The Antimicrobial Activity of Lipophilic Green Tea Polyphenols on S. mutans and the ESKAPE Pathogens

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Abstract:

Dental caries, or cavities, is a highly prevalent disease affecting societies of different economic and geographic status across the globe. This disease is caused by dental decay by bacteria fermenting the carbohydrates in food and producing acid end-products that dissolve the enamel. Though there are a few different bacteria that can cause cavities, *Streptococcus mutans* is the main etiological cause of dental caries. The earliest stages of cavity formation are typically marked with biofilm development, in which the thick slime layer confers bacteria greater adherence to the enamel surface, the ability to adapt to more adverse conditions, and quorum sensing for communicative defense and regulation. *S. mutans* utilizes sugary foods, such as those containing sucrose, and releases acid end-products that overwhelm the buffering capacity of saliva and drop the pH of the oral cavity to 5.0 or lower. At this point, the enamel begins wearing away and cavities form.

The ESKAPE pathogens represent another healthcare issue, as they are a group of bacteria that are heavily implicated in nosocomial infections and resistance to common antibiotics. The group is made up of the bacteria *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* bacteria species. Due to inappropriate and excessive use of antibiotics over the years, this group is primarily responsible for hospital-acquired infections and treatments have been more challenging to come by due to their resistance mechanisms. These mechanisms include drug inactivation or alteration, modification of drug binding sites, reduced intracellular drug accumulation, and biofilm formation.

Green tea, produced from the plant *Camellia sinensis*, is a popular beverage throughout the world, and the polyphenolic compounds of the plant have been reported to have many health benefits, including antimicrobial and anti-cariogenic properties. The green tea polyphenols are comprised of several catechins, but Epigallocatechin Gallate (EGCG) is the most abundant. Due to the instability of EGCG in aqueous solution and its tendency to readily oxidize, more stable derivatives must be studied instead. Epigallocatechin Gallate-Sterate (EGCG-S) and Palmitoyl-Epigallocatechin Gallate (P-EGCG) are esterified, stable derivatives. P-EGCG is of particular interest due to its clearing by the FDA in China as a safe food preservative, and thus it could potentially be an oral treatment in the case of *S. mutans*. 
In this study, the inhibitory effects of EGCG-S and P-EGCG at concentrations of 250 µg/mL and 500 µg/mL were studied against the cariogenic *S. mutans* bacterium. These effects were studied through colony forming unit assays, time course studies, disk diffusion assays, and post-application treatments of the polyphenolic compounds. The time course study was performed in artificial saliva with different concentrations of sucrose (0%, 0.1%, 0.5%, and 1%) in order to simulate the effects of the treatment in the oral cavity filled with sugar. Four different mouthwash products were used in the disk diffusion assay and post-application treatment in order to compare the effects of the mouthwashes and P-EGCG/EGCG-S. The next objective of the study was to investigate the effects of the green tea polyphenol treatments on the growth of *S. mutans*. To do so, Congo Red assays, Resazurin assays, and Crystal Violet assays were conducted. A separate study was conducted to investigate the effects of EGCG-S against the ESKAPE pathogens, and disk diffusion assays and colony forming unit assays were performed. The disk diffusion assays were conducted synergistically with the tea polyphenolic compounds and several antibiotics in order to study if the effects of the antibiotics could be made more potent by adding EGCG-S.

The results of the study of *S. mutans* growth suggests that EGCG-S and P-EGCG do inhibit the growth, but with mixed effectiveness. This treatment is comparable to the mouthwashes, although Chlorohexidine appears to be the most effective. *S. mutans* biofilm formation was inhibited by P-EGCG in the Congo Red assay, but the results of the Resazurin and Crystal Violet assays were inconsistent and inconclusive due to apparent cloudiness and precipitation. EGCG-S appeared to inhibit the growth of the ESKAPE pathogens as evidenced in the colony forming unit assay, although the degree of inhibition was mixed among organisms and concentrations of EGCG-S. The disk diffusion assay demonstrated that EGCG-S made the ESKAPE pathogens more sensitive to several of the antibiotics. Overall, the study suggests that P-EGCG and EGCG-S are effective in inhibiting the growth of *S. mutans* and the ESKAPE pathogens, but future studies must be conducted in order to ascertain the degree of inhibition and its effectiveness in synergistic treatment with mouthwashes/antibiotics.
Montclair State University

The Antimicrobial Activity of Lipophilic Green Tea Polyphenols on *S. mutans* and the ESKAPE Pathogens

by

Mathew Abtahi

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A THESIS

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INTRODUCTION

a. Dental Caries

Dental caries is a highly prevalent, multifactorial disease affecting societies of different economic and geographic status throughout the world (Gao et al., 2016). This disease is one of the costliest as well, and though it typically does not have mortality implications, it is a significant issue for health service providers and proper oral health (Forssten et al., 2010). Dental caries, more commonly referred to as cavities, is dental decay caused by bacteria fermenting carbohydrates in foods, producing acid as an end product and subsequently dissolving the enamel of the oral cavity (Featherstone, 2008). Tooth decay is most problematic in undeveloped and underprivileged areas, but it is also highly common amongst privileged societies as a result of diets high in carbohydrates, including sucrose (Marsh, 2004; Downer et al., 2005). Food industries have been mass producing processed foods due to technological advances, and these foods are often loaded with sugar to make the foods more appetizing. Dental caries is the most common of all childhood diseases and is estimated to occur five times more frequently than asthma, which is the second most common illness in children (Peterson et al., 2013). There are several factors which contribute to the formation of dental caries, including diet and the level of immune response, but the most influential factor is the acidity of the oral cavity induced by the microbial flora (Taylor et al., 2005). The oral cavity typically maintains a relatively neutral pH of approximately 6.8, and cavity development typically arises when the cavity becomes more acidic, which ultimately promotes the demineralization of the enamel and results in decay (Aframian et al., 2006).

The issue of dental caries in the world cannot be understated, as it is extremely prominent amongst all ages, sexes, and classes. According to the World Health Organization, 60-90% of
children and approximately 100% of adults are victims to this disease worldwide (Gao et al., 2016). The DMFS (decay-missing-filled-surface) index describes the state of dental caries in an individual and has been a key measure of dental decay for the past 70 years. The DMFS ranges from 0 to 148 with the higher values correlating to worse oral health. Research conducted on oral health demonstrated that 32 children participating in a study consuming over 163 grams of sugar daily developed a score of 5 on the index, whereas another group of 32 children consuming less than 78 grams of sugar daily scored a 3.2 on the DMFS index within a two-year period (Rugg-Gunn et al., 1984). This study is one of several suggesting that sugar intake is strongly correlated to the development of dental caries. Tooth brushing is one of the most widely accepted and effective methods for preventing the formation of dental caries, and several studies have shown a strong correlation between the frequency of brushing and the development of caries (Mclaren et al., 2016; Tinanoff., 2017). However, individual brushing techniques are variable among different social positions and education levels. One study demonstrated that an individual’s education level is correlated to his or her oral condition (Rebelo et al., 2015). Similarly, an individual’s net income may also play a factor in dental cavity formation, as lower-income demographics displayed higher cavity rates than higher-income groups (Nomura et al., 2002). There is a direct link between personal oral hygiene and cavity formation, and those with greater health education and greater wealth are better equipped to properly take care of their oral health.

The increase in acidity of the oral cavity leading to dental caries pathogenesis begins with the production of a biofilm and includes several steps. The biofilm, which is a slime layer composed of millions of bacterial cells, shares many properties with dental plaque and is formed in a three-stage process: docking, locking and maturation (Forssten et al., 2010). In the initial docking stage, the first bacteria adhere to the tooth surface. In the second step, the bacteria
encase themselves into an extracellular matrix and then transition to the final stage of the process where bacterium multiply into high cell densities (Kolenbrander et al., 2007). Biofilm formation provides an advantage to bacteria, as it renders them capable of adjusting to various environments and increases their rates of adherence, conjugation, and growth, as well as allowing for a process of communication between bacterial cells termed quorum sensing (Saini et al., 2011). This method of communication has conferred bacteria the ability to adapt to and survive fluctuations in their environments, while also allowing them to promote their pathogenicity through the upregulation of virulence factors (Cvitkovitch et al., 2003). The biofilm can grow exponentially thicker and form dental plaque, which can provide a site for the colonization and growth of many bacterial species, particularly *Streptococcus mutans* (Forssten et al., 2010). This bacterium is a gram-positive, facultative anaerobic cocci that is common in the oral cavity and is one of the main etiological causes of dental caries, partly due to its ability to metabolize sucrose at a significantly faster rate than other organisms (Forssten et al., 2010; Oda et al., 2015; Loesche, 1986; Minah et al., 1977). *S. mutans* is most effective at temperatures between 18-40°C and is an α-hemolytic streptococci species with eight stereotypes, thick cell walls, and a propensity to aggregate in pairs of short chains (Ajdic et al., 2002). This bacterium contains circular DNA and encompasses three distinct plasmids that confer resistance to antibiotics and heavy metals, produce bacteriocin, and have mechanisms for conjugation-like transfer activities (Shigeyuki et al., 1986). As a gram-positive bacterium, the quorum-sensing system of *S. mutans* is made up of a signal peptide, a membrane-bound histidine kinase sensor, and an intracellular response regulator which control several physiological functions, including competence development and gene transfer in *S. mutans* (Cvitkovitch et al., 2003). The quorum sensing capabilities of *S. mutans* may also play a critical role in the development of biofilm as
well, which allows *S. mutans* to effectively combat adverse environmental conditions and host defense mechanisms. Typically, the appearance of *S. mutans* in the oral cavity is followed by the formation of dental caries after 6-24 months (Forssten et al., 2010).

Many of our dietary options across the globe utilize carbohydrates, and these carbohydrates, particularly sucrose, are one of the main reasons for the high cavity rate in developed countries (Sheiham, 1984). These dietary carbohydrates result in the production of additional acid which may exceed both the ability of saliva to expunge acid end-products and the neutralizing capability of the salivary/plaque buffer system (Trahan et al., 1985). This leads to more frequent acidification of the plaque. Sugars, like sucrose, feed the bacteria in our oral cavity and utilize it to produce glucosyltransferases. Glucosyltransferase produced by *S. mutans* plays an essential role in the virulent dental plaque development and is also vital in the formation of extracellular polysaccharides, namely glucans from sucrose, which allow for bacterial adhesion to the tooth enamel and to each other (Krzyściak et al., 2013). Because sucrose is a low-molecular-weight disaccharide, it can be rapidly fermented and utilized by the plaque microbes, especially *S. mutans*. The process of the fermentation of the sucrose causes a drastic drop in the pH to 5.0 or lower in the oral cavity due to the release of acid end-products such as lactic acid (Loesche, 1996). Typically, sucrose ingestion is met with sufficient saliva which acts as a buffer to prevent pH and maintain the pH of the oral cavity. Saliva typically reduces the incidence of cavities in a multitude of ways, such as through action as a mechanical cleansing agent that prevents plaque build-up, by reducing the solubility of enamel with calcium, phosphate and fluoride ions, and by acting as a buffer against the acid end-products produced by cariogenic bacteria (Mandel, 1974). The acid end-products of carbohydrate fermentation by bacteria like *S. mutans* results in an increase in free hydrogen ions in the oral cavity, which
lowers the pH. Saliva contains bicarbonate ions and the enzyme carbonic anhydrase, which catalyzes the reaction between the hydrogen ions and the bicarbonate ions. As this reaction continues to occur, resulting in water and carbon dioxide gas end products, the pH rises and the environment returns to its neutral pH levels (Makawi et al., 2017). However, it has been discovered that it is not the content or quantity of carbohydrates in a meal that necessarily dictates cavity formation, but also the frequency of their intake (Krzyściak et al., 2013). When the pH of the plaque decreases to approximately 5.0 or lower, the salivary buffers are rendered ineffective, lactic acid diffuses into the tooth, the enamel begins to erode, and calcium and phosphate ions are released from the sites beneath the enamel. Though the saliva typically replenishes these minerals and acts as a buffer against the acid, frequent consumption of carbohydrates, particularly sucrose, prolongs the time period in which the plaque is acidic and thus overwhelms the salivary buffer (Loesche, 1996).

