2 formulations and bleach.

D. Combination of Bacteria: *Pseudomonas aeruginosa*+ *Staphylococcus aureus*+ *Staphylococcus epidermidis*

EGCG-S and P-EGCG tea polyphenols were used with wash and the antibiotics, Polymyxin B and Bacitracin to see how they affect the formation of biofilm when treated against a combination of bacteria, which consists up of *P. aeruginosa, S. aureus and S. epidermidis*. Figure 41 shows the experimental crystal violet plate and a template that shows the treatments that each well was given. When EGCG-S was used with Polymyxin B together, there was a percent inhibition of 67.58 percent. Interestingly, when EGCG-S, wash, Polymyxin B and Bacitracin were combined as a treatment to treat the combination bacteria, 83.89 percent of biofilm formation was inhibited, shown in Figure 42. The use of antibiotics with EGCG-S and P-EGCG increases the percent inhibition of biofilm formation, shown in Figures 42 and 43, respectively. The EGCG-S, Polymyxin B, Bacitracin and Wash combined treatment worked the best at inhibiting biofilm formation against the combination bacteria.



Well 1: Control	**Well 2**: EGCG-S	**Well 3:** P-EGCG	**Well 4**: Wash	**Well 5**: Polymyxin	**Well 6:** Bacitracin
Well 7: EGCG-S+Polymyxin	**Well 8:** EGCG-S+ Bacitracin	**Well 9:** EGCG-S+ Polymyxin+ Bacitracin	**Well 10:** EGCG-S+ Wash+ Polymyxin+ Bacitracin	**Well 11:** Wash+ Polymyxin+ Bacitracin	**Well 12:** Polymyxin + Bacitracin
Well 13: P-EGCG+ Polymyxin	**Well 14:** P-EGCG+ Bacitracin	**Well 15:** P-EGCG+ Polymyxin+ Bacitracin	**Well 16**: P-EGCG+ Wash+ Polymyxin+ Bacitracin	**Well 17**: Bleach	**Well 18:** Empty-TSB
Well 19: P-EGCG (no cells)	**Well 20:** Polymyxin (no cells)	**Well 21:** Bacitracin (no cells)	**Well 22**: P-EGCG+ Polymyxin+ Bacitracin (no cells)	**Well 23:** CF1	**Well 24:** CF2

Figure 41: Combination Bacteria (*Pseudomonas aeruginosa+ Staphylococcus aureus+ Staphylococcus epidermidis)* Crystal Violet Quantitative Assay Experimental Plate with the template.