In addition to extracellular polysaccharides, many plaque bacteria like \textit{S. mutans} produce intracellular polysaccharides from various sugars. In the absence of exogenous sugar, \textit{S. mutans} may use these stored intracellular polysaccharides as a means of initiating its pathogenicity (Berman et al., 1967; Durso et al., 2014). The pathogenesis of this bacterium induces acid production in which the pH of dental plaque drops below 5.0, and thus \textit{S. mutans} must be able to adapt to these conditions and become tolerant of its environment (Bender et al., 1986; Grisworld et al., 2004). \textit{S. mutans} utilizes several mechanisms for increased tolerance to acidity, such as upregulation of the DNA repair pathway and downregulation of proton penetration into the cell membrane, but the key feature of this bacterium’s increased acid tolerance is the membrane-bound F\textsubscript{1} F\textsubscript{0} –ATPase (Quivery et al., 2001). Membrane bound F\textsubscript{1} F\textsubscript{0} –ATPase can maintain the internal pH value due to its ability to pump protons out of the cells (Bender et al., 1986).
b. ESKAPE pathgens

Similarly, the ESKAPE bacteria group are also costly and present issues and concerns for people across the globe. ESKAPE is an acronym for a group of bacteria comprised of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* bacteria species. These bacteria are grouped together due to their common role in life-threatening nosocomial infections, as well as their potential drug resistance mechanisms (Peneş et al., 2017). A 2011 survey of nosocomial infections in the United States reported 722,000 cases of infections leading to 75,000 patient fatalities (Magill et al., 2014). The number of pathogenic bacteria capable of drug resistance mechanisms and the potential of resistance is growing rapidly, ultimately placing a significant cost on healthcare systems and functions. The methicillin resistant strain of *Staphylococcus aureus*, MRSA, is one of several hospital acquired bacterial infections with new cases appearing outside of a hospital setting, suggesting that this antibiotic resistant strain is becoming an increasing threat to public health. In 2000, MRSA infections were reported in high school and college athletic programs, and there have been several reports of outbreaks in NFL locker rooms in the past 15 years. There are several mechanisms by which the ESKAPE pathogens that allow them to resist antibiotics, and these genes that confer this resistance may be carried on the chromosome, plasmid, or transposons of the bacteria (Giedraitiene et al., 2011). The mechanisms by which these bacteria resist drugs include drug inactivation or alteration, modification and regulation of the binding sites of the antibiotics, reduction of the intracellular drug accumulation, and the formation of biofilm (Wright, 2005; Li, 2004; Wilson, 2014). Some pathogenic bacteria are becoming more resistant to antibiotics due to overuse and inappropriate
use and they are no longer confined to hospitals, suggesting the need for new, investigational alternatives.

The ESKAPE pathogenic bacteria are among the most heavily implicated organisms in both nosocomial infections and their continually developing antibiotic resistance. *Enterococcus* species are gram-positive facultative anaerobes typically found in the gut of humans and animals (Santajit et al., 2016). *Enterococcus faecalis* is perhaps the most clinically relevant, as reports have indicated rising resistance to ampicillin and vancomycin leading to infections in healthcare facilities. In the Netherlands, the average case of invasive ampicillin-resistant enterococcal infections in university hospitals spiked from 10 infections in 1999 to 50 in 2005 per hospital (Top et al., 2008). *S. aureus* is a gram-positive, cocci bacterium that is a typical feature of skin flora, particularly of the nose and perineum of humans and animals (Santajit et al., 2016). Historically, infections induced by *Staphylococcus* species have been controlled effectively by penicillin treatment, but excessive use of these antibiotics have led to the emergence of β-lactamase-producing Staphylococcus, with 65-85% of this species now also resistant to penicillin G (Bodonakik et al., 1984). Reports of MRSA emerged as recently as the 1960s, and now 25% of this species are comprised of the methicillin resistant strain, with the number being as high as 50% in certain regions (Indrawattana et al., 2013). *Klebsiella pneumonia*, a member of the *Enterobacteriaceae* family, is a nonfastidious, gram-negative bacillus that has acquired the capacity for β-lactamase enzymes. These enzymes are capable of disrupting the chemical structure of β-lactamase antibiotics including penicillins, cephalosporins, and carbapenems, resulting in infections in healthcare settings (Indrawattana et al., 2013). *Acinetobacter baumannii* are widely present in the environment and are capable of persisting for a relatively long period on human hands, potentially causing high rates of cross contamination in nosocomial infections.
(Houang et al., 1998). It is a gram-negative bacterium that may cause infections at a wide range of sites, including the urinary and respiratory tracts. Like the other ESKAPE pathogens, strains of this bacterium have developed resistance to several antibiotics, including colistin and imipenem. *Pseudomonas aeruginosa* is a gram-negative, rod-shaped, facultative anaerobe typically found in the normal gut flora that is particularly troublesome for immunocompromised patients (Santajit et al., 2016). Its antibacterial resistance emanates from enzyme production and porin change, and patients typically become infected through exogenous sources, such as through contact with the environment (Fukuoka et al., 1993). *Enterobacter* species, nonfastidious gram-negative rods, represent the last of the ESKAPE pathogens, and they demonstrate resistance to a wide range of antimicrobial drugs through high levels of mutations, with the exception of tigecycline and colistin (Boucher et al., 2009). Due to the rising antibiotic resistance of this group of bacteria, it is essential that alternative solutions are found to limit the rate of nosocomial and exogenous infections.

c. **Green tea polyphenolic compounds**

Tea is a popular beverage throughout the world and is made from the infusion of dried *Camellia sinensis* leaves. Eastern cultures, including China and India, are known to use tea as a medicinal treatment based on its many health benefits. Previous studies have demonstrated that *Camellia sinensis*, particularly the non-fermented form known as green tea, has several medical advantages, including anti-inflammatory, antifungal, antiviral, antioxidant, anti-mutagenic, ant carcinogenic, anti-diabetic, protein-denaturing, and antibacterial characteristics (Biswas et al., 2015; Hamilton-Miller., 2001; Hirasawa et al., 2002; Hirasawa and Takada., 2004; Makimura et al., 1993; Nakane and Ono., 1989; Nakayama et al., 1990; Otake et al., 1991; Sakanaka et al., 1989; Wu-Yuan et al., 1988; Xu et al., 2012). Research into the topic of the remedial effects of
green tea have found that the benefits are likely to be a result of the polyphenolic catechins present in green tea, and these catechins may also play a key role in the inhibiting the growth of S. mutans both in vitro and in vivo (Biswas et al., 2015; Hirasawa et al., 2006; Mankovskaia et al., 2013). Shown in Figure 1, three specific catechins, epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG), are implicated as the prominent factors in the antibacterial features of green tea.

EGCG is the most active catechin of these three, and it makes up 59% of the catechins found within green tea (Taylor et al., 2005). However, EGCG can be relatively unstable in aqueous solution and has been found to oxidize readily, and thus an esterified derivative of EGCG, called epigallocatechin-3-gallate-sterate (EGCG-S), has been synthesized (Chen et al., 2009; Chen et al., 2003). Additionally, another esterified derivative of EGCG, named Palmitoyl-epigallocatechin-3-gallate, has been approved by the FDA in China for safe consumption as a preservative and may have implications as an ingestible antibacterial agent. The molecular structures for EGCG-S and P-EGCG are shown in Figures 2 and 3, respectively. These green tea components have been suggested to have anticariogenic effects through in vivo research in both animal and human participants, as well (Biswas et al., 2015; Hamilton-Miller., 2001; Xu., 2010).

![Figure 1. Structures of various green tea catechins (Hashimoto et al., 1999).](image1)

![Figure 2. Molecular structure of EGCG-S obtained from the Global Ingredient Archival System via NIH.](image2)
Though there have been several studies suggesting that green tea polyphenols are effective in reducing *S. mutans* and the ESKAPE pathogens, there is limited research concerning the efficacy of P-EGCG against *S. mutans*. As an FDA approved substance in China, P-EGCG has the potential to serve as an ingestible remedy for the cariogenic bacterium. With regards to the ESKAPE pathogens, the effects of EGCG-S against this group may serve as a potential alternative for antibiotics considering the resistance these bacteria have developed and continue to develop. Therefore, the objectives of this study are 1) to determine whether utilizing EGCG-S or P-EGCG (utilizing 250 and 500 µg/mL of both) may inhibit the growth and biofilm formation of *S. mutans*, 2) to observe if P-EGCG can limit the growth of *S. mutans* in artificial saliva containing different concentrations of sucrose, 3) to compare the effectiveness of P-EGCG and EGCG-S against several brands of commercial mouth wash against the growth and proliferation of *S. mutans*, 4) to study the effects of EGCG-S against the ESKAPE pathogens, 5) to study the effects of EGCG-S against ESKAPE pathogens compared to and synergistically with several antibiotics. The bacteria representing the A of the ESKAPE acronym, *Acinetobacter baumannii*, was ordered but never received by the lab, and thus the experiments conducted on the ESKAPE pathogens exclude this bacterium. Future studies should aim to include this bacterium.
MATERIALS & METHODS

1) Pre/Post-Experimental Protocols

Prior to each experiment, all materials and media were autoclaved or exposed to ultraviolet light utilizing the labs in the CELS building at Montclair State University for 10 minutes and all surfaces were cleaned with a 10% bleach solution. Following each experiment, the hood was exposed to ultraviolet light for 10 minutes.