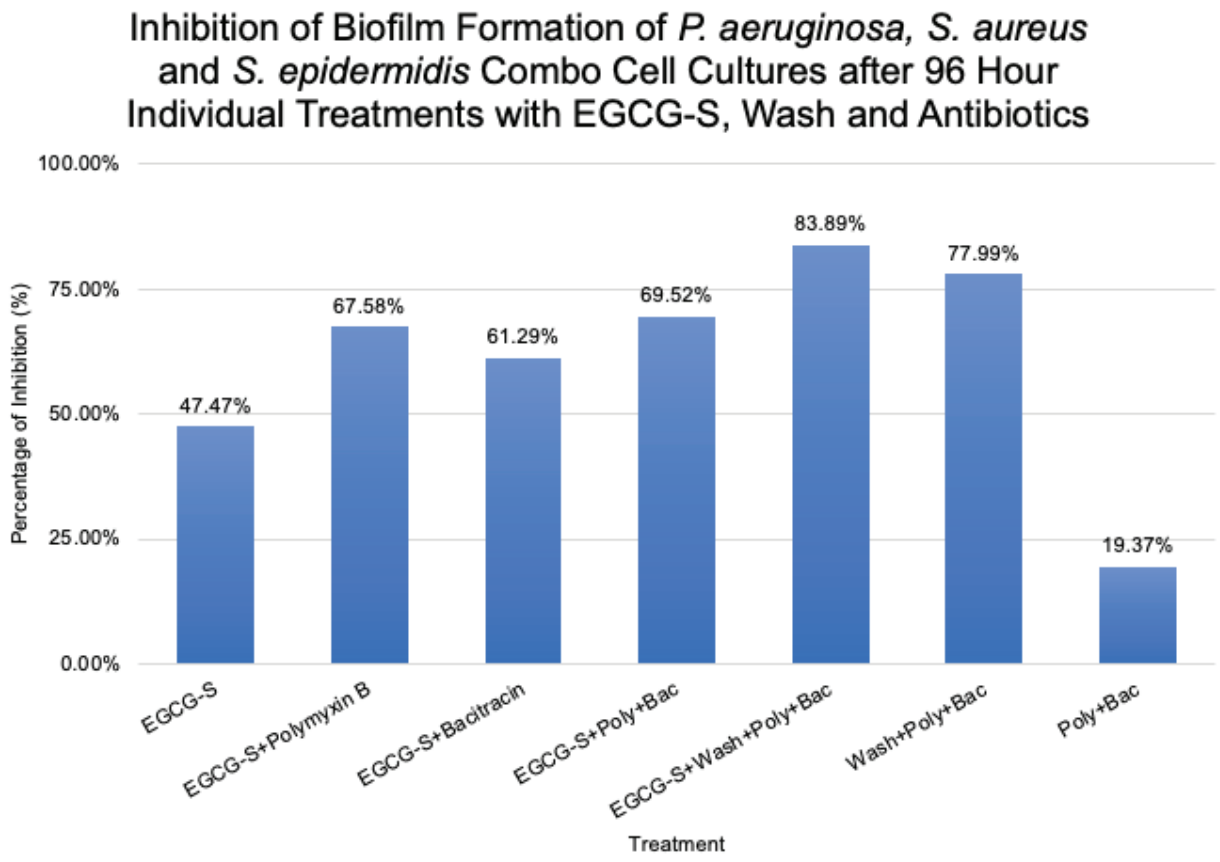


Figure 42: Inhibition of Biofilm Formation of *P. aeruginosa, S. aureus* and *S. epidermidis* Combination Bacterial Cell Cultures after 96 Hour Treatments with EGCG-S, Wash and Antibiotic Combinations