2) Culturing Bacteria

The bacteria were grown and cultured on tryptic soy agar (TSA) plates, nutrient agar plates, or Mueller-Hinton agar plates. The cultures were incubated at 37°C for 24 hours and were subsequently stored in a cold room or refrigerator set at 4°C for future experimental use. The stock plates were frequently restocked and replaced to ensure the bacteria were pure and growing, and the purity of stock cultures was also routinely checked via microscope.

a) Bacteria Isolation

Stock culture plates were plated through the discontinuous streaking method shown in Figure 4. After confirming the purity of an isolated colony of the bacteria, the remainder of the colony was used to make a stock culture using a continuous streaking method, as shown in Figure 5. Both streaking methods were then incubated overnight at 37°C, and were then wrapped in parafilm and stored in the refrigerator/cold room.
b) Overnight cultures

Tryptic soy broth was prepared and 10 mL were pipetted into 15 mL plastic tubes. These tubes were exposed to ultraviolet light for 10 minutes prior to use. Before the night of an experiment, the purity of the stock plates was confirmed through a simple stain. A wired loop or sterile cotton swab was used to inoculate a small portion of the bacteria off of the stock
plate into the tryptic soy broth within the plastic tube, and then the mixture was vortexed and mixed to ensure optimal inoculation. Next, it was placed in a shaking incubator overnight at 250 rpm and at 37°C. Lastly, to ensure the culture remained pure overnight, the purity was checked with a simple stain before the start of the experiment.

3) Media Preparation

a) **Tryptic Soy Broth**

Tryptic Soy Broth from Difco™ was prepared by adding 30 grams of medium and 1 liter of deionized water into a 2-liter flask. The flask was stirred with a sterile glass rod, over a hot plate if necessary, to ensure proper mixing of the medium and water. The solution was subsequently autoclaved at 121°C, and then allowed to cool. The flask was then placed into the refrigerated cold room at 4°C until needed.

b) **Nutrient/Mueller-Hinton/Tryptic Soy/Congo Red Agar Preparation**

Nutrient Agar from Difco™ was prepared using 30 grams of medium and 1 liter of deionized water. Mueller-Hinton Agar from Difco™ was prepared using 38 grams of medium and 1 liter of deionized water. Tryptic Soy Agar (TSA) from Remel™ was prepared using 40 grams of medium and 1 liter of deionized water. Tryptic Soy Agar (TSA) from Remel™ was prepared by mixing 40 grams of the commercial medium and 1 liter of deionized water. Both solutions were mixed thoroughly with a sterile glass rod, using a hot plate if necessary, and then autoclaved. Next, the solutions were poured into individual sterile plates and left to solidify. The plates were then exposed to ultraviolet light for 20 minutes. Lastly, they were placed back into plastic sleeves, sealed with tape, labeled, and put into the refrigerated cold room at 4°C until needed.
Congo Red Agar from Sigma-Aldrich was prepared following the protocol outlined by Schwartz et al (2012). Media and Congo Red were prepared in two separate flasks and mixed together after being sanitized through the autoclave. The first medium contained 9.25 grams of Brain Heart Infusion broth medium, 12.5 grams of sucrose (5%), 5 grams of bacto agar, and 250 mL of deionized water. This flask was then stirred and mixed with a sterile glass rod, using a hot plate if necessary, until the contents were mixed homogenously. The second solution contained 0.4 grams of Congo Red powder (50X) and 10 mL of deionized water. Both preparations were autoclaved at 121°C and set aside to cool until they reached a temperature between 55-60°C. 5 mL of the Congo Red solution was then added to the broth solution and mixed thoroughly, and the solution was then pipetted in accordance to the well plate being used. These plates were then allowed to solidify at room temperature. Fresh Congo Red media was prepared prior to each experiment.

c) Artificial Saliva preparation

The artificial saliva was generated within the lab following the formula listed by Klimek et al., 1982. The formula is as follows:

\[0.33g \text{KH}_2\text{PO}_4, 0.34g \text{Na}_2\text{HPO}_4, 1.27g \text{KCl}, 0.16g \text{NaSCN}, 0.58g \text{NaCl}, 0.17g \text{CaCl}_2, 0.16g \text{NH}_4\text{Cl}, 0.2g \text{urea}, 0.03g \text{glucose}, 0.002g \text{ascorbic acid}, \text{and} 2.7g \text{mucin dissolved in} 1000 \text{mL distilled water. This solution maintains a pH of 7.}\]

d) Preparation of Green Tea Polyphenols

All green tea polyphenols were purchased from Camellix LCC, Augusta, GA.

a. EGCG-S
EGCG-S was prepared using 200 proof ethanol, and the stock concentrations were prepared and diluted depending on the concentration needed for each experiment. The calculation of the master stock solution is as follows:

\[ 2.5 \text{ mg/mL} = 0.0025 \text{g tea EGCG-S} + 1\text{mL EtOH} \]

b. P-EGCG

P-EGCG was prepared using 200 proof ethanol, and the stock concentrations were prepared and diluted depending on the concentration needed for each experiment.

4) Cytological Staining

a) Simple Stain

A smear preparation was performed by first sterilizing a wire loop over a flame until it appeared orange in color. Once the loop cooled down, so as not to kill the bacterial cells, it was used to obtain a small loop of bacteria from the stock culture. The loopful of bacteria was then inoculated onto a sterile glass slide. A drop of methylene blue dye was then placed on top of the smear, followed by a cover slip. The slide was then blotted with bibulous paper, and then observed under oil immersion at 1000X total magnification (Lee et al., 2015).

b) Gram-Stain

A smear preparation was prepared using the same technique as performed for the simple stain. The smear was then allowed to air dry and was then covered with crystal violet dye for 20 seconds and subsequently rinsed with deionized water. Next, the sample was covered with Gram’s iodine for 1 minute, exposed to Gram’s decolorizer for 10-20 seconds, and then washed with deionized water. The smear was then counter stained with safranin for 1 minute before being washed with deionized water. Lastly, a cover slip was placed upon the smear,
blotted with bibulous paper, and observed under oil immersion at 1000X total magnification (Lee et al., 2015).

**Study of the Effects of Lipophilic Green Tea Polyphenolic Compounds on *S. mutans***

5) Monitoring the Growth of *S. mutans*

a) **Standard Growth Curve**

To monitor the growth of *Streptococcus mutans*, a standard growth curve was performed over a 24-hour period. The overnight culture in tryptic soy broth was first diluted to 0.1, and the culture was then placed into an incubator at 37°C. Readings were taken at every hour at 600 nm for 10 hours and a final reading taken at 24 hours. This experiment was conducted using a spectrophotometer.

b) **Growth Curve in different concentrations of Sucrose**

The cariogenic effects of *S. mutans* has in the presence of carbohydrates in the oral cavity has been reported in various studies over the year, and thus a growth curve in artificial saliva with sucrose concentrations of 0%, 0.1%, 0.5%, and 1% was conducted. Overnight cultures were prepared in each of these artificial saliva conditions was diluted to an OD\(_{600}\) reading of approximately 0.1. Readings were taken every other hour at 600 nm for 10 hours and a final reading was taken at 24 hours. This experiment was conducted using a spectrophotometer.

6) The Effect of EGCG-S and P-EGCG against the growth of *S. mutans*

a) **Colony Forming Units (CFU)**

A CFU assay was conducted using P-EGCG 250 µg/mL and P-EGCG 500 µg/mL. An overnight culture of *S. mutans* in tryptic soy agar and then diluted to an OD\(_{600}\) reading of 0.1.
Once this reading was obtained, 90 µL of this diluted overnight culture was aliquoted to a new conical tube along with 10 µL of either 250 µg/mL or 500 µg/mL P-EGCG. The controls were prepared by adding 10 µL of diH2O to the 90 µL of the diluted overnight. These treatments were then left to sit for two hours. These separate treatments were then diluted to reach the appropriate dilutions. A 10⁻² dilution was achieved by aliquoting 10 µL of this mixture and adding 990 µL of diH₂O. Each subsequent dilution was achieved by sequestering 10 µL of the previous dilution and adding 990 µL of diH₂O. Once the correct dilutions were prepared, 100 µL were pipetted onto the appropriately labeled nutrient agar plate and then inoculated through the continuous streaking method.

Once each plate was inoculated, they were then incubated at 37°C for 24 hours. After 24 hours, the plates were removed from the incubator and the individual plaques on the plate were counted to determine the colony forming units. For plates in which there were a substantial amount to count, the plates were divided to make counting easier and the number of colonies was then multiplied to give an approximate count of the entire plate. Once the colony forming units of each plate was counted, the percent of inhibition of each tea treatment was calculated to identify the effect of the tea against *S. mutans*. The following equation was utilized:

\[
\text{Percent of Inhibition} = \left( \frac{\text{CFU}_{\text{Control}} - \text{CFU}_{\text{Treated}}}{\text{CFU}_{\text{Control}}} \right) \times 100
\]

b) Time Course Study

A time course study was conducted in order to determine the effectiveness of the tea polyphenols at different time points. The time points chosen for this study were 30 seconds, one minute, and 5 minutes. Overnight cultures of *S. mutans* were prepared in artificial saliva of different concentrations of sucrose: 0% sucrose, 0.1% sucrose, 0.5% sucrose, and 1%
sucrose. Thus, there were four separate overnight cultures needed for this experiment. *S. mutans* was inoculated into 5-6 mL of each artificial saliva condition and then incubated for 24 hours. Prior to the time course study, the overnight cultures were plated to try and determine the best dilution factor for the time course experiment itself. The dilution factors $10^{-2}$ and $10^{-4}$ were selected for the experiment.

After preparing the overnight and determining the dilution factor, 50 µL of each overnight culture was then centrifuged for 5 minutes at 7000 RPM in order to form a pellet in which the bacteria have aggregated towards the bottom of the microcentrifuge tube. After forming the pellet, the pellet was either resuspended in 50 µL of 250 µg/mL P-EGCG, corresponding to the treated samples, or 50 µL of diH$_2$O, corresponding to the control of the experiment. After the pellet was resuspended in the tea, 10 µL of the treated samples were removed at each time point of 30 seconds, 1 minute, and 5 minutes and placed into 990 µL of diH$_2$O. This dilutes the sample to a factor of $10^{-2}$. A further dilution was then conducted by taking 10 µL of the $10^{-2}$ dilution and adding it to 990 µL of diH$_2$O. The same process was done with the control in order to get the same dilutions.

After the proper dilutions were made, 100 µL of each sample was plated onto the designated nutrient agar plate. The samples were then inoculated onto each plate using sterile, dispensable plastic loops through the continuous streaking method, and were then incubated at 37°C for 24 hours. After 24 hours, the plates were removed from the incubator and the CFU count of each plate was determined. The CFU count was then used to determine the percent of inhibition of the tea at each time point.
c) Disk Diffusion

Prior to the experiment, 15 mm disks were autoclaved within a glass petri dish in order to sterilize them. Nutrient agar plates were then labeled and sectioned off according to the treatment conditions to be used in each section. *S. mutans* from an overnight culture were continuously streaked onto these plates using a sterile cotton swab. The sterile disks were then soaked in the antiseptic/disinfectant/tea, and then aseptic forceps were used to transfer the disks to the corresponding nutrient agar plate. The treatments used in this disk diffusion study include the commercial mouthwashes Chlorohexidine, Colgate, Dentiste Plus White, and Listerine, and the tea treatments were EGCG-S 250 and 500 µg/mL and P-EGCG 250 and 500 µg/mL. The disks were placed in the center of the plate, and then the plates were inverted and incubated for 48 hours at 37°C. After incubation, the radius of the zone of inhibition was measured in millimeters using a ruler from the edge of the disk to the edge of the growth, as shown in Figure 6. The measurement corresponds to the ability of the treatment to inhibit the bacterial growth. The mouthwashes used are demonstrated in Figure 7, and the ingredients and flavors of these mouthwashes are demonstrated in Table 1.

![Figure 6. Demonstration of a disk diffusion plate and the zone of inhibition. The grey in each section represents the disk soaked in treatment, and the white represents the clearing zone in which no bacteria have grown. The red line represents the zone of inhibition measured in millimeters.](image-url)
Figure 7. The mouthwash brands included in this study. From left to right, the mouthwashes are the 100% natural Dentiste Plus White Perfect mouthwash, the commercial mouthwash brands Listerine and Colgate, and the prescription rinse Chlorhexidine mouthwash.

<table>
<thead>
<tr>
<th>Mouthwash Brand</th>
<th>Flavor</th>
<th>Active Ingredients</th>
<th>Inactive Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dentiste Plus White Perfect</td>
<td>Mint</td>
<td>Sage Extract, clove oil, xylitol, Funnel extract, Chamomile extract, Glycyrrhina extract, Vitamin C, Eucalyptus</td>
<td>Water, alcohol (21.6%), sorbitol solution flavoring, poloxamer 407, benzoic acid, sodium saccharin, sodium benzoate, FD&amp;C Green No. 3</td>
</tr>
<tr>
<td>Listerine</td>
<td>Cool Mint</td>
<td>Eucalyptol 0.092% Menthol 0.042% Methyl Salicylate 0.060% Thymol 0.064%</td>
<td></td>
</tr>
<tr>
<td>Colgate Total Gum Health</td>
<td>Clean Mint</td>
<td>Cetylpyridinium chloride 0.075% (Ceepryn)</td>
<td>Ater, glycerin, propylene glycol, sorbitol, poloxamer 407, flavor, potassium sorbate, citric acid, sodium saccharin, sucralose, FD&amp;C green no.3</td>
</tr>
<tr>
<td>Chlorhexidine Gluconate 0.12% Oral Rinse</td>
<td>Spearmint</td>
<td>Chlorhexidine Gluconate (1,1'-hexamethylene bis [5-(p-chlorophenyl) biguanide]di-D-gluconate)</td>
<td>Water, 11.6% alcohol, glycerin, PEG-40 sorbitan disostearate, flavor, sodium saccharin, FD&amp;C Blue no.1</td>
</tr>
</tbody>
</table>

Table 1. A list of the flavors and ingredients of the four mouthwashes used in several experiments in this study.
d) Post-Application of EGCG-S and P-EGCG

A post-application study was conducted in order to determine the effect of EGCG-S and P-EGCG as disinfectants against *S. mutans*. Overnight cultures were prepared and controls were prepared to dilution factors of $10^{-4}$, $10^{-6}$, and $10^{-8}$ by diluting with water and the commercial mouthwash products were diluted to a factor of $10^{-2}$. The dilutions were prepared utilizing diH$_2$O. Once the dilutions were prepared, 100 µl of each dilution was plated on TSA plates and then left to air dry for five minutes.

Once the plates dried, the P-EGCG, EGCG-S, and commercial mouthwashes were added to the designated plates. 100 µL of P-EGCG (250 and 500 µg/mL), EGCG-S (250 and 500 µg/mL), Chlorohexidine, Listerine, Colgate, and Dentiste Plus White were pipetted onto the designated plate for each treatment and the plate was air-dried for an hour afterwards. Following drying, the plates were incubated at 37°C for 24 hours. All plates were incubated at optimal temperature overnight and the CFU was counted, followed by calculation of the percent of inhibition. Due to the high count of CFU on certain plates, a division method was used to count in which the plates were divided into 16 in order to obtain a more countable section, and then the value of this section was multiplied by 16 in order to give an approximation of the total CFU value on the plates.

7) Study the Effect of P-EGCG on Biofilm formation

a) Congo Red Assay

Overnight cultures were prepared and diluted to 0.1 optimal density at 600 nm. 250 µL of the overnight of *S. mutans* was pipetted onto two Congo Red wells of a 24 well plate. The
experimental groups were prepared the same way, but the tea treatment was added as well. 250 µg/mL and 500 µg/mL P-EGCG were used for treatment. Two wells were treated with 50 µL of 250 µg/mL P-EGCG and another two wells were treated with 100 µL of 250 µg/mL P-EGCG. The same was done for 500 µg/mL P-EGCG. The Congo Red plates were then incubated for 4 days at 37°C. Pictures were taken after the four days to monitor the growth of the biofilm. The positive results were observed as dark precipitation on the red agar plates, and negative results were observed as a lack of change from the strong red color observed before inoculation.

b) Resazurin Assay

Overnight cultures were prepared in the four different sucrose concentrations of artificial saliva, and the cultures were then diluted to an absorbance range of approximately 0.3 OD at 600nm using a spectrophotometer. The cultures were then incubated overnight at 37°C, and were then plated in a 48 well plate. The experiment was conducted using P-EGCG, and three repeating trials were prepared to test for the effects of 250 µg/mL P-EGCG, 500 µg/mL P-EGCG, and a control using diH2O for each of the artificial saliva conditions. Following the set-up of the plate, it was incubated at 37°C for 3-4 days to allow for biofilm to develop. Following this incubation, the supernatant of each cell was removed while being cautious so as not to disrupt the biofilm by touching the bottom of the well with the pipette. After removing the supernatant, each well was rinsed with 100 µL of 10X PBS, and the supernatant was removed following the wash. Next, 200 µL of Resazurin working solution, prepared from 400 µL stock resazurin and 20 mL of PBS, was pipetted into each well. After adding the Resazurin working solution, the fluorescence was measured at excitation of 560 nm and emission of 590 nm using the multi-plate reader in order to measure the growth of biofilm.
c) Crystal Violet assay

The procedure of making the overnight culture in artificial saliva of different sucrose concentrations, preparing the 48 well plate, and incubating the plate for 3-4 day follows the protocol of the Resazurin assay. After incubating for 3-4 days, the supernatant was removed from each well and the wells were then washed with 250 µL of 10X PBS. The supernatant was removed, and 200 µL of Crystal Violet dye was then added to each well. The treatment was then left to sit for 30 minutes, and the Crystal Violet was subsequently removed from each well. The plate was then left to air-dry overnight by turning the plates upside down. The following day, 500 µL of acetic acid was added to each well, and the absorbance of the wells was measured at 595 nm in order to measure the growth of biofilm formation.

Study of the Effects of Lipophilic Green Tea Polyphenolic Compounds on the ESKAPE pathogens

8) Effects of EGCG-S on the growth of the ESKAPE pathogens

a) Disk Diffusion assay

Prior to the experiment, 6 mm blank disks were autoclaved within a glass petri dish in order to sterilize them. These blank disks were used as controls throughout the experiment. Mueller-Hinton agar plates were then labeled and sectioned off according to the treatment condition to be used in each section. Cultures of the ESKAPE bacteria were prepared in Tryptic Soy Broth and incubated overnight at 37°, diluted to an OD_{600} reading of 0.1, and then the bacteria from each culture was continuously streaked onto the appropriate plates using a sterile cotton swab. Aseptic forceps were then used to place the 6 mm disks of either the control or the antibiotics used in the experiment. The antibiotic disks used in this
experiment included Erythromycin, Chloramphenicol, Penicillin, Tetracycline, Vancomycin, Kanamycin, Ampicillin, Streptomycin, Bacitracin, Cephalothin, and Polymyxin. After placing the disks in the center of each section, each disk was treated with 100 µL of 500 µg/mL EGCG-S. The plates were then incubated for 24 hours at 37°C. Following incubation, the diameter of the zone of inhibition was measured in millimeters using a ruler from one edge of the inhibitory zone to the other. This measurement corresponds to the ability of the treatment to inhibit the bacterial growth. The inhibitory effect of the combination of the tea polyphenols and the antibiotic were compared with the effect of the individual antibiotics, and the percent inhibition of the tea polyphenols was calculated with the following equation:

\[
\text{Percent of Inhibition} = \left( \frac{\text{ZOI}_{\text{Control}} - \text{ZOI}_{\text{Treated}}}{\text{ZOI}_{\text{Control}}} \right) \times 100
\]

b) Colony Forming Unit assay

A CFU assay was conducted using EGCG-S 250 µg/mL and EGCG-S 500 µg/mL. Overnight cultures of the ESKAPE bacteria were prepared in tryptic soy broth and then diluted to an OD$_{600}$ reading of 0.1. Once this reading was obtained, 90 µL of this diluted overnight culture was aliquoted to a new conical tube along with 10 µL of either 250 µg/mL or 500 µg/mL EGCG-S. The controls were prepared by adding 10 µL of diH$_2$O to the 90 µL of the diluted overnight. These treatments were then left to sit for two hours in order to allow the cells to be adequately exposed to the tea polyphenols. These separate treatments were then diluted to reach the instructed dilutions. A $10^{-2}$ dilution was achieved by aliquoting 10 µL of this mixture and adding 990 µL of diH$_2$O. Subsequent dilutions were prepared by either sequestering 10 µL of the previous dilution and adding 990 µL of diH$_2$O in order to achieve two further dilutions or by sequestering 100 µL of the previous dilution and adding 900 µL of diH$_2$O to achieve one further dilution. Once the correct dilutions were prepared,
100 µL were pipetted onto the appropriately labeled nutrient agar plate and then inoculated through the continuous streaking method with a sterile cotton swab.

Once each plate was inoculated, they were then incubated at 37°C for 24 hours. After 24 hours, the plates were removed from the incubator and the individual plaques on the plate were counted to determine the colony forming units. For plates in which there were a substantial amount to count, the plates were divided to make counting easier and the number of colonies was then multiplied to give an approximate count of the entire plate. Once the colony forming units of each plate was counted, the percent of inhibition of each tea treatment was calculated to identify the effect of the tea against *S. mutans*.

**RESULTS & DISCUSSION**

The Effect of EGCG-S and P-EGCG against the growth of *S. mutans*

1) Cytological Staining

Simple and gram-stains were conducted in order to characterize *S. mutans* and identify its morphology and cell surface. The results of these stains are demonstrated in Figures 8 and 9. The simple stain confirms that the bacteria present are in fact *S. mutans*, as evidenced by the circular, cocci shape typically found in chains. The gram-stain technique showed purple-stained cells, confirming that *S. mutans* is a gram-positive bacterium. Gram-positive bacteria have a thick outer peptidoglycan cell wall layer, and this layer takes up the crystal violet and confers the purple stain.
Figure 8. Simple stain of *S. mutans*, confirming the coccic shape of the bacterium and its propensity to form chains.

Figure 9. Gram-stain of *S. mutans*. The purple color demonstrates that the thick outer peptidoglycan cell wall layer took up the crystal violet dye, which is characteristic of gram-positive organisms.

### 2) Monitoring the Growth of *Streptococcus mutans*

The growth of *S. mutans* was monitored for a period of 24 hours. The absorbance was read every hour for the first 10 hours of the growth in nutrient broth, and the absorbance was...
read every two hours for the first 10 hours for the growth in artificial saliva with different concentrations of sucrose. In both cases, a final reading was taken at the 24th hour.