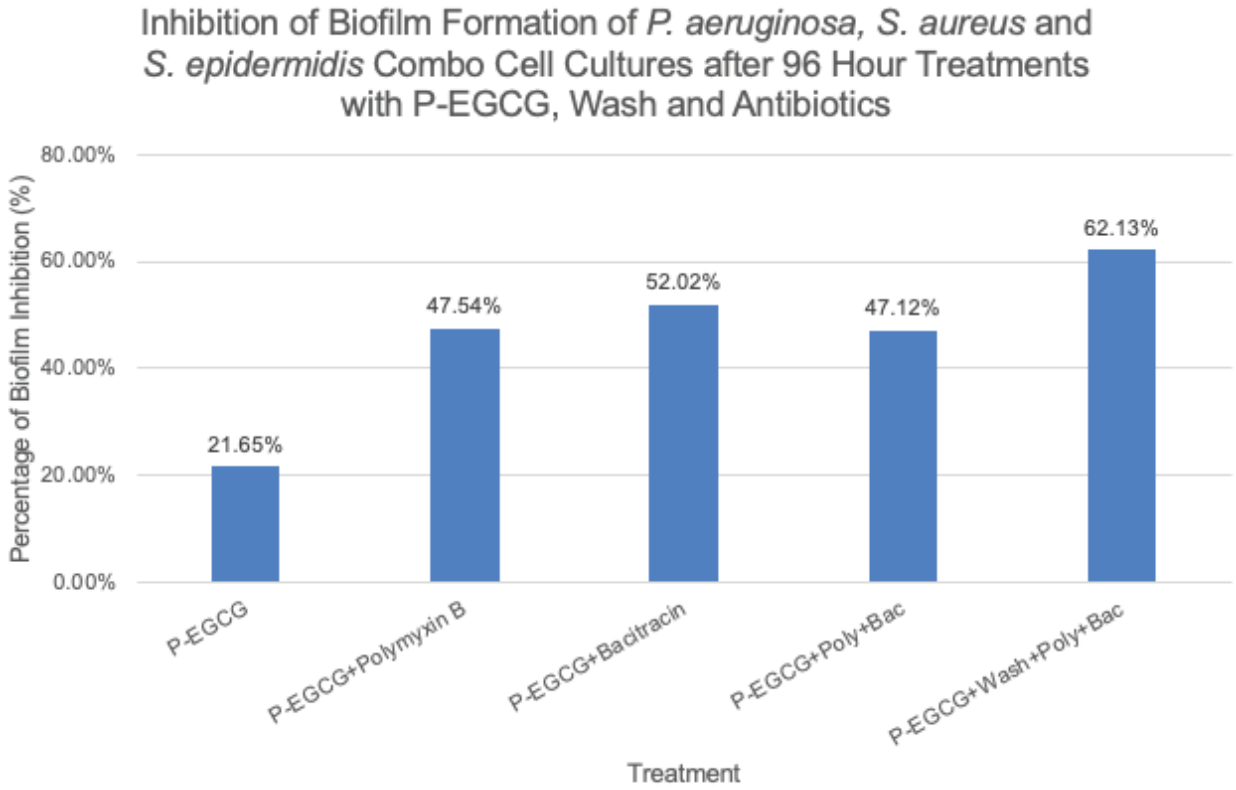


Figure 43: Inhibition of Biofilm Formation of *P. aeruginosa, S. aureus* and *S. epidermidis* Combination Bacterial Cell Cultures after 96 Hour Treatments with P-EGCG, Wash and Antibiotic combinations.

Summary:

EGCG-S by itself works really well for inhibiting biofilm formation for *P. aeruginosa, S. aureus,* and *S. epidermidis*. Bacitracin works better as a treatment alone than Polymyxin B for all three of the bacteria, especially for *S. epidermidis*. For *P. aeruginosa*, EGCG-S + Polymyxin B+ Bacitracin as a treatment works the best at inhibiting biofilm formation, but not for *S. aureus* and *S. epidermidis*. The single EGCG-S treatment for *S. aureus* worked better than the combination treatments with antibiotics. EGCG-S+ Polymyxin B+ Bacitracin+ Wash worked the best for the combination bacteria.

Percentage of Inhibition (%)										
	EGCG-S	P-EGCG	Bacitracin	Polymyxin B	EGCG-S+ Bac	EGCG-S+ Poly	EGCG-S+ Poly+ Bac	CF1	CF2	Bleach
PA	95.11%	64.70%	92.97%	82.95%	90.56%	98.10%	99.32%	94.13%	98.97%	99.89%
SA	93.38%	70.42%	89.89%	85.77%	68.25%	50.69%	68.09%	81.02%	79.58%	99.55%
SE	87.44%	44.59%	98.90%	97.64%	98.65%	94.16%	68.86%	25.10%	80.70%	98.92%
Combo	47.47%	21.65%	-	-	61.29%	67.58%	69.52%	-	-	-

Figure 44: Summary of Crystal Violet Quantitative Biofilm Assay. PA: *Pseudomonas aeruginosa;* SA: *Staphylococcus aureus*; SE: *Staphylococcus epidermidis*; Combo: Combination Bacteria (*Pseudomonas aeruginosa*+ *Staphylococcus aureus*+ *Staphylococcus epidermidis*).

Discussion

Gram negative bacteria are characterized by having a thin peptidoglycan layer along their cell wall compared to Gram-positive bacteria, which have a thick peptidoglycan layer. Since Gram-negative bacteria have an outer membrane protecting them along with the peptidoglycan layer, they are known to be more pathogenic and virulent than Gram-positive bacteria. The reason that performing a gram stain to differentiate if bacteria of interest is gram-positive or gram-negative is because treatments could work differently on the two categories of bacteria. DNA extracting, sequencing and a BLAST search will be performed to confirm the identity of the bacteria.

There are two ways that the tea polyphenols EGCG-S and P-EGCG regarding treating bacterial infections, either in the wash step or in the antibiotic steps. From this study, it shows that EGCG-S and P-EGCG combined with any of the two antibiotics gives promising results, but further studies will be needed. In order to get a higher efficacy, the concentration of the tea polyphenols could be increased. A concentration study could be performed in the future going from 250 μg/mL to 500 μg/mL to see the effectiveness of the green tea polyphenols. Treatments on *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis* individually showed similar results. The treatments did not work as well on the combination bacteria. The combination of the three bacteria are harder to treat. This could be due to the interaction and competition of these microorganisms. 5 μL of overnight culture was used for each of the treatments regarding the individual bacteria. For the combination bacteria, equal volumes of 5 μL of overnight culture from each microorganism was mixed into an eppendorf tube and 5 μL was taken out from the eppendorf tube and used for each respective treatment. Since the three microorganisms could have different generation times, the number of bacterial cells may not be

the same at the beginning of the treatment. Studies in the future could involve bacterial cell populations that have identical optical densities. Treatments can be applied to the bacterial cell populations to see if they are density dependent. A gram stain can also be performed to check the proportion of rod shaped bacteria to cocci shaped bacteria. This profiling study is the initial step in searching for the best combination treatments. Further experiments with the best treatment combinations should be carried out in many repeatings. The multiple readings will allow statistical analysis to be used to determine the significance of the different treatments.