A standard growth curve typically displays four distinct stages: the lag phase, the exponential growth phase, the stationary phase, and the death phase. The growth of *S. mutans* in nutrient agar demonstrated the first three of these phases over the observed 24 hour period. As demonstrated in Figure 10, the lag phase was observed after 1 hour. Then, the exponential phase with a constant generation time was observed between 2 and 7 hours, which corresponds to the large slope of the graph at this juncture. The stationary phase can be observed from hours 7 to 24, as represented by the plateau on the graph. The optical density readings at these time points are very similar, suggesting a near equal proportion of cellular growth and cellular death. This is due to the nutrients being exhausted by the bacteria in the previous hours, causing the cells to die with lack of nourishment. Under optimal conditions, the generation time of *S. mutans* was determined to be 67.5 minutes, based on the equation: 

\[ g = \frac{\log_{10} N_1 - \log_{10} N_0}{\log_{10} 2} \]

This suggests that 67.5 minutes is the time in which this bacterium doubles in population size.

The growth of *S. mutans* in artificial saliva with different concentrations of sucrose (0%, 0.1%, 0.5%, 1%) demonstrated that the bacteria grow most effectively in the presence of sucrose. This supports the idea that the cariogenicity of *S. mutans* corresponds to the consumption of carbohydrates, particularly sucrose. The generation time was calculated to be 170.6 minutes in artificial saliva with 0% sucrose, 76.89 minutes in artificial saliva with 0.1% sucrose, 94.38 minutes in artificial saliva with 0.5% sucrose, and 89.6 minutes in artificial saliva with 1% sucrose. These results are demonstrated in Figure 11. The graph has some irregularities compared to a normal growth curve, which may correspond to
fluctuations in the nutrients and the uptake of nutrients compared to the standard growth curve performed. Further trials should be conducted to understand the reason for some of these irregularities, as well as further experiments observing the growth of *S. mutans* in artificial saliva with different concentrations of sucrose.

**Figure 10.** Standard growth curve for *Streptococcus mutans* in nutrient broth. The generation time was calculated to be 67.5 minutes for the bacterial growth to double. The results were from the mean of three repeating trials, and the growth was read at OD$_{600}$ using a spectrophotometer.
The Effects of Green Tea Polyphenolic Compounds on the Cariogenic *S. mutans*

a) Colony Forming Unit Assay

A CFU assay was performed with the goal of comparing the experimental treatment using the tea polyphenols and the control treatment at countable ranges, and using the count of colony forming units in order to calculate the percent of inhibition of the green tea polyphenols. The experiment was conducted using P-EGCG 250 μg/mL and P-EGCG 500 μg/mL, and diH₂O was utilized as a control. The results demonstrate that P-EGCG at both concentrations (250 μg/mL and 500 μg/mL) inhibit the growth of *S. mutans*, albeit at different effectiveness. The percent of inhibition of 250 μg/mL P-EGCG was calculated at 64.87%, and the percent of inhibition of 500 μg/mL P-EGCG was calculated at 80.58%. These results suggest that the green tea polyphenolic compounds are effective in inhibiting the growth of the bacteria as opposed to the control which simply contained the cells, tryptic.

**Figure 11.** Growth curve for *Streptococcus mutans* in artificial saliva with different sucrose concentrations of 0%, 0.1%, 0.5%, and 1%. The generation time was calculated to be 170.6 minutes in artificial saliva with 0% sucrose, 76.89 minutes in artificial saliva with 0.1% sucrose, 94.38 minutes in artificial saliva with 0.5% sucrose, and 89.6 minutes in artificial saliva with 1% sucrose.
soy broth, and diH₂O. Dilutions of $10^{-4}$ and $10^{-5}$ were prepared for the control, and yielded average colony forming unit counts of 242 and 31, respectively. Dilutions of $10^{-2}$, $10^{-3}$, and $10^{-4}$ were prepared for the conditions utilizing tea polyphenolic treatments.

In both the 250 µg/mL and 500 µg/mL, the $10^{-2}$ and $10^{-3}$ dilutions yielded extremely high counts. The plaque count for 250 µg/mL P-EGCG $10^{-2}$ averaged at nearly 1000 plaques over two trials, and the count for the $10^{-3}$ dilution averaged at 589 plaques over two trials. The plaque count for 500 µg/mL P-EGCG $10^{-2}$ dilution also averaged at nearly 1000 plaques, and the count for the $10^{-3}$ dilution averaged at nearly 489 plaques. It appears that $10^{-4}$ was the appropriate dilution to utilize, as the values were far more countable. For 250 µg/mL P-EGCG, the plaque count at $10^{-4}$ averaged at 86 over two trials. For 500 µg/mL P-EGCG, the plaque count at $10^{-4}$ averaged at 47 plaques over two trials. The plaque counts for the control and treatment plates are demonstrated in Table 2 and Figure 13. The calculated percent of inhibition of the 250 µg/mL P-EGCG and 500 µg/mL P-EGCG, compared to the controls at this dilution, were 64.87% and 80.58%, respectively, as demonstrated in Figure 12. These results suggest that at similar dilutions, P-EGCG strongly inhibits the bacteria and that 500 µg/mL P-EGCG inhibits the bacteria more effectively than 250 µg/mL P-EGCG. These results are shown in Figures 4A and 4B.
<table>
<thead>
<tr>
<th>Treatment/Control for S. mutans</th>
<th>Plaque count, Trial 1</th>
<th>Plaque count, Trial 2</th>
<th>Average</th>
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<tbody>
<tr>
<td>Control $10^{-4}$</td>
<td>244</td>
<td>240</td>
<td>242</td>
</tr>
<tr>
<td>Control $10^{-5}$</td>
<td>30</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>250 P-EGCG $10^{-2}$</td>
<td>~1000</td>
<td>~1000</td>
<td>~1000</td>
</tr>
<tr>
<td>250 P-EGCG $10^{-3}$</td>
<td>567</td>
<td>610</td>
<td>589</td>
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<tr>
<td>250 P-EGCG $10^{-4}$</td>
<td>87</td>
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<tr>
<td>500 P-EGCG $10^{-4}$</td>
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<tr>
<td>% inhibition of 250 P-EGCG</td>
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<td>64.87%</td>
</tr>
<tr>
<td>% inhibition of 500 P-EGCG</td>
<td></td>
<td></td>
<td>80.58%</td>
</tr>
</tbody>
</table>

*Table 2.* The plaque counts and calculated percent of inhibition of the colony forming unit assay. Two trials were conducted, and the averages of each trial were used to calculate the percent of inhibition. 500 µg/mL P-EGCG inhibits *S. mutans* more effectively than 250 µg/mL P-EGCG, as they inhibit 80.58% and 64.87% of the bacteria, respectively.

*Figure 12.* The percent of inhibition of both concentrations of P-EGCG (250 µg/mL and 500 µg/mL). P-EGCG 500 µg/mL is more effective in inhibiting *S. mutans* than P-EGCG 250 µg/mL.
b) Time Course Study

The goal of the time course study was to determine the effectiveness of P-EGCG at various time points, as a means of getting insight into the effectiveness of this green tea polyphenolic compound as an additive or main ingredient of a potential oral cleanser. Though dilutions of $10^{-2}$ and $10^{-4}$ were prepared, the results for $10^{-4}$ suggested that this was too much of a dilution, as all of the plates lacked countable plaques. Thus, the results are entirely of the dilution factor $10^{-2}$. The results of the time course study suggest that P-EGCG 250 $\mu$g/mL inhibits *S. mutans* growth within artificial saliva with different concentrations of sucrose, but the results are mixed. It appears that the inhibitory effects of the treatment increase as time goes on, and this difference is especially pronounced in artificial saliva without sucrose. After 30 seconds, the green tea compounds inhibited 13.79% of the bacteria, but after five minutes this value increased to 72.4% of bacteria. The effectiveness of the
treatment in this time course study varies between sucrose conditions. The effectiveness is limited in the three conditions containing sucrose, as after five minutes only 40% of bacteria in saliva with 0.1% sucrose is inhibited, 56.4% of bacteria in saliva with 0.5% sucrose is inhibited, and 48.24% of bacteria in saliva with 1% sucrose is inhibited. These values are low; repeated trials and different concentrations of both P-EGCG and EGCG-S are required to find the optimal treatment for this study. The colony forming unit count is demonstrated in Table 3 and the percent of inhibition at each time point is demonstrated graphically in Figure 14. The plates at each dilution are demonstrated in Figures 15-19. The main takeaway from this experiment is that the P-EGCG does inhibit the bacterial growth in artificial saliva with different concentrations to some extent, and this inhibitory effect becomes more potent as time goes on. It appears that the beneficial effects of sucrose on the growth of S. mutans may outweigh the inhibitory effects of 250µg/mL P-EGCG, as the bacteria in greater concentrations of sugar are not inhibited nearly as effectively as the bacteria grown without sugar. Future studies must also focus on conducting the same study with commercial mouthwashes, both independently and in combination with the green tea polyphenolic compounds in order to find the most effective oral cleanser. The goal of this study was to ultimately compare the effectiveness of mouthwashes to tea treatments at the time points studied and then to potentially do a synergistic study of the combined effects of mouthwash and tea polyphenols. Although the latter half of the goal was not accomplished, future studies should focus on doing so.
<table>
<thead>
<tr>
<th>Treatment (10⁻²)</th>
<th>30 seconds</th>
<th>1 minute</th>
<th>5 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial Saliva 0% Sucrose Control</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artificial Saliva 0% Sucrose Treated</td>
<td>25</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>% inhibition= 13.79%</td>
<td>% inhibition= 31.03%</td>
<td>% inhibition = 72.4%</td>
</tr>
<tr>
<td>Artificial Saliva 0.1% Sucrose Control</td>
<td>750</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artificial Saliva 0.1% Sucrose Treated</td>
<td>570</td>
<td>540</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>% inhibition= 24%</td>
<td>% inhibition = 28%</td>
<td>% inhibition= 40%</td>
</tr>
<tr>
<td>Artificial Saliva 0.5% Sucrose Control</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artificial Saliva 0.5% Sucrose Treated</td>
<td>656</td>
<td>496</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>% inhibition= 34.4%</td>
<td>% inhibition = 50.4%</td>
<td>% inhibition= 56.4%</td>
</tr>
<tr>
<td>Artificial Saliva 1% Sucrose Control</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artificial Saliva 1% Sucrose Treated</td>
<td>67</td>
<td>53</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>% inhibition= 21.18%</td>
<td>% inhibition = 37.65%</td>
<td>% inhibition= 48.24%</td>
</tr>
</tbody>
</table>

Table 3. The CFU count and calculated percent of inhibition of the bacteria in different concentrations of sucrose. The dilution factor throughout was 10⁻²

Figure 14. A graphical representation of the percent of inhibition of 250 µg/mL P-EGCG at different time points of 30 seconds, 1 minute, and 5 minutes during the time course study
Figure 15. The controls used in the study with a dilution factor of $10^{-2}$. The image on the top left corresponds to the bacteria grown in 0% sucrose, the image on the top right corresponds to the bacteria grown in 0.1% sucrose, the image on the bottom left corresponds to the bacteria grown in 0.5% sucrose, and the image on the bottom right corresponds to the bacteria grown in 1% sucrose.

Figure 16. The plates of the bacteria treated with 250 µg/mL P-EGCG at different time points in artificial saliva with 0% sucrose. From left to right, this figure shows the effect of 250 µg/mL P-EGCG on *S. mutans* grown in artificial saliva without sucrose after 30 seconds, 1 minute, and 5 minutes. The treatment is more effective over time.
Figure 17. The plates of the bacteria treated with 250 µg/mL P-EGCG at different time points in artificial saliva with 0.1% sucrose. From left to right, this figure shows the effect of 250 µg/mL P-EGCG on *S. mutans* grown in artificial saliva with 0.1% sucrose after 30 seconds, 1 minute, and 5 minutes. The treatment is more effective over time.

Figure 18. The plates of the bacteria treated with 250 µg/mL P-EGCG at different time points in artificial saliva with 0.5% sucrose. From left to right, this figure shows the effect of 250 µg/mL P-EGCG on *S. mutans* grown in artificial saliva with 0.5% sucrose after 30 seconds, 1 minute, and 5 minutes. The treatment is more effective over time.
Figure 19. The plates of the bacteria treated with 250 µg/mL P-EGCG at different time points in artificial saliva with 1% sucrose. From left to right, this figure shows the effect of 250 µg/mL P-EGCG on S. mutans grown in artificial saliva with 1% sucrose after 30 seconds, 1 minute, and 5 minutes. The treatment is more effective over time.

c) Disk Diffusion Assay

A disk diffusion assay was conducted in order to compare the inhibitory effects of EGCG-S and P-EGCG at both 250 µg/mL and 500 µg/mL to the effects of the four aforementioned mouthwash products. A disk diffusion assay results in the clearing of the bacterial lawn, and the greater the size of this clearing, the more effective the treatment is at inhibiting bacterial growth. The results of this disk diffusion assay demonstrate that the commercial mouthwash product Chlorohexidine is most effective against S. mutans, with a ZOI measured at 5 mm. Colgate demonstrated a ZOI of 2.5 mm, Dentiste Plus White had a ZOI of 1 mm, and Listerine proved to be least effective with a ZOI of 0 mm. The tea treatments had similar effectiveness compared to the mouthwashes besides chlorohexidine, as EGCG-S 250 µg/mL had a ZOI of 2 mm, EGCG-S 500 µg/mL had a ZOI of 1 mm, P-EGCG 250 µg/mL had a ZOI of 1 mm, and P-EGCG 500 µg/mL had a ZOI of 0 mm. These
results are demonstrated in Table 4 and Figure 20. The results suggest that EGCG-S 250 µg/mL has a comparable inhibitory effect as Colgate does against *S. mutans*, and that EGCG-S 500 µg/mL and P-EGCG 250 µg/mL had a comparable inhibitory effect as Dentiste Plus White mouthwash. It also appears EGCG-S is a more effective tea treatment than P-EGCG. Future studies should focus on repeated trials, as well as the synergistic effect of the different teas and different concentrations of these teas with the mouthwash. Together, these treatments could perhaps produce a more effective oral health care mouthwash than the majority of the commercially available ones. Listerine particularly demonstrates the greatest need for antibacterial additives, such as EGCG-S and P-EGCG, as it did not inhibit the growth of *S. mutans* in this particular assay. The reason P-EGCG has been a treatment of focus throughout this study is due to its clearance by the FDA in China as a safe preservative in food. This implies that the green tea polyphenolic treatment could perhaps be an effective, consumable product for antimicrobial infections, but these results do not suggest its effectiveness as an individual product. Future studies should specifically focus on combining this treatment with others in hopes of creating an effective, safe to consume product.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorohexidine</td>
<td>5</td>
</tr>
<tr>
<td>Colgate</td>
<td>2.5</td>
</tr>
<tr>
<td>Dentiste Plus White</td>
<td>1</td>
</tr>
<tr>
<td>Listerine</td>
<td>0</td>
</tr>
<tr>
<td>EGCG-S 250 µg/mL</td>
<td>2</td>
</tr>
<tr>
<td>EGCG-S 500 µg/mL</td>
<td>1</td>
</tr>
<tr>
<td>P-EGCG 250 µg/mL</td>
<td>1</td>
</tr>
<tr>
<td>P-EGCG 500 µg/mL</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4. The ZOI results for the commercial mouthwash brands used as well as the different concentrations of EGCG-S and P-EGCG. Chlorohexidine appeared to have the greatest inhibitory effect against the bacterium, whereas EGCG-S was comparable or more effective than the rest of the mouthwash brands. P-EGCG 500 250 µg/mL had a comparable ZOI to Dentiste Plus White, and P-EGCG 500 µg/mL and Listerine did not inhibit the growth of the bacteria.
Figure 20. Images of the plates used to conduct the disk diffusion assay utilizing different brands of commercial mouthwash and different concentrations of EGCG-S and P-EGCG. The plate on the left is divided into 4 distinct sections. The section on the top left corresponds to Chlorohexidine, the top right corresponds to Listerine, the bottom left corresponds to Colgate, and the bottom right corresponds to Dentiste Plus White. This plate demonstrates that Chlorohexidine cleared the greatest proportion of bacteria, followed by Colgate. The plate on the right contains disks soaked in different concentrations of EGCG-S and P-EGCG. The section on the top left corresponds to EGCG-S 250 µg/mL, the section on the right corresponds to EGCG-S 500 µg/mL, the section on the bottom left corresponds to P-EGCG 250 µg/mL, and the section on the bottom right corresponds to P-EGCG 500 µg/mL.

**d) Post-Application of P-EGCG and EGCG-S**

The post application experiment was conducted in order to observe the effects of the green tea polyphenols after the growth of S. mutans on the agar plates. This perhaps has more clinical relevance, as it demonstrates the efficacy of the green tea polyphenols after S. mutans has already colonized a site and thus may give insight into the ability of the tea polyphenols to treat infection. The results of the post-application suggest great effectiveness of both the mouthwashes and the tea treatments against S. mutans growth. Prior to any treatment, the plates had a plaque count of 180,000. This value remained constant post-diH2O treatment in the control, but the values dropped significantly in each of the treatments. The results are shown in Figure 5A-E. Chlorohexidine was the most effective in inhibiting 99.9996% of the bacteria on the plate, followed by Colgate, Protect CT, and Listerine, all of which still
inhibited at a rate greater than 99.98%. The tea treatments were nearly equally effective, with 
EGCG-S 250 µg/mL and P-EGCG 250 µg/mL inhibiting at over 99% as well. The results of 
this post-application study are reported in Table 5 and the percent of inhibition is 
demonstrated graphically in Figure 21. Figures 22, 23, and 24 demonstrate the plates after the 
application of the green tea polyphenolic compounds. These results indicate great 
effectiveness of the tea polyphenolic compounds, and possibly suggest effectiveness of a 
synergistic treatment of both the mouthwashes and the tea polyphenols.

Future studies should focus on conducting a post-application study utilizing the 
mouthwashes and tea polyphenolic compounds synergistically to study whether they inhibit 
the bacteria more effectively than the individual treatment of antibiotics or tea polyphenolic 
compounds. A log reduction calculation was also obtained through an online website. The 
log reduction shows the relative number of bacteria eliminated from the agar plates by the 
treatments. A 1-log reduction corresponds to a ten-fold reduction in bacteria, a 2-log 
reduction corresponds to a 100-fold reduction in bacteria, a 3-log reduction corresponds to a 
1000-fold reduction in bacteria, and so on. Chlorhexidine appears to be the ideal 
mouthwash to test for synergistic effects with the tea polyphenolic compounds, as it has 
proven over several trials of this experiment and the disk diffusion experiment to be the most 
effective of the mouthwashes against S. mutans. The disk diffusion experiment and this post-
application experiment both reveal that 250 µg/mL EGCG-S is more effective than either 
concentration of P-EGCG and that P-EGCG has limited effectiveness compared to the 
mouthwash treatments. Both experiments suggest that perhaps P-EGCG is best suited as an 
additive, and future studies should further investigate this.
<table>
<thead>
<tr>
<th>Treatment (10⁴)</th>
<th>CFU prior to treatment</th>
<th>CFU post-treatment</th>
<th>Percent of Inhibition</th>
<th>Log Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>180,000</td>
<td>180,000</td>
<td>0%</td>
<td>0</td>
</tr>
<tr>
<td>Chlorohexidine</td>
<td>180,000</td>
<td>0.8</td>
<td>99.99955556%</td>
<td>5.35</td>
</tr>
<tr>
<td>Colgate</td>
<td>180,000</td>
<td>8.32</td>
<td>99.99537778%</td>
<td>4.34</td>
</tr>
<tr>
<td>Protect CT</td>
<td>180,000</td>
<td>16.32</td>
<td>99.99093333%</td>
<td>4.04</td>
</tr>
<tr>
<td>Listerine</td>
<td>180,000</td>
<td>32.32</td>
<td>99.98204444%</td>
<td>3.75</td>
</tr>
<tr>
<td>EGCG-S 250 µg/mL</td>
<td>180,000</td>
<td>510</td>
<td>99.71666667%</td>
<td>2.55</td>
</tr>
<tr>
<td>EGCG-S 500 µg/mL</td>
<td>180,000</td>
<td>2000</td>
<td>98.888888889%</td>
<td>1.95</td>
</tr>
<tr>
<td>P-EGCG 250 µg/mL</td>
<td>180,000</td>
<td>1456</td>
<td>99.19111111%</td>
<td>2.09</td>
</tr>
<tr>
<td>P-EGCG 500 µg/mL</td>
<td>180,000</td>
<td>2256</td>
<td>98.74666667%</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Table 5.** Results of the post-application treatment of different mouthwashes and tea polyphenols. The results include the CFU count of each plate before and after treatment, the calculated percent of inhibition of each treatment, and the log reduction of each treatment. Chlorohexidine is the most effective mouthwash against *S. mutans*, inhibiting over 99.999% of the bacteria. EGCG-S 250 µg/mL is the most effective tea treatment, inhibiting over 99.7% of the bacteria.

**Figure 21.** Graphical representation of the percent of inhibition of the treatments used in the post-application.
**Figure 22.** Each of the control plates used. The controls were diluted to $10^{-4}$, $10^{-6}$, and $10^{-8}$, and they were not treated with either mouthwash or tea polyphenols. The controls were instead treated with diH$_2$O.

**Figure 23.** Mouthwash treated plates at a dilution factor of $10^{-2}$. Chlorhexidine inhibited the bacteria most effectively, followed by Colgate, Protect CT, and Listerine.