CF1 and CF2 are very good antimicrobial agents. They are really potent and have a very high efficacy. Further studies need to be performed to determine the log reduction. A higher density of cells should be used and with more serial dilutions. Since the CF1 and CF2 formulations have such a high efficacy, future experiments should be carried out with 1:2, 1:5 and 1:10 dilutions of these CF1 and CF2 formulations. If lower concentrations of these formulations can obtain the same results, that will be cost-effective for commercializing these formulations.

A Congo red qualitative assay is fast, and it provides very clear results. All three of the microorganisms can form biofilm within 24 hours. These microorganisms are very good biofilm formers. In order to look at their biofilm forming ability, the experiment should be carried out again and the observation time should be extended from 12 to 96 hours. This study makes it easy to understand which agents regarding treatments worked. EGCG-S, P-EGCG with antibiotics as treatments worked at inhibiting biofilm formation against all three bacteria: *Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermidis*. Also, it is interesting to work on pre-formed biofilm. It has been reported that pre-formed biofilm are hard to treat with

antibiotics, so future studies can be focused on that and treating pre-formed biofilm. Even though the most efficient and effective approach to controlling bacterial infections associated with biofilms is to inhibit the formation of biofilm, joint prosthetic medical devices that can have virulent bacterial biofilms on them make it hard to treat the bacterial infections. It has recently been shown that biofilm can be eliminated by using antibiofilm compounds (Wolfmeier et al., 2017). Therefore, in order to improve treatment efficacy on persistent biofilms, antibiofilm compound that have a high bioavailability could be used. Whether or not the synergism of EGCG-S and antibiotics can extend to interfere with matured biofilms is still elusive and of interest to further investigate.

The crystal violet quantitative biofilm assay showed that Bacitracin and Polymyxin B alone worked pretty well against *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis*. The EGCG-S, Polymyxin B and Bacitracin combination treatment worked the best for *Pseudomonas aeruginosa* bacterial infections, but not for *Staphylococcus aureus* and *Staphylococcus epidermidis*. This could be due to some interaction with the EGCG-S and antibiotics, so the experiment needs to be repeated a few more times. Performing a crystal violet biofilm assay is very time consuming and technical. The sides of the wells have to be cleaned really well otherwise background noise will affect the absorbance reading of the samples. This quantitative biofilm assay, because of the process can possibly give you very high standard errors. There are other biofilm assays like the resazurin fluorescence biofilm assay, which gives better quantitative data, and the background noise is also reduced with this type of assay. In future studies, a resazurin biofilm assay can be performed with the same treatments used in the crystal violet biofilm assay and the results can be compared to each other.

Conclusion

In this study, EGCG derivatives were used to see if they could overcome the antibiotic resistance problem. EGCG-S and P-EGCG were used in various combinations with wash and the two antibiotics, Polymyxin B and Bacitracin to see if they can inhibit the growth of *Pseudomonas aeruginosa, Staphylococcus aureus and Staphylococcus epidermidis*. With all of the combination profiling studies that were performed, they suggested that EGCG-S and P-EGCG can enhance the efficacy of the wash step and the antibiotics (Polymyxin B and Bacitracin) step. The study indicated that the CF1 and CF2 formulations are excellent antimicrobial agents and they inhibit the growth of all three of the bacteria in 30 seconds with near 100 percent inhibition and a very high log reduction. The Congo Red qualitative biofilm assay, which is a combination profiling study indicated that all of the EGCG-S and P-EGCG containing combination treatments worked. Polymyxin B did not work on any of the bacteria, and this could be why the current treatment method is not as effective. The Crystal Violet quantitative biofilm assay showed that EGCG-S by itself worked really well and that it also worked well with the two antibiotics. This study shows that the EGCG-S and P-EGCG tea polyphenols can be used as a treatment option against bacteria that cause joint infections.

Future Studies

The mechanism in which the EGCG derivatives, EGCG-S and P-EGCG target bacteria and prevent the formation of biofilm will have to be further researched. This was an initial profiling study, so the most effective combination treatments will be further analyzed. The Crystal Violet Biofilm Assay should be carried out again to get conclusive results or another method like a resazurin assay should also be used to do a quantitative study. The bacteria will have to be analyzed through PCR as well as observation through a fluorescence microscope. To have a better understanding of the biofilm involved in the Congo Red Assay, live and dead assays will need to be performed. In the future, the treatments that were found to be the most effective in this study should be used on prosthetic joint materials and further analyzed.

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