**Figure 24.** The post-application plates utilizing EGCG-S 250 and 500 µg/mL and P-EGCG 250 and 500 µg/mL. EGCG-S and P-EGCG 250 µg/mL were most effective in inhibiting the bacteria, as they inhibited over 99% of the bacteria.
4) Treatment of Biofilm with Green Tea Polyphenolic Compounds

a) Congo Red Assay

A Congo Red assay was conducted in order to test for the growth of biofilm formation on this agar with and without treatment of P-EGCG at both 250 µg/mL and 500 µg/mL. Biofilm formation is a typical marker of cavity formation and confers S. mutans several advantages in cariogenicity. The results of this qualitative experiment demonstrate that both concentrations of P-EGCG, at both 50 µL and 100 µL, inhibited the growth of the biofilm effectively. The results of this experiment are demonstrated in Figure 25. The controls demonstrate the dark colored accumulation indicative of a positive result of biofilm formation. Congo Red is an amyloid binding dye, and when biofilms are produced, amyloid or amyloid-like proteins are produced, which bind to the dye and turn the dye black in color. Both concentrations of P-EGCG appear to have completely inhibited biofilm formation, as evidenced by the strong red color of the treated wells. This suggests that P-EGCG actively prevents the formation of the slime layer which allows for further adhesion of bacterial cells along with increased resistance to suboptimal conditions. Thus, P-EGCG may be able to prevent the formation of S. mutans biofilm in the oral cavity, which is typically one of the first signs of dental cavity formation. Future studies should evaluate the effects of the different mouthwashes used throughout this study on the growth of S. mutans biofilm on Congo Red agar, as the prevention of biofilm development is highly significant and would highlight the significance of using P-EGCG as an additive to oral cleansers if they are unable to inhibit this growth as effectively as the tea polyphenolic compound individually.
Resazurin and Crystal Violet Assay

Resazurin and Crystal Violet Assays were conducted several times as another means of observing the impact of the green tea polyphenols on the ability of *S. mutans* to form biofilm. Despite the repeated attempts to conduct these experiments, the results were consistently false-positives, inconsistent, and counter-intuitive. Before performing each experiment again, a qualitative analysis was made of each plate after adding 250 µg/mL and 500 µg/mL of EGCG-S and P-EGCG into the plates. This revealed that both concentrations of P-EGCG and 500 µg/mL EGCG-S resulted in cloudiness and precipitation, which may have interfered with the results of each experiment and resulted in the general inconsistency. The cloudiness of
the wells treated with both concentrations of P-EGCG and 500 µg/mL EGCG-S can be observed in Figure 26, and the precipitation can be observed in the crystal violet dyed wells in Figure 27. In Figure 27, the wells with the greatest accumulation at the bottom are those treated with P-EGCG, whereas the controls that were not treated with tea had virtually no accumulation.

The reason for the cloudiness and precipitation need to be investigated further, although it appears that these conditions only occur when both concentrations of P-EGCG and 500 µg/mL of EGCG-S were added to the liquid media. Future studies should look to study the effects of the green tea polyphenols on biofilm formation using different studies, such as potentially through a fluorescence scope study. This may prove to work more effectively due to the fact that solid agar is used in the experiment, similar to the solid agar that proved effective in the Congo Red assay.

**Figure 26.** The cloudiness of the wells demonstrates that the tea polyphenolic compounds may be precipitating and accumulating in the solution. This may explain the inconsistencies in the results.
Figure 27. Demonstration of the accumulation of the bottom of the crystal violet plates. There is accumulation on the plates treated with P-EGCG, but no accumulation on the control plates. This gave inconsistent results, because the tea polyphenols should inhibit the growth of biofilm but the absorbance scan showed that the treated wells had greater accumulation.

5) Effect of EGCG-S on ESKAPE Pathogens

a) Gram-Stain Results

A gram-stain was conducted of the ESKAPE organisms to characterize each bacterium. The results confirm that Enterococcus faecalis and Staphylococcus aureus are gram-positive bacterium, as evidenced by the dark purple stain observed in Figure 28A and 28B. The gram-stain also confirmed that Klebsiella pneumoniae, Pseudomonas aeruginosa, and Enterobacter species are gram-negative species, as evidenced by the light pink hue of the stained cells. These results are shown in Figure 28 C-E. The gram-negative bacteria are stained pink because they have an extra layer made up of lipopolysaccharides and proteins, and thus they take up the safranin, resulting in the pink color.
b) Disk Diffusion Results

The disk diffusion experiments were conducted on each of the ESKAPE bacteria in order to test for the synergistic effects of EGCG-S with the antibiotics. Considering these bacteria are associated with increasing antibiotic resistance, any treatment that could increase the effectiveness of the antibiotics against these bacteria is worth investigating. The results of the disk diffusion assay of the ESKAPE bacteria indicate that 500 µg/mL EGCG-S is able to inhibit the growth of each individual bacteria, as evidenced by the comparison of the blank disk and the blank disk treated with EGCG-S. The results of the synergistic effects of the
antibiotics and 500 µg/mL EGCG-S vary for each organism. An organism’s response to an antibiotic can be described as being resistant, intermediately resistant, or sensitive, and the synergistic treatment of the tea conferred increase resistance to certain antibiotics in each organism compared to the effects of the antibiotic alone. Each organism’s resistance profile to the antibiotics is based on the zone of inhibition, and thus the ability of EGCG-S to synergistically create a larger zone of inhibition than the antibiotic alone correlates to the bacteria being more sensitive to this treatment.

For *E. faecalis*, the status of this bacterium with regards to Kanamycin changed from resistant to intermediately resistant and from resistant to sensitive with regards to Streptomycin. These results for *E. faecalis* are shown in Figures 29-30. The bacterium *S. aureus* appeared sensitive most of the antibiotics without the treatment of green tea polyphenols, and thus the synergistic treatment only slightly increased the effectiveness of the antibiotics. The most promising effect of EGCG-S was in changing the status of *S. aureus* from intermediately resistant to Vancomycin to sensitive to it. The results for *S. aureus* are demonstrated in Figures 31-32. The effect of EGCG-S on the antibiotic resistance of *K. pneumoniae* and *P. aeruginosa* was far more pronounced. *K. pneumoniae* was initially resistant to Erythromycin, Penicillin, and Bacitracin, but treatment with the antibiotic and 100 µL of EGCG-S made this bacterium only intermediately resistant. The combined treatment also changed *K. pneumoniae* from being resistant to Vancomycin and Cephalothin to being fully sensitive to the treatment. The results for *K. pneumoniae* are reported in Figures 33-34. Though *P. aeruginosa* showed resistance to Chloramphenicol, Penicillin, Ampicillin, Streptomycin, and Bacitracin, the combined treatment with EGCG-S changed the status of this bacterium to intermediately resistant to these antibiotics. This bacterium also
changed from being resistant to Vancomycin and Polymyxin individually to being completely sensitive to both antibiotics when treated with the tea polyphenols. The results for *P. aeruginosa* are shown in Figure 35-36. Lastly, *Enterobacter* species showed intermediate resistance to Chloramphenicol and Polymyxin individually, but when synergistically treated with these antibiotics and 500 µg/mL EGCG-S, they showed sensitivity to both. The results for *Enterobacter spp.* are shown in Figures 37-38.

The combined treatment of EGCG-S and the antibiotics proved effective in more cases than the aforementioned examples, but they did not result in changes to each bacteria’s drug susceptibility. However, it is still significant that EGCG-S increase the effectiveness of those antibiotics. Ultimately, the disk diffusion study demonstrates that EGCG-S is effective in increasing the impact of several antibiotics against a group of organisms becoming increasingly resistant to drugs. Should this resistance increase even further in the coming years, there will be an increasing demand for different approaches to inhibit the growth and proliferation of bacteria, particularly the ESKAPE pathogens. Thus, EGCG-S can perhaps offer a solution to control ESKAPE infections and limit the principal cause of nosocomial infections. Future studies should aim to conduct repeating trials, include more antibiotics, and also utilize more concentrations of EGCG-S in order to find the optimal treatment to use synergistically with the antibiotics. The results for *Enterobacter spp.* were inconsistent, and future studies should focus on this bacterium specifically.
Figure 29. A graphical representation of the inhibitory effect of the antibiotics, individually (bar on the left) and synergistically with 500 µg/mL EGCG-S (bar on the right). The greatest impact of the synergistic treatment is observed in Kanamycin and Streptomycin, as the bacteria changed from resistant to intermediately resistant (Kanamycin) and sensitive (Streptomycin).

Figure 30. A graphical representation of the percent of increase/decrease of the zone of inhibition of the synergistic treatment compared with the individual treatment of antibiotics for *E. faecalis.*
Figure 31. A graphical representation of the inhibitory effect of the antibiotics, individually (bar on the left) and synergistically with 500 µg/mL EGCG-S (bar on the right). The bacterium was sensitive to all of the antibiotics except Vancomycin, and the synergistic treatment caused *S. aureus* to be sensitive to Vancomycin as well.

Figure 32. A graphical representation of the percent of increase/decrease of the zone of inhibition of the synergistic treatment compared with the individual treatment of antibiotics for *S. aureus*. 
Figure 33. A graphical representation of the inhibitory effect of the antibiotics, individually (on the left) and synergistically with 500 µg/mL EGCG-S (bar on the right). *K. pneumoniae* was resistant to Erythromycin, Penicillin, Bacitracin, Vancomycin, and Cephalothin, but the synergistic treatment made the bacterium more sensitive to these. The synergistic treatment made the bacterium intermediately resistant to Erythromycin, Penicillin, and Bacitracin, and sensitive to Vancomycin and Cephalothin.

Figure 34. A graphical representation of the percent of increase/decrease of the zone of inhibition of the synergistic treatment compared with the individual treatment of antibiotics for *K. pneumoniae*.
**Figure 35.** A graphical representation of the inhibitory effect of the antibiotics, individually (bar on the left) and synergistically with 500 µg/mL EGCG-S (bar on the right). *P. aeruginosa* was resistant to Chloramphenicol, Penicillin, Ampicillin, Streptomycin, Bacitracin, Vancomycin, and Polymyxin, but the synergistic treatment made the bacterium more sensitive to these. The synergistic treatment made the bacterium intermediately resistant to Chloramphenicol, Penicillin, Ampicillin, Streptomycin, and Bacitracin, and the treatment made the bacterium sensitive to Vancomycin and Polymyxin.

**Figure 36.** A graphical representation of the percent of increase/decrease of the zone of inhibition of the synergistic treatment compared with the individual treatment of antibiotics for *P. aeruginosa*.
**Figure 37.** A graphical representation of the inhibitory effect of the antibiotics, individually (bar on the left) and synergistically with 500 µg/mL EGCG-S (bar on the right). The results were mixed, but the bacteria changed from being intermediately resistant to Chloramphenicol and Polymyxin to sensitive to both when synergistically treated with these antibiotics and EGCG-S.

**Figure 38.** A graphical representation of the percent of increase/decrease of the zone of inhibition of the synergistic treatment compared with the individual treatment of antibiotics. Further trials need to be conducted to ascertain the results, as it appears that the synergistic treatment worked less effectively than several individual antibiotics, including Vancomycin, Ampicillin, Bacitracin, and Cephalothin.
c) Colony Forming Unit Assay

The results of the colony forming unit assay demonstrate that both 250 and 500 µg/mL EGCG-S are effective in inhibiting the growth of the ESKAPE pathogens, though the degree to which they inhibit the bacteria is mixed. A $10^{-2}$ dilution factor was utilized throughout, as the countable range was ideal. The effects of the green tea polyphenols were not very potent against *E. faecalis* as compared to its effects on other bacteria, as both concentrations inhibited 32% of the bacterial growth. The percent of inhibition is demonstrated graphically in Figure 39. The results for the use of EGCG-S against *S. aureus* suggest that 250 µg/mL EGCG-S is more effective than 500 µg/mL EGCG-S, as these concentrations of EGCG-S inhibited 77.1% and 70.15%, respectively. The results for the percent of inhibition of *S. aureus* is reported graphically in Figure 40. However, the opposite observation was observed for *K. pneumoniae*, as 500 µg/mL EGCG-S was more effective in inhibiting bacterial growth than 250 µg/mL EGCG-S. The percent of inhibition for 250 µg/mL EGCG-S was 32%, and the percent of inhibition for 500 µg/mL EGCG-S was 43%. These results are demonstrated in Figure 41. The use of 250 µg/mL and 500 µg/mL EGCG-S against *P. aeruginosa* inhibited 55.55% and 61.73% of bacterial growth, respectively. These results are shown in Figure 42. Lastly, the use of 250 µg/mL EGCG-S was the most effective on *Enterobacter* species compared to any of the concentrations of EGCG-S used on the ESKAPE pathogens, inhibiting 78.50% of bacterial growth. These results are reported in Figure 43.

Though the results do report bacterial growth inhibition, repeating trials should be conducted to confirm these results. With regards to *Enterobacter spp.*, 500 µg/mL EGCG-S should be utilized to study its effectiveness compared to 250 µg/mL EGCG-S, which was relatively effective.
Figure 39. A graphical representation demonstrating the percent of inhibition of each concentration of EGCG-S against the growth of *E. faecalis*. Both concentrations limited growth at equal strength, inhibiting 32% in both cases.

Figure 40. A graphical representation demonstrating the percent of inhibition of each concentration of EGCG-S against the growth of *S. aureus*. 250 µg/mL EGCG-S inhibited growth more effectively than 500 µg/mL EGCG-S, with the percent of inhibition being 77.1% and 70.15%, respectively.
Figure 41. A graphical representation demonstrating the percent of inhibition of each concentration of EGCG-S against the growth of *K. pneumoniae*. 500 µg/mL EGCG-S inhibited growth more effectively than 250 µg/mL EGCG-S, with the percent of inhibition being 43 EGCG % and 32%, respectively.

Figure 42. A graphical representation demonstrating the percent of inhibition of each concentration of EGCG-S against the growth of *P. aeruginosa*. 500 µg/mL EGCG-S inhibited growth more effectively than 250 µg/mL EGCG-S, with the percent of inhibition being 61.73% and 55.55%, respectively.
Conclusions

In conclusion, it appears that EGCG-S and P-EGCG are effective in inhibiting the growth of *S. mutans*, as demonstrated in the colony forming unit assay, the time course study, the disk diffusion assay, and the post-application treatment of the green tea polyphenolic compounds. The colony forming unit assay demonstrated that 250 µg/mL P-EGCG inhibited 64.87% of *S. mutans* growth and 500 µg/mL P-EGCG inhibited 80.58% of bacterial growth. A time course study was conducted in order to observe the effects of P-EGCG over different time points of 30 seconds, 1 minute, and 5 minutes, and the results were mixed. Though the experiment did demonstrate that the green tea polyphenol treatment was more effective at the later time points, suggesting that the treatment needs longer exposure to the bacterial cells to increase effectiveness, the inhibition of the bacteria was not very pronounced. The treatment was most effective after 5 minutes in the artificial saliva with 0% sucrose condition, suggesting that the beneficial effects of added sucrose
on bacterial growth may be greater than the inhibitory effects of P-EGCG against \textit{S. mutans}.

However, because cloudiness and precipitation were observed when P-EGCG was inoculated into the media mixture of artificial saliva and tryptic soy broth in the case of the Resazurin and Crystal Violet assays, it is possible that this phenomenon occurred and skewed these results. One of the goals of the experiments with \textit{S. mutans} and the green tea polyphenols was to observe its effectiveness compared to several mouthwash brands with the hopes of P-EGCG being more effective than the mouthwash brands or potentially suitable as an additive to them. The disk diffusion assay and post-application treatment investigated this phenomenon, and revealed that Chlorohexidine was the most effective treatment of all in both experiments. It inhibited over 99.7\% of bacterial growth in the post-application experiment and created a zone of inhibition twice as large as the next best treatment, Colgate. 250 \(\mu\text{g/mL}\) EGCG-S demonstrated similar effectiveness to Colgate and proved more effective than both Listerine and Dentiste Plus White, and 250 \(\mu\text{g/mL}\) P-EGCG was more effective than Listerine in the disk diffusion assay. However, the results of these two studies suggest that P-EGCG is not more effective as an individual agent than the tested mouthwashes, and thus it may be better suited as an additive to other oral cleansing agents as opposed to an individual treatment.

The results of the Congo Red, Resazurin, and Crystal Violet assays were mixed. The control wells of the Congo Red assay demonstrated dark accumulation on the wells, suggesting the growth of biofilm after incubation for 3-4 days. The wells treated with both \textit{S. mutans} cultures and both concentrations of EGCG-S and P-EGCG were a deep red color, suggesting complete inhibition of biofilm formation. This is very promising considering that biofilm formation is an early marker of dental cavity formation. However, the results of the Resazurin and Crystal Violet assays were inconsistent, and a qualitative analysis of the wells showed
cloudiness and precipitation. This may have been due to the reaction of P-EGCG and 500 µg/mL EGCG-S with the liquid media, and this was observed over several trials. The reason for this precipitation is unknown at this point, and must be investigated further.

*S. mutans* is the main etiological cause of dental caries due to its ability to utilize carbohydrates like sucrose and produce acid end-products that wear away at the enamel. The experiments conducted sought to limit the growth of *S. mutans* with P-EGCG, an esterified derivative of EGCG that has been approved by the FDA in China for consumption. Experiments were conducted in tryptic soy broth, in artificial saliva containing different concentrations of sucrose, and in comparison to four different mouthwash products. Overall, the results of the *S. mutans* study suggest that P-EGCG is effective in inhibiting the bacterial growth, but EGCG-S may be more effective and neither treatment appears to be more effective than available mouthwash treatments. Though these treatments do have benefits as organic, herbal remedies, they do not appear to be better suited as individual treatments. However, their role as synergistic agents may be more impactful.

The results of the ESKAPE study suggest that EGCG-S is effective in inhibiting the growth of each organism, as evidenced by the colony forming unit study. However, the degree of inhibition varied greatly between each organism, and repeated trials are necessary to ascertain the extent to which EGCG-S impacts the growth of each organism. The issue with the ESKAPE pathogens is their growing resistance to antibiotics, and thus the disk diffusion assay investigated whether 500 µg/mL EGCG-S could increase each organism’s sensitivity to antibiotics. As each organism becomes more resistant to antibiotics, alternative treatments are needed in order to control this issue, as the ESKAPE pathogens are a principal cause of nosocomial infections and threaten to become more problematic as time goes on. The results of the disk diffusion assay
demonstrate that treatment of the antibiotics with EGCG-S increases each organism’s sensitivity
to select antibiotics. *E. faecalis* was originally resistant to Kanamycin and Streptomycin, but
treatment with EGCG-S made this organism intermediately resistant to Kanamycin and sensitive
to Streptomycin. Treatment with EGCG-S changed the susceptibility of *S. aureus* to Vancomycin
from intermediately resistant to sensitive. *K. pneumoniae* was originally resistant to
Erythromycin, Penicillin, Bacitracin, Vancomycin, and Cephalothin, but after treatment with
EGCG-S, the bacterium became intermediately resistant to Erythromycin, Penicillin and
Bacitracin, and became sensitive to Vancomycin and Cephalothin. *P. aeruginosa* was resistant to
Chloramphenicol, Penicillin, Ampicillin, Streptomycin, Bacitracin, Vancomycin, and
Polymyxin, but treatment with EGCG-S made this bacterium intermediately resistant to
Chloramphenicol, Penicillin, Ampicillin, Streptomycin and Bacitracin, and sensitive to
Vancomycin and Polymyxin. Lastly, *Enterobacter* species was intermediately resistant to
Chloramphenicol, but treatment with the green tea polyphenolic compound made this bacterium
sensitive to this drug. These results suggest that EGCG-S may be an effective additive to
antibiotics to combat the drug resistance development of the ESKAPE bacterium.

**Future Studies and Implications**

With regards to the study on the growth of *S. mutans*, future studies should focus on
repeating trials of each experiment in order to ascertain that the results are accurate. Though
three trials were typically conducted for each, it is worth investigating further. Future studies
should also aim to further compare the effectiveness of mouthwash products (both in terms of the
mouthwashes used in this study and more mouthwash products). The purpose of the experiments
with the mouthwash was to observe if the green tea polyphenolic compounds, particularly P-
EGCG, could perhaps serve as a more effective alternate to commercial brands of mouthwash. Thus, further experimentation should be performed to see if this is the case, and future studies should also focus on synergistic treatments of mouthwash and P-EGCG. Chlorohexidine appears to be the most effective mouthwash product that was tested and Listerine inhibited the growth least effectively of the mouthwashes, but perhaps both products could be more effectively with P-EGCG included as an additive. The time course study in particular should continue to be tested, as this experiment could give the most insight into the effects of P-EGCG as an individual or synergistic oral cleansing agent. Overall, more synergistic studies utilizing both P-EGCG and mouthwash products need to be conducted in the hopes of discovering a more effective oral cleansing product than commercially available ones. Further experiments must also be conducted within artificial saliva at different concentrations of sucrose to emulate the oral cavity after sugar consumption. The viability of P-EGCG as a safe-to-consume product must also be investigated further, as well as its effects in vivo to determine if the effects of P-EGCG are as promising within the oral cavity as they appear to be within a lab setting.

Future experiments should aim to investigate the phenomenon observed during the Resazurin and Crystal Violet assays for analyzing the growth of biofilm. Whereas the Congo Red experiment demonstrated that P-EGCG inhibited the growth of S. mutans biofilm, the Resazurin and Crystal Violet assays gave inconclusive, inconsistent results due to apparent cloudiness and precipitation. It appears that both concentrations of P-EGCG and 500 µg/mL EGCG-S react poorly with the liquid media, whether it be the artificial saliva or the tryptic soy broth, and the reason for this cloudiness and precipitation must be understood. Other experiments to study the effects of P-EGCG and EGCG-S on biofilm growth should be conducted using solid agar, such as a fluorescence scope study.
Future studies on the ESKAPE pathogens should include repeated trials of both the colony forming unit assay and the disk diffusion experiment, as well as a greater range of experiments, potentially including synergistic CFU experiments including EGCG-S and antibiotics. A greater range of antibiotics could also be investigated. The purpose of experimenting with antibiotics and EGCG-S was to study if EGCG-S could make the ESKAPE bacteria more sensitive to antibiotics. Though the disk diffusion studies did demonstrate the effectiveness of EGCG-S in making the bacteria more sensitive to various antibiotics, it is worth investigating this phenomenon over repeated trials, over more experiments, and with a greater range of antibiotics.
References:


