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Green Tea Polyphenols Inhibit the Growth of the Dental Cavity Causing Bacteria Streptococcus mutans

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GREEN TEA POLYPHENOLS INHIBIT THE GROWTH OF THE DENTAL CAVITY

CAUSING BACTERIA *STREPTOCOCCUS MUTANS*

by

Amy Lynn Melok

A Master's Thesis Submitted to the Faculty of

Montclair State University

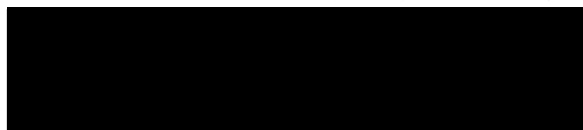In Partial Fulfillment of the Requirements

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Montclair, New Jersey

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Abstract:

An often overlooked disease, dental caries, or cavities, is one of the most prevalent infectious diseases across the globe. *Streptococcus mutans (S. mutans)* is the main etiological bacteria present in the oral cavity that leads to dental caries. All of the *S. mutans* in the oral cavity try to pool together to form an intelligent biofilm that adheres to the surfaces of teeth. *S. mutans* thrive on the sugary foods we eat and release acid by products that aid in the wearing away of the teeth's enamel. As this enamel begins to wear away, the decaying process begins and cavities are formed.

A popular drink among many cultures, green tea contains polyphenols that have been previously reported to possess antioxidant, antimicrobial, antimutagenic, cancer preventing and anti-cariogenic properties. These green tea polyphenols have been studied in biofilm inhibition, but its role in cariogenic biofilm inhibition is not yet well known.

In this study, the stability of EGCG and EGCG-S were studied to determine its antimicrobial properties over a period of 5 months. Next, the effect of EGCG-S on *S. mutans* was examined through Colony Forming Units (CFU), Live/ Dead ® Assay and Scanning Electron Microscopy (SEM). Once the antimicrobial effect of EGCG-S was determined to be successful in inhibiting the growth of *S. mutans*, its effect to inhibit biofilm was studied through testing various sugar environments, Congo Red Assay and Crystal Violet Assay. The next objective of this study was to determine the possible synergistic effect of EGCG-S with common mouthwashes through disk diffusion, 96-well micro-titer plate assay, time course study, and Live/Dead ® Assay. Finally, The growth of *S. mutans* was monitored on three types of dental materials: Bruxzir®, PMMA, and EVA to see if treatment with EGCG-S inhibited its growth on these surfaces. This study suggests that EGCG-S is effective in reducing *S. mutans* growth, inhibiting its biofilm formation, enhances current mouthwashes antimicrobial properties, and has potential to reduce *S. mutans* growth on various dental materials.

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Introduction

a. Dental caries

Dental caries, or tooth decay, is a multifactorial disease that affects a large percentage of today's society (Gao et al., 2016; Aas et al., 2005). While it is obvious that dental caries are extremely problematic in undeveloped and underprivileged areas, this disease is also seen extensively among privileged societies as well (Marsh et al.,2003; Downer et al., 2005). Dental caries is an infection of the oral cavity that is caused by bacteria wearing away the outer layer of the tooth surface, the enamel. These bacteria feed off of different factors that can increase their damaging effects. While diet and the immune response of the host play a large role in inducing dental caries, the most influential factor is the acidic environment of the oral cavity caused by the microbial flora (Taylor et al., 2005). Of the thousands of resident bacteria present in the oral cavity, they maintain a relatively neutral pH around 6.8 (Aframian et al,. 2006). Problems arise when this pH drops to a more acidic value, which promotes the demineralization of the enamel resulting in tooth decay.

The acidic conditions that lead to dental caries pathogenesis involves several steps starting with the formation of a biofilm. A biofilm is defined as a community of bacteria that attach to a surface. While dental plaque is moderately specialized, it still shares the main properties of all biofilms. Biofilm formation is a three-stage process: docking, locking and maturation (Blanco et al., 2005). First, the docking step is described as an initial contact stage where the first bacterium attaches to the tooth surface. Next, the locking stage is when the bacteria encase themselves into an extracellular matrix before heading into the final stage of maturation where the bacteria multiply into high cell

densities (Kolenbrander et al., 2007). Bacteria that are packed together into biofilms are able to accomplish more than that of a single bacterium. Biofilms are capable of adjusting to different environments, increasing rates of adherence, conjugation, and growth, as well as having a distinctive method of communication between cells called quorum sensing (Saini et al., 2011).

Our world is filled with dietary options filled with sugars. When we consume these sugars, like sucrose, we are feeding the bacteria in our oral cavities. The resident bacteria of the mouth take in the sucrose and produce glucosyltransferases. These glucosyltransferases, or *gtfs*, produce a sticky glycocalyx film, or pellicle, which helps the bacteria to adhere to the surface of the teeth better (Taylor et al., 2005). A byproduct of this reaction is the release of acid. When this acid is released, the pH of the oral cavity drops initiating the erosion of the nearby teeth's enamel.

Biofilms of the oral cavity can be divided into two locations: tooth associated and soft tissue associated sites (Kolenbrander et al., 2007). Soft tissue associated sites refer to the epithelia of the oral cavity. These soft tissue associated sites must be continuously recolonized in order to be successful because epithelial tissue is prone to shedding (Kolenbrander et al., 2007). In contrast to the soft tissue associated sites, tooth associated sites have a unique advantage because they are one of the few non-shedding surfaces found in the human body (Kolenbrander et al., 2007). Although there is no shedding, each tooth is different than the one next to it. An individual tooth is exposed to different amounts of wear by tongue (lingual) and cheek (buccal) contact and the amount of saliva that passes over it. The only surfaces of the teeth that are immune to lingual and buccal wear are the interproximal regions, which are located in between the teeth.

Saliva is present in both locations of oral biofilm and function as a bacterial nutrient, as well as an enzymatic antibacterial agent. The majority of oral saliva secrete from the parotid, submandibular, and sublingual glands. Each gland's saliva varies slightly in composition. The parotid gland secretes mainly amylase, while the submandibular and sublingual glands secrete primarily mucins. Mucins have antimicrobial properties, which are suggested to delay bacterial invasion in the oral cavity (Scannapienco et al., 1994). The flowing of saliva through the oral cavity may reduce the rate of adherence of bacteria, but it also provides the bacteria dietary nutrients to aid in the development of biofilm (Homer et al., 1996; Palmer et al., 2001). To succeed best in the oral cavity, bacteria must be able to overcome the flow of saliva and avoid being swallowed by having extreme adherence capabilities.

While there are thousands of bacteria present in the oral cavity, three of the most commonly found are *Streptococcus mutans (S. mutans), Enterococcus faecalis (E. faecalis),* and *Lactobacillus acidophilus (L. Acidophilus)*. *S. mutans* often gets the most attention in dental studies because it has been previously shown to favor attachment to tooth enamel (Duchin and Van Houte, 1978; Kolenbrander et al., 2000). As the primary biofilm colonizer of tooth enamel and a major contributor to oral pH reduction, *S. mutans* has been recognized to be the principle etiological agent of tooth decay (Beighton, 2005; Clarke, 1924; Loesche, 1986).

S. mutans is a gram-positive, non-motile, acid-producing, facultative anaerobic bacterium. Most effective in temperatures of 18 to 40°C, this α-hemolytic streptococci species has eight stereotypes, thick cell walls, and aggregates in pairs of short chains. The *S. mutans* UA159 serotype c strain genome has already been sequenced and is

instinctively proficient by containing all of the genes essential for biofilm formation and quorum sensing (Ajdic et al., 2000). This bacterium is made up of circular DNA and has three distinctive plasmids that (1) function in resistance to antibiotics and heavy metals, (2) produce bacteriocin, and (3) have mechanisms for conjugation-like transfer activities (Shigeyuki et al., 1986).

From the sequenced genome of the UA159 strain, virulence factors have been identified that are associated with extracellular adherent glucan production, adhesins, acid tolerance, proteases, and hemolysins (Ajdic et al., 2000). *S. mutans* is often recognized for its *gtf* production. These *gtf* enzymes function in production of the intracellular and extracellular polysaccharides essential to biofilm formation (Hamada et al., 1980). Specifically, on the tooth-associated sites, the extracellular polysaccharides facilitate the attachment of *S. mutans* to tooth surfaces (Schilling et al., 1992). In conjunction with the other bacteria of the oral cavity, like *E. faecalis* and *L. acidophilus*, initiates the biofilm formation process and release lactic acid in the saliva thus inducing the demineralization of tooth enamel.

b. Dental Materials

Since many people have various dental materials present in their mouth for aesthetic, health and protective reasons, it is important to assess *S. mutans* behavior on these different surfaces as well. Used in both dental bridges and implants Bruxzir ® is a solid zirconia that has become very popular amongst dentists today. Bruxzir ® is often chosen for its durability, translucency (Bunek et al., 2014) and ability to withstand wearing away for years (Christensen, 2014). Polymethyl methacrylate (PMMA) was first

introduced into dentistry as a base for dentures and is still used today (Frazer, 2005).

PMMA is often used in making dentures because it is easy to manipulate and alter for the

perfect fit for a patient. Shock Doctor mouthguards are advertised as "the #1 mouthguard

in the world," and athletes in many different sports use mouthguards to prevent the loss

or damage of teeth. However, an often overlooked factor of mouthguards is the ability of

bacteria to accumulate on its surfaces if they are not properly taken care of. Made

primarily of ethylene vinyl acetate (EVA), these mouthguards are made to be heated up

in boiling water and molded to the athletes bite.

c. Properties of Green Tea Polyphenols

A popular drink around the world, tea is made from the infusion of dried *Camellia*

sinensis leaves. Eastern cultures, such as China and India, are known to use tea

medicinally based on its many beneficial health care uses. Previous literature has

established that *Camellia sinensis*, especially the non-fermented type commonly known

as green tea, has a numerous amount of medical advantages. These preceding studies

have recognized green tea to have anti-inflammatory, antiviral, antifungal, antioxidant,

protein-denaturing, anti-mutagenic, anti-diabetic, anti-carcinogenic, and antibacterial

characteristics (Biswas et al., 2015; Elvin-Lewis et al., 1980; 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). The remedial affects of green tea are thought to be a result of

the polyphenolic catechins present in green tea (Biswas et al., 2015). Three specific

catechins, whose structures are shown in Figure 1, epicatechin gallate (ECG),

epigallocatechin (EGC) and epigallocatechin-3-galate (EGCG), are thought to be the main factors in green tea's antibacterial properties (Biswas et al., 2015; Yam et al., 1997; Hara, 2001). The most active catechin of the three, EGCG, makes up most of the content of the catechins at 59% (Taylor et al., 2005). While EGCG may seem like the most ideal catechin to use when treating bacteria, it can be relatively unstable under certain conditions. Thus, a derivative of EGCG called epigallocatechin-3-gallate-sterate, EGCG-S, whose structure is shown in Figure 2, has been synthesized. EGCG-S is the esterified version of EGCG, and has been shown to have inhibitory effects on various types of bacteria, including endospore-forming bacteria (Lee et al., unpublished results). Additionally, some natural forms of modified polyphenols, such as a mixed form of green tea polyphenols (GTP) and the lipophilic version of green tea polyphenols (LTP), have also been noted to have therapeutic benefits.

These green tea components are known to have antibacterial properties, it has been determined that these bioactive components are also anticariogenic. Dental research completed *in vivo* in both animal and human participants demonstrated that green tea reduces carious incidents (Biswas et al., 2015; Hamilton-Miller, 2001; Xu, 2010). Previous literature determined that in short-term experimentation, green tea extracts have demonstrated anti-plaque capabilities (You, 1993). A solution containing 0.2% green tea wash used by the participants when brushing their teeth daily, and resulting in a dramatic decrease in the overall plaque index (You, 1993). Similarly, a 0.25% green tea mouthwash as well as green tea dissolvable tablets also had significant results decreasing the plaque indices of its participants (Kaneko et al., 1993; Liu and Chi, 2000). While these results are significant, they are only short-term findings. Long-term findings in the

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dental field are hard to attain due to the various aspects that can affect the results, including diet and health conditions.

While determining how many cups of green tea a day prevents caries from occurring is not an easy task, some researchers have simply compared dental health between green tea drinkers and non green tea drinkers. In one study, children that drank one cup of tea per day displayed a significantly lower occurrence of caries compared to the control group that did not drink tea daily (Onisi et al., 1981). In similar studies, children who drank 1 to 3 cups of tea daily did not have as many caries or develop them as frequently as children who did not (Elvin-Lewis and Steelman, 1986; Jones et al., 1999).

The leading cariogenic bacteria, *S. mutans*, is negatively impacted by green tea polyphenols in many ways. The green tea polyphenols have been shown to not only reduce the proliferation of *S. mutans*, but also obstruct their initial attachment to the tooth's surface. Pools of oral bacteria, including *S. mutans*, were previously shown to have minimum inhibitory concentrations (MIC) in the range of 50 to 1,000 $\mu g/mL$ of the following mixed tea polyphenols: EG, EGC and EGCG (Sakanaka et al., 1989; Kawamura and Takeo, 1989). Other significant findings include short exposure to 1$\mu g/mL$ of EGCG produces a bactericidal effect, as well as inhibiting amylase secretion from *S. mutans* (Rasheed and Haider, 1998; Zhang and Kashket, 1998). Based on these findings, tea polyphenols are capable of inhibiting the growth of *S. mutans* and potentially reducing the amount dental caries. This study will use two lipophilic tea polyphenols not yet studied to reduce the growth and production of biofilm in *S. mutans*.
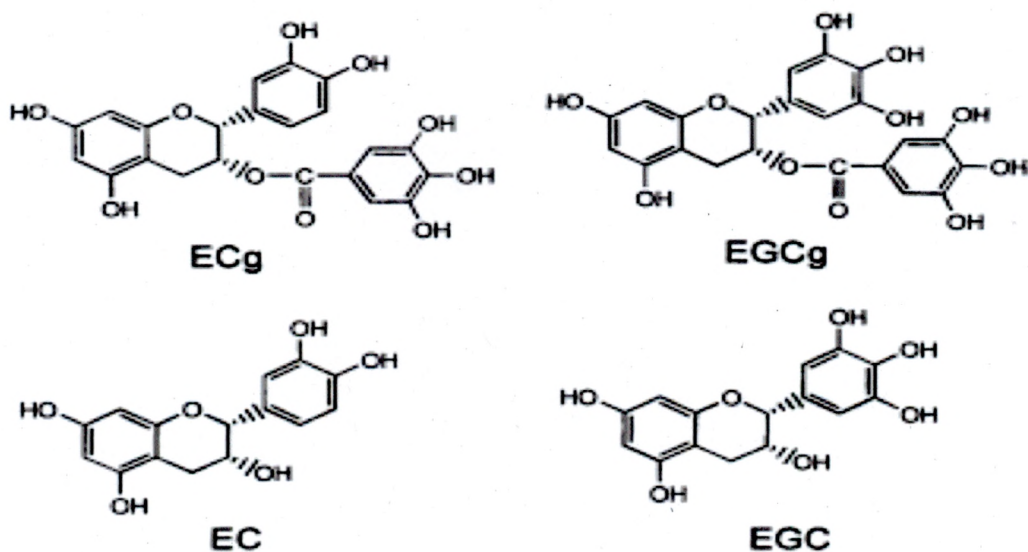
Figure 1. Molecular Structures of ECg, EC, EGCg and EGC
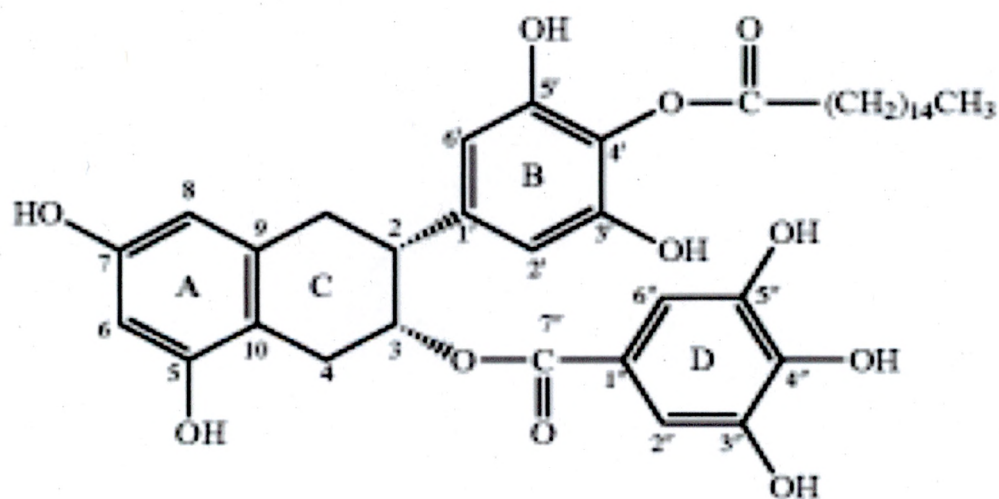
Figure 2. Molecular structure of EGCG-Sterate

Objectives of Study:

I. Identify & Monitor the Growth of *S. mutans*

II. Determine the Stability of EGCG & EGCG-S as Antimicrobial Agents

III. Observe the Effect of EGCG-S on *S. mutans*

IV. Study the Effect of GTPs on Biofilm formation in *S. mutans*

V. Identify Possible Synergistic Relationships of EGCG-S with Oral Care Products

VI. Observe the effect of *S. mutans* growth on various dental materials and the effect of

EGCG-S on these materials

Materials and Methods

1. Pre/ Post- Experiment Set up

Before beginning an experiment, all materials were autoclaved or exposed to ultraviolet light for 20 minutes and all surfaces were cleaned with a 10% bleach solution.

2. Culturing Bacteria

Streptococcus mutans was grown and maintained on tryptic soy agar plates, nutrient agar plates, or Mueller-Hinton agar plates. Stock plates were maintained and stored in a cold room or refrigerator at 4°C for future experimental usage. Stock plates were routinely restocked and replaced to ensure the bacteria were fresh and growing. Before each experiment, the purity of the stock cultures was checked. If contamination was present, a bacterial isolation was performed.

a. Bacteria Isolation

Pure stock culture plates were isolated using a discontinuous streaking method shown in Figure MM1. Once the purity of an isolated colony was confirmed, the rest of the colony was used to make a stock culture using a continuous streaking method, shown in Figure MM2, on a separate agar plate. Both streaking methods require an overnight incubation period at 37°C. After incubation, the plates were then wrapped in parafilm and stored in the refrigerator until needed.
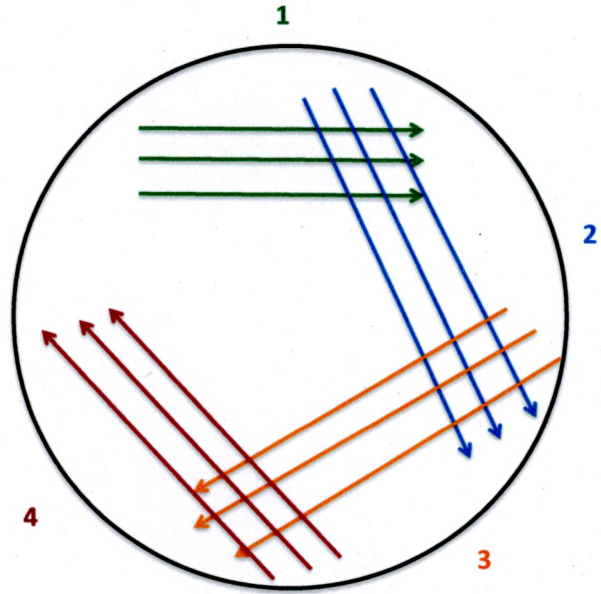
Figure 3. Discontinuous streaking method used for isolation of bacterial colonies.
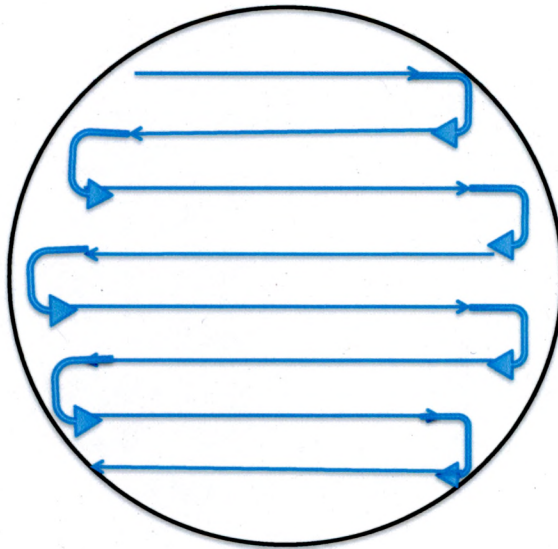
Figure 4. Continuous streaking method used in the making of stock cultures.

b. Overnight Cultures

One liter of tryptic soy broth was prepared and 6mL was pipetted into 20mL glass test tubes. These glass tubes were prepared in a test tube rack and autoclaved through the liquid cycle at 121°C for 15 minutes. Upon completion of autoclaving, the rack was stored in the refrigerated cold room at 4°C.

Before the night of an experiment, the purity of the stock plates were checked through a simple stain. If the stock plate was pure, it could be used to prepare an overnight culture. To prepare an overnight culture, a wired loop or cotton swab was used to streak a small portion of bacteria off of the stock plate and aseptically mixed into the prepared broth. Every time the cap was removed from the test tube containing the broth culture it was flamed over the Bunsen burner to prevent other organisms from contaminating the culture. Each overnight culture was labeled with the name of the bacteria, as well as the date in which it was cultured. After inoculation, it was left in a shaking incubator overnight at 250rpm and 37°C. To ensure the culture was not contaminated overnight, the purity was checked with a simple stain before the start of that day's 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 placed on a magnetic stir bar hot plate, at the lowest temperature setting to ensure the medium and water were mixed thoroughly. The solution was then autoclaved at

121°C for 15 to 20 minutes. Upon completion of autoclaving, the medium was allowed to cool for ~30 minutes. It was then labeled with the type of medium and date and 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[TM] was prepared using 30 grams of medium and 1 liter of deionized water. Mueller-Hinton Agar from Difco[TM] was prepared using 38 grams of medium and 1 liter of deionized water. Tryptic Soy Agar (TSA) from Remel[TM] was prepared using 40 grams of medium and 1 liter of deionized water. The media were mixed thoroughly using the magnetic stir bar hot plate, at the lowest temperature setting. Next, the mixture was autoclaved at 121°C for 15 to 20 minutes. After the mixture were autoclaved, they were poured into individual sterile plates and left to solidify. Once solidified, they were spread out in the hood and exposed to ultraviolet light for 20 minutes. The plates were then placed back into plastic sleeves, sealed with tape, labeled, and stored in the refrigerated cold room at 4°C until needed.

Congo Red Agar from Sigma-Aldrich was prepared according to the procedure outlined by Schwartz et al (2012). Media and Congo Red were prepared in two separate flasks and then mixed together after being autoclaved. The first medium contained 9.25grams of Brain Heart Infusion broth medium, 12.5 grams of sucrose (5%), 5 grams of bacto agar, and 250mL of deionized water. This flask was placed on a magnetic stir bar hot plate until all contents were mixed evenly, the solution looked clear, and was boiling. 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 for 15 to 20

minutes, and set aside to cool until they reached a temperature between 55-60°C.

5mL of the Congo Red solution was added to the broth solution and mixed

thoroughly. This solution was then pipetted according to the type of well plate

being used. For 6 well plates 2mL was used and for 24 well plates 1mL was used.

These plates were allowed to solidify at room temperature. Fresh Congo Red

media was made before each experiment.

4. Simple Stain, Gram Stain, and Morphological Arrangement Analysis

a. Purity Check (Simple Stain)

A smear preparation was aseptically performed by first sterilizing a wire

loop over a Bunsen burner until it appears orange in color. After the wire loop

cools down, it was used to obtain a small loop of bacteria from the stock plate.

The loopful of bacteria was then placed onto a sterile glass slide. The smear was

heat fixed and then a drop of methylene blue dye was placed on top of the smear.

A cover slip was then place on top of the dye before blotting the sample dry with

bibulous paper. The sample was observed under oil immersion at 1000x total

magnification (Lee et al, 2015).

b. Gram Stain and Morphological Arrangement Analysis

A smear preparation was prepared using the same technique as in the

simple stain. After the smear was allowed to dry, it was first covered with crystal

violet dye for 20 seconds and then washed with deionized water for 2 seconds.

Next, the sample was covered with Gram's iodine for 1 minute, exposed to

Gram's decolorizer indirectly for 10-20 seconds, and then washed with deionized water for 2 seconds. Then the smear was counter stained with safranin for 1 minute before being washed with deionized water indirectly for 2 seconds. Finally, a cover slip was placed on top of the smear, blotted dry with bibulous paper, and observed under oil immersion at 1000x total magnification (Lee et al, 2015).

5. Preparation of Green Tea Polyphenols

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

a. EGCG-S

EGCG-S was prepared using 200 proof ethanol. Stock concentrations were prepared and diluted to the required concentration needed for each experiment. Below is the calculation of the master stock solution:

- 2.5mg/mL= 0.0025g tea EGCG-S+ 1mL of EtOH

b. LTP

LTP stock (2.5 mg/mL) was prepared using the same technique and calculations as in EGCG-S.

6. Monitoring the Growth of Bacteria

a. Standard Growth Curve

A standard growth curve was generated for *Streptococcus mutans* by first diluting an overnight culture with tryptic soy broth to 0.1 at 650_{nm}. This culture was then placed in an incubator at 37°C. Readings were taken at every hour at 650_{nm} for 10 hours and a final reading was taken at hour 15. This experiment

was done in triplicate and the average of the readings was used.   Standard deviation (SD) was calculated.

b. Effect of Sugar Content on Biofilm Formation

To determine which environment best induces the development of biofilm, a preliminary study examing the growth of *S. mutans* in the presence of different sugars at various concentrations was done. Both dextrose and sucrose were examined at the concentrations of 0.1%, 1% and 5%. Overnight cultures were prepared, checked for purity, and diluted to an OD_{650} of 0.3. Sterile glass cover slips were placed at the bottom of 6 well plates.   The control sample contained 2mL of overnight culture. The experimental groups contained either 1400uL of the designated sugar broth and 600uL of overnight culture, or 1200uL of the designated sugar broth, 600uL of overnight culture and 200uL of artificial saliva. Three sets of plates were made. One set was incubated for 24 hours, one set was incubated for 48 hours, and the final set containing the artificial saliva was incubated for 24 hours as well.

After the incubation period, the cover slips were aseptically removed from the wells and placed on top of a labeled glass cover slide. In a dark room, 10uL of Syto®9 dye from the LIVE/DEAD® Baclight[TM] Kit was placed on top of the coverslip and another coverslip was placed on top of the fluorescent dye. These slides were wrapped in aluminum foil and left to incubate in the dark at room temperature for at least 20 minutes. Samples were viewed with a ZEISS fluorescence microscope under 1000x total magnification.

7. Stability Study of Green Tea Polyphenols on *Streptococcus mutans*

a. Colony forming Unit (CFU) assay for Determining the Viability of Cells

The overnight cultures of *Streptococcus mutans* were treated with EGCG-S or EGCG that have been incubated alone in 4°C, 25°C, 37°C for 0 day, 6 days, 12 days, 3 month and 5 month respectively. Untreated sample and samples treated with proper storage EGCG-S and EGCG were used as controls. After two-hour treatment with 250 ug/ml of EGCG-S or EGCG, serial dilutions were carried out and plated on nutrient agar plates and incubated for 24 hours. The colonies were counted and colony-forming unit (CFU) were determined. The percentage of inhibition was calculated using the following equation:

$$\% \text{ of Inhibiton} = \left(\frac{CFU_{Control} - CFU_{Treated}}{CFU_{Control}}\right) X\ 100$$

b. Live/Dead Microscopic Observation

The LIVE/DEAD® Baclight^{TM} Bacterial Viability Kit was used for this experiment. In the kit (L13152), separate dye solutions can be made by dissolving the contents of Component A or B and pipetted into 2.5mL of filter-sterilized deionized water. The separate solutions were prepared at different ratios, and then added to the bacterial suspension as a 1-to-1 ratio. Each fluorescent dye component was transferred into Eppendorf tubes and covered in aluminum foil to avoid exposure to light. Before using the dyes, they were vortexed to ensure that the dyes were evenly mixed. The dyes were stored in the freezer until needed. 10 uL of cultures of *Streptococcus mutans* treated with EGCG and EGCG-S at varying times and temperatures were stained with 10 uL of live/dead fluorescence dye mixture (Components A and B). The sample was pipetted up and down on the slide to ensure that the bacteria and dyes were mixed.

Aluminum foil was then used to wrap each slide to reduce the chance of light exposure while the samples were allowed to incubate for 20 minutes at room temperature. Each slide was viewed under a ZEISS fluorescent microscope at 1000x total magnification or confocal microscopy. Green stain indicates that the cell membrane is still intact suggesting the cell is alive. Red stain indicates that the cell membrane is no longer intact, which suggests cell death.

8. Study the Effect of Green Tea Polyphenols on Biofilm Formation

a. Congo Red Assay

Overnight cultures were prepared and checked for purity before beginning the experiment. The overnight cultures were first diluted to 0.3 optical density at 650_{nm}. *S. mutans* was swabbed aseptically onto the agar for the positive controls. The negative controls had no bacteria or solutions added to the agar. The experimental groups were prepared as follows: 600uL of tryptic soy broth, 300uL of bacteria, and 100uL of the designated treatment. 24 well plates were used and . the solution was mixed and swabbed onto the agar before being incubated for 4 days at 37°C. Pictures were taken of the plates every day for qualitative analysis. The positive results were observed as dark precipitation on the red agar plates.

b. Crystal Violet Assay

Overnight cultures were prepared and checked for purity before beginning the experiment. The overnight cultures were then diluted 0.3 optical density at 650_{nm}. Before beginning the experiment, 24 well plates were labeled according to the samples to be placed in each well. Positive controls and negative controls,

were included with the experimental groups. All groups tested were tested in triplicate. The positive control wells contained 700uL of tryptic soy broth and 300uL of the overnight culture. The negative control contained 1000uL of tryptic soy broth. The experimental groups contained 600ul of tryptic soy broth, 300uL of overnight culture, and 100uL of the designated tea polyphenol. Each well had a total volume of 1mL. The 24 well plate was labeled, parafilmed, and allowed to incubate for 4 days at 37°C.

On the 4th day, the plates were aspirated, washed with 1x PBS, and stained with 0.1% crystal violet for 30 minutes. The crystal violet was then aspirated, the plates were inverted on absorbent pads and allowed to dry overnight. The next day, sterile cotton swabs were used to remove any excess dye on the sides of the well to ensure it did not affect the readings. 1mL of 30% acetic acid was placed into each of the 24 wells. The spectrophotometer was set at $OD595_{nm}$, blanked, and then each of the 24 samples were read making sure to blank the spectrophotometer between each sample. After each reading, the OD_{595} was recorded. These readings were then used in the following equation to determine the percentage of biofilm inhibition:

$$\% \text{ of inhibiton} = \left(\frac{\text{Control } OD_{595} - \text{Treated } OD_{595}}{\text{Control } OD_{595}}\right) \text{ X } 100$$

9. Study the Effect of Green Tea Polyphenols on Cell viability

a. Colony Forming Units

Overnight cultures were prepared and checked for purity before beginning the experiment. Cultures were then serially diluted 10^{-8} before being diluted to 0.3 optical density. 1.5mL Eppendorf tubes were prepared with 300uL of

overnight culture, 600uL of broth, and 100uL of the designated concentration of each tea polyphenol. The Eppendorf tubes were vortexed to mix all contents before pipetting 100uL of the mixture onto nutrient agar plates aseptically. All plates were incubated overnight at 37°C. Colony Forming Units were recorded and the percentage of inhibition was calculated using the following equation:

- % of Inhibition $= \left(\frac{CFU_{Control} - CFU_{Treated}}{CFU_{Control}} \right)$ X 100

10. Microscopic Observation

a. Scanning Electron Microscopy

Overnight cultures were prepared and checked for purity before beginning the experiment. Cultures were then diluted to 0.3 optical density at 650_{nm}. A sterile 6 well plate was used for this experiment. Coverslips were exposed to ultraviolet light for 10 minutes on each side to ensure sterility. Afterwards, the coverslips were placed at the bottom of each well. The positive control group's wells contained 700uL of tryptic soy broth and 300uL of the diluted overnight culture. The experimental groups contained 600uL of tryptic soy broth, 300uL of overnight culture, and 100uL of the designated concentration of tea polyphenol. The 6 well plate was then incubated for 4 days at 37°C.

A second SEM study was done to assess the growth of *S. mutans* on the following dental materials: (1) Bruxzir®, (2) Poly(methyl methacrylate) or PMMA and (3) Ethylene-vinyl acetate or EVA. Bruxzir® and PMMA were autoclaved before use and EVA was exposed to UV light for 10 minutes on each side. The same preparation was used as above, by replacing the coverslip with the desired dental material.

Samples were then brought to the Microscopy & Microanalysis Research Laboratory (MMRL) for further preparation. Each sample was arpirated and rinsed with 1x PBS for five minutes. Next, the samples were fixed with 2.5% glutaraldehyde in 0.1M of cacodylate buffer and incubated for 30 minutes at room temperature. All of the samples were then washed with 0.1M Sodium cacodylate buffer for 10 minutes. Next, the samples were incubated with 1% osmium tetroxide in 0.1M cacodylated buffer for 30 minuts at 4°C . The samples were then rinsed with 0.1M cacodylate buffer for 10 minutes. This step was repeated for a total of three washes. Then the samples were dehydrated in a series of ethanol washes. The percentage of ethanol increased each wash from 30% to 50% and then 70%. Each wash lasted 10 minutes. Samples were stored overnight in 70% ethanol at 4°C. The next day, the samples were continually dehydrated with a series of ethanol concentrations of 80%, 90%, and 100% (twice) for 10 minutes at each concentration. Then the samples were transferred into microporous vials and immersed in 100% ethanol. The vials were then placed into the Denton Critical Point Dryer and the samples were dried using liquid CO_2 at 1072psi and 31°C. Finally, the samples were mounted onto a stub and coated with a thin layer of metal film using the Denton IV Sputter Coater before microscopic observation.

b. Fluorescence Microscopy

Overnight cultures were prepared and checked for purity before beginning preparation for the experiment. Overnight cultures were diluted to 0.3 OD_{650}. For the positive control groups, 700uL of tryptic soy broth and 300uL of overnight

cultures were aseptically transferred into a 24 well plate. Experimental groups consisted of 600uL of tryptic soy broth, 300uL of overnight culture, and 100uL of the designated tea polyphenol treatment. The 24 well plate was incubated for 4 days at 37°C.

The LIVE/DEAD® Baclight™ Bacterial Viability Kit was used for this experiment. Methods for this kit was explained in section 7b.

The 24 well plate was removed from the incubator after 4 days and 50uL of each sample was individually transferred onto a sterile, labeled glass slide. Next, 20uL of the fluorescent dye mixture (Components A and B) were added to the glass slide. The sample was pipetted up and down on the slide to ensure that the bacteria and dyes have mixed. Aluminum foil was then used to wrap each slide to reduce the chance of light exposure while the samples were allowed to incubate for 20 minutes at room temperature. Each slide was viewed under a ZEISS fluorescent microscope at 1000x total magnification.

## 11. Study the Potential Synergistic Effect of Tea Polyphenols on Current Mouthwash treatments

a. Disk Diffusion

Before the experiment, 6mm disks were autoclaved in a glass petri dish to ensure their sterility. Plates were sectioned off and labeled based on the treatment to be placed in that section. Bacteria from an overnight culture were continuously streaked onto nutrient agar plates. A 24 well plate was prepared with 1mL of each treatment to be tested. The treatments included H_2O (a negative control), EGCG-S 250ug/mL, LTP 250ug/mL, Listerine, Cepacol, Scope, Crest,

Chlorohexidine Gluconate, Colgate, and the combination of each mouthwash with either EGCG-S 250ug/mL or LTP 250ug/mL. One disk was submerged in each treatment in the 24 well plate for 1 minute and then allowed to dry until no contents dripped off of the disk. Each disk was placed in the center of a section of the agar plate and gently pressed on with sterile forceps. The forceps were flamed in between the placement of each disk. Once all of the disks were in place, the plates were inverted and incubated overnight at 37°C.

The next day, measurements of the zone of inhibition, ZOI, were taken. The ZOI was measured from the edge of the disk to the end of the clearing zone as shown in Figure MM3. Measurements were taken in millimeters and the average of 4 measurements per disk was calculated. These values were used to calculate the percent inhibition of every treatment tested.
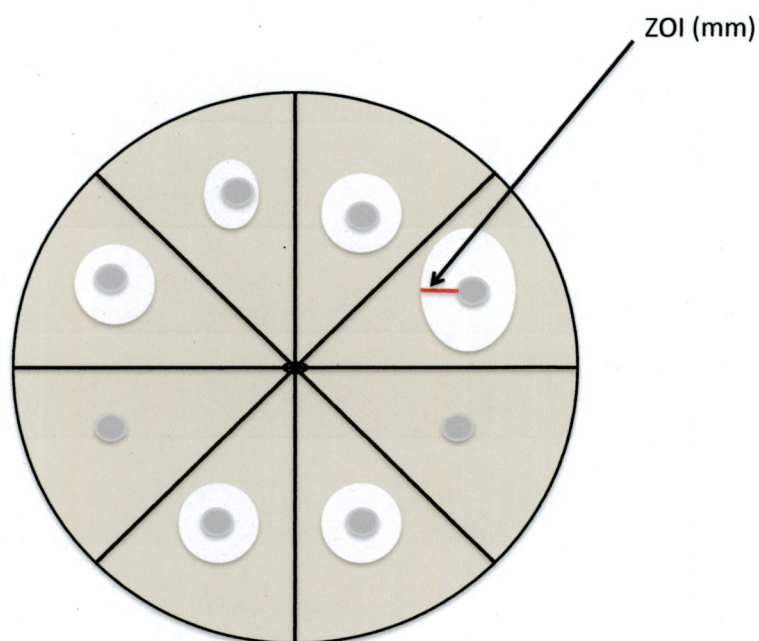
Figure 5. Example of a Disk Diffusion plate. The white represents the clearing zone, in which no bacteria have grown. The gray is the disk soaked in treatment, while the tan color represents the bacterial growth on the agar plate. The red line represents the zone of inhibition (ZOI), which is measured in millimeters to help calculate the percent inhibition.

b. Study the effect of mouthwash and tea polyphenols alone or in combination on the growth of *S. mutans* via 96 well micro-titer plate assay

To monitor the effect of tea polyphenols, mouthwashes, and the combinations of tea polyphenols and mouthwashes on the growth of *S. mutans*, a 96 well micro-titer plate assay was carried out. The overnight culture was diluted to an OD_{650} of 0.1 using tryptic soy broth. The samples were then treated accordingly. The positive control contained 70uL of broth and 30uL of overnight culture, while the treated samples contained 60uL of broth, 30uL of overnight culture and 10uL of treatment in one well of a 96 well plate. The growth was monitored for 15 hours, with readings being taken at OD_{650} every hour for the

first 10 hours, and a final reading at the 15th hour. While readings were not being taken, the plates were kept in the incubator at 37°C. This study was done in triplicate and the mean and standard deviation of the readings at each time point were also calculated.

c. Time Course Study

To study the time that it takes for treatments to become effective, a Time Kill study was carried out. To mimic the time that mouthwash is present in the mouth, as well as the long term effects, the times selected were 5s, 10s, 15s, 30s, 1min, 5min, 1hr, and 24hr. Overnight cultures were prepared, checked for purity, and diluted to an OD_{650} of 0.3. 1mL of overnight was placed into microcentrifuge tubes, which were then centrifuged for 5 minutes at 5,000 rpm. The supernatant was discarded, while the pellet of bacteria was kept for experimental use. The pellet was exposed to either 1mL of mouthwash alone or the combination of 900 of mouthwash and 100uL of 10X EGCG-S 250ug/mL. At each time point listed above, 100uL of the sample was spread plated out onto tryptic soy agar plates. All plates were incubated overnight at 37°C and colony forming units (CFU) were counted the next day.

d. Live/ Dead

Overnight cultures were prepared and checked for purity before beginning preparation for the experiment. Overnight cultures were diluted to 0.3 OD_{650}. 1mL of overnight culture was centrifuged for 5 minutes at 5,000rpm(?).The LIVE/DEAD® Baclight™ Kit was prepared using the same method described in section 7b. 25uL of Component A and 25uL of Component B were mixed with

each individual sample. The 96 well plate was wrapped in aluminum foil and allowed to incubate for 10 minutes at room temperature. After the incubation period, 50uL of each sample was placed onto a glass slide, a glass cover slip was placed on top, and were wrapped in aluminum foil again to incubate for another 10 minutes. Finally the samples were viewed under the ZEISS fluorescence microscope at 1000x total magnification.

Results and Discussion:

1. Identification & Monitoring the growth of *Streptococcus mutans*

To characterize *S. mutans*, the simple and gram stain were carried out to identify the morphology and it cell surface. A simple stain and gram stain of *S. mutans* are shown in Figure 6. In Figure 6A, the simple stain confirms that the bacteria present are circular, cocci shaped bacteria that are typically present in chains. In Figure 6B, a gram stain technique was done and the cells stained dark purple, which confirms that *S. mutans* is a gram-positive bacterium.

The growth of *S. mutans* was monitored for a period of 16 hours. The absorbance was read every hour for the first 10 hours at an optical density of 650nm. A final reading was done at the 15th hour. A standard growth curve displays four distinct stages: the lag phage, exponential growth, stationary phase, and the death phase. The first three stages were observed during this time period. In Figure 7, the first hour after inoculation, a lag phase was observed, then an exponential phase with a constant generation time was observed between 2 to 10 hours. The stationary phase can be observed between hours 10 -15. The optical density readings between these time periods are very similar, and also indicate that at these time points the amount of cellular growth is equal to the amount of cellular death. The bacterial culture was grown in nutrient broth. Once these nutrients are exhausted the bacteria will start to die off rapidly because of the lack of essential nutrients. Under optimal growth conditions, the generation time of *S. mutans* was determined to be 8hrs, based on the following equation: $g = (\log_{10} N_1 - \log_{10} N_0) / \log_{10} 2$. This indicates that *S. mutans* takes 8 hours to double in population size.
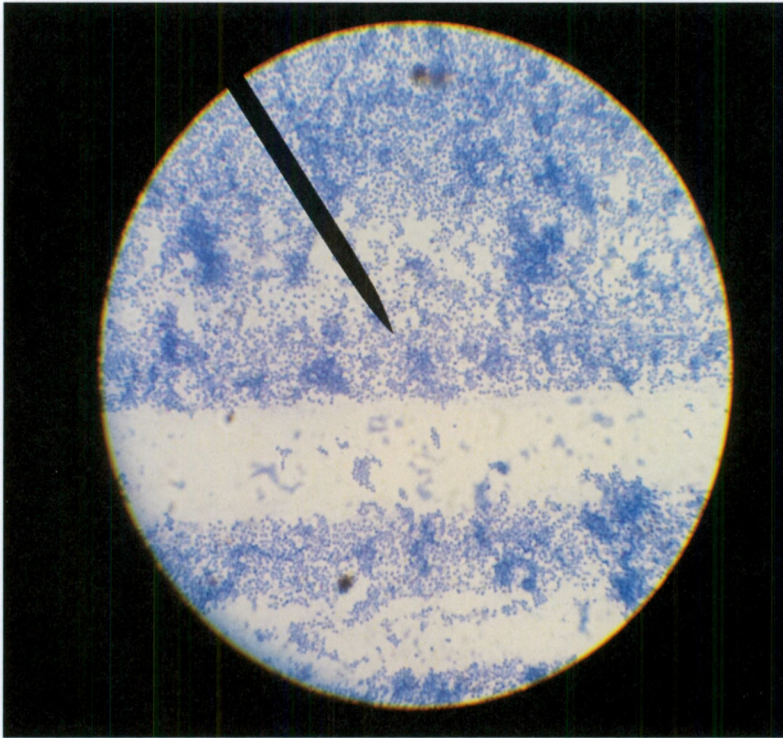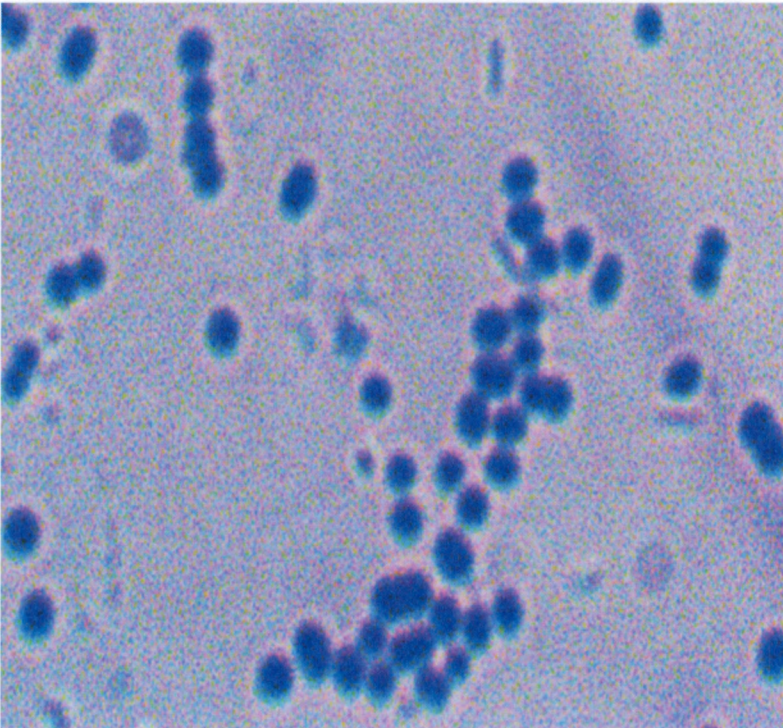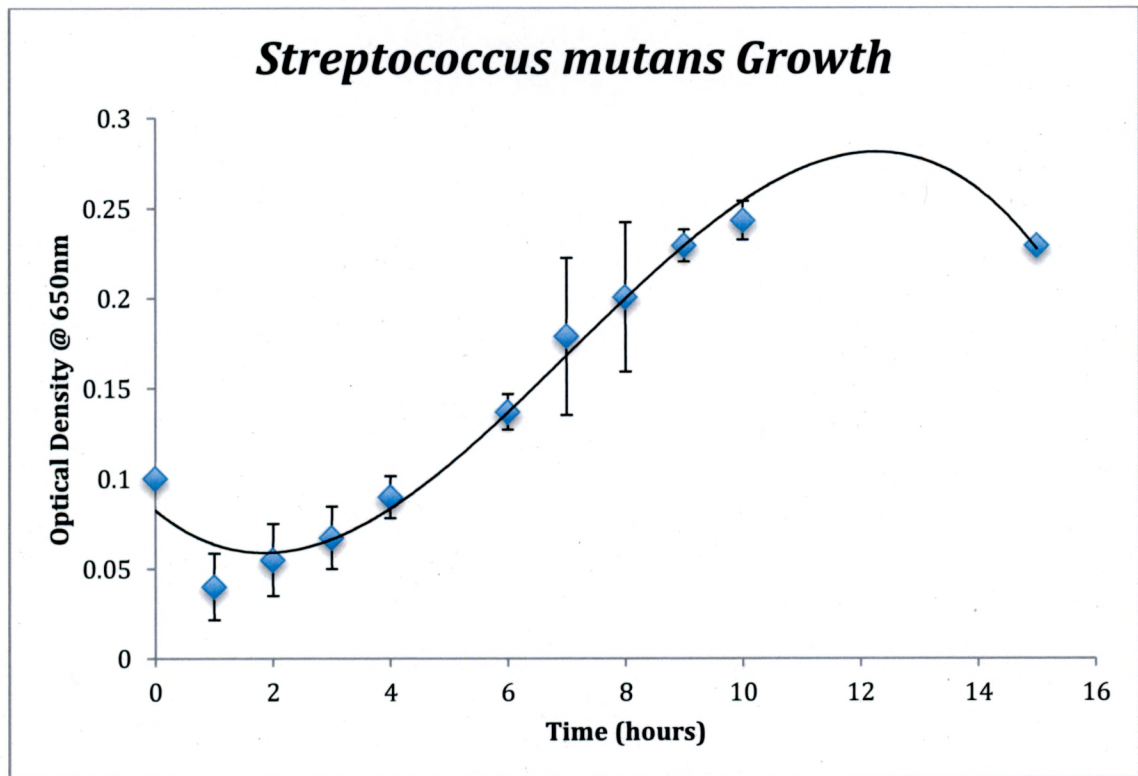
Figure 6. **(A)** Simple stain of *Streptococcus mutans* from a stock culture viewed under 400x magnification. **(B)** Gram positive stain of *Streptococcus mutans* from a stock culture viewed under 1000x magnification.

Figure 7. Standard growth curve for *Streptococcus mutans*. The generation time was calculated to be 8hrs. The growth curve results were from the mean of three repeatings and the y axis bars represent the standard deviation (SD). The growth was read at OD_{650} using a 96 well micro-titer plate reader. Samples were kept in a 37°C incubator for the duration of the experiment.

2. Stability Study of Green Tea Polyphenols EGCG and EGCG-S as Antimicrobial Agents

Stability tests were conducted in order to formulate green tea derivatives into therapeutic preparations. Stock solutions (2mg/mL) of EGCG and EGCG-S were prepared and stored at 4°C, 25°C, and 37°C for durations of 2 hours, 6 days, 12 days, 3 months and 5 months consecutively. At every time point, EGCG and EGCG-S were added to the bacterial culture *Streptococcus mutans* at a final concentration of 200 µg/mL and treated for 1 hour. To evaluate the effect of time and temperature on the antimicrobial activity of these polyphenols, two methods were used. The viable count was determined by the quantification of colony forming units (CFU), as shown in Figure 8. Cell viability was assessed by using the LIVE/DEAD® BacLight™ Assay Kit, in combination with fluorescence microscopy. The two dyes used in this kit, Syto®9 and propidium iodide, are picked up by the cells based on whether or not the cell membrane is still intact. Cell membranes that have been destroyed pick up the propidium iodide, stain red, and are thus considered dead. Cell's whose membranes are still intact pick up the Syto®9 dye, appear green, and are considered alive. Upon binding to DNA, Syto®9 dye excites green fluorescence, allowing you to visualize them under the microscope.

The results from the CFU study are shown in Figure 9 for EGCG-S and Figure 10 for EGCG. It is indicated that incubation for 1 and 6 days at all temperatures did not affect EGCG or EGCG-S's antimicrobial activity. EGCG-S incubated for 12 days did not affect its antibacterial activity at 4°C, 25°C, and 37°C with a percentage of inhibition of 97.92%, 97.87% and 97.91% respectively. EGCG-S incubated for 3 months and 5 months at 25°C yielded results similar to the control with a percentage of inhibition of

93% and 92% respectively; at 37°C incubation EGCG-S showed a percentage of inhibition reduced to 81% in both 3 months and 5 months of incubation. Very different results were obtained with EGCG in the same conditions after 6 days incubation. A significant decrease of activity was observed in EGCG at 25°C and 37°C at day 12. The percentage of inhibitions were 11.95% and 9.56% respectively. After 3 months of incubation, the percentage of inhibition was 40% for 25°C and 42% for 37°C. For 4 months of incubation, the percentage of inhibitions for 25°C, and 37°C were 27% and 41% respectively. The LIVE/DEAD® BacLight[TM] Assay Kit was also used to evaluate cell viability under fluorescence microscopy as shown in Figures 11-16. The results correlated very well with CFU study; when tea polyphenols have strong antibacterial activity, the majority of cells were dead (red), and when the polyphenols lost their antimicrobial activity, many viable cells (green) were observed. Thus EGCG-S under different incubation periods still maintain its ability to kill and most cell were not viable. Many of the EGCG treated cells, under different incubation periods, were still viable. The results suggested that modified EGCG-S is structurally and functionally more stable in high temperature settings, and as a result, a better molecule to be used as a potential therapeutic agent to combat bacterial infection.

Further studies have to be carried out with more replicates and much higher temperatures, such as 45 °C, 65°C and 72°C, to ensure that EGCG-S can withstand higher temperatures including the temperature for pasteurization. This can also heighten the possibility of using EGCG-S as an alterative organic food preservative.
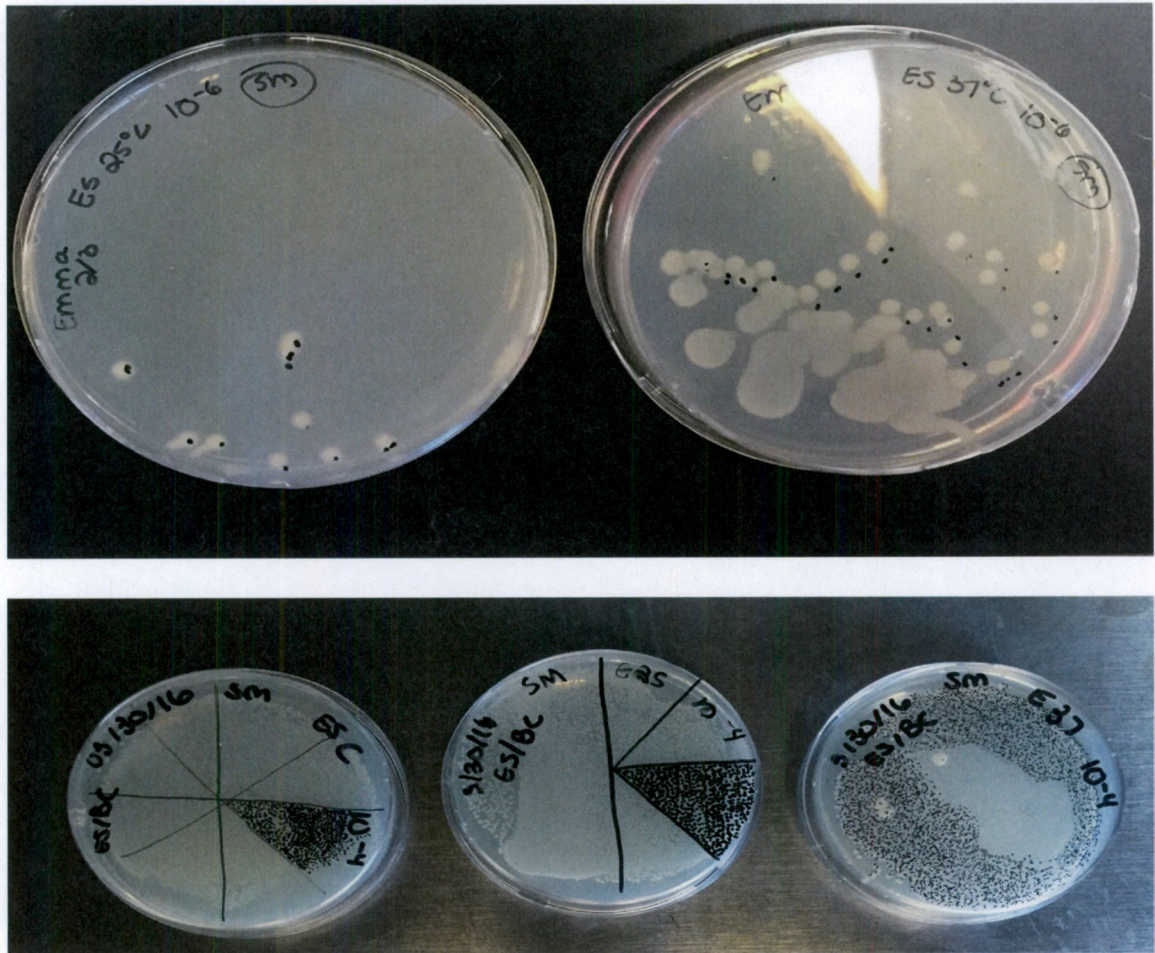
Figure 8. Examples of the plates used for the quantification of Colony Forming Units (CFU).
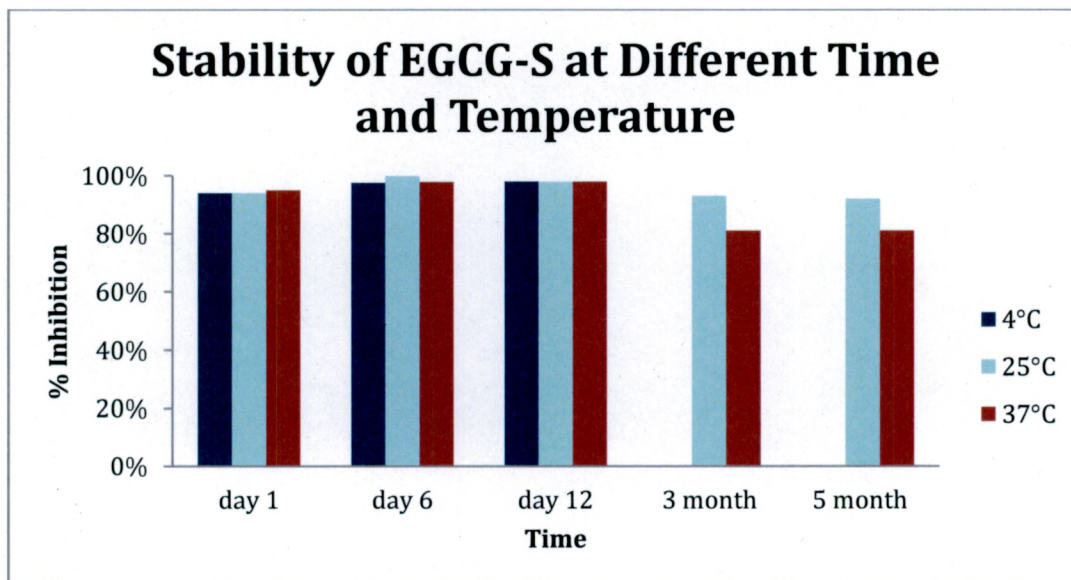
**Figure 9.** The percentage of inhibition of EGCG-S over a period of 5 months.
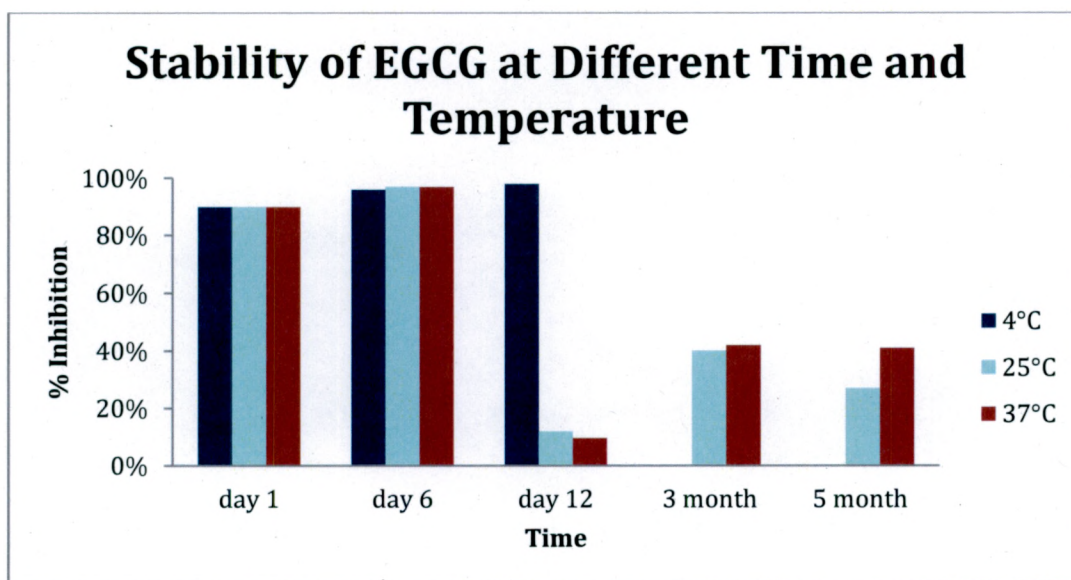
Figure 10. The percentage of inhibition of EGCG over a period of 5 months.

	Percentage of Inhibition				
	Day 1	Day 6	Day 12	3 months	5 months
EGCG-S 4°C	94%	97.5%	97.92%		
EGCG-S 25°C		99.8%	97.87%	93%	92%
EGCG-S 37°C		97.8%	97.91%	81%	81%
EGCG 4°C	90%	96%	97.95%		
EGCG 25°C		97%	11.95%	40%	27%
EGCG 37°C		97%	9.56%	42%	41%

Table 1. The percentage of inhibition was calculated from the CFU for both EGCG-S and EGCG at various temperatures at different time intervals over a period of 5 months.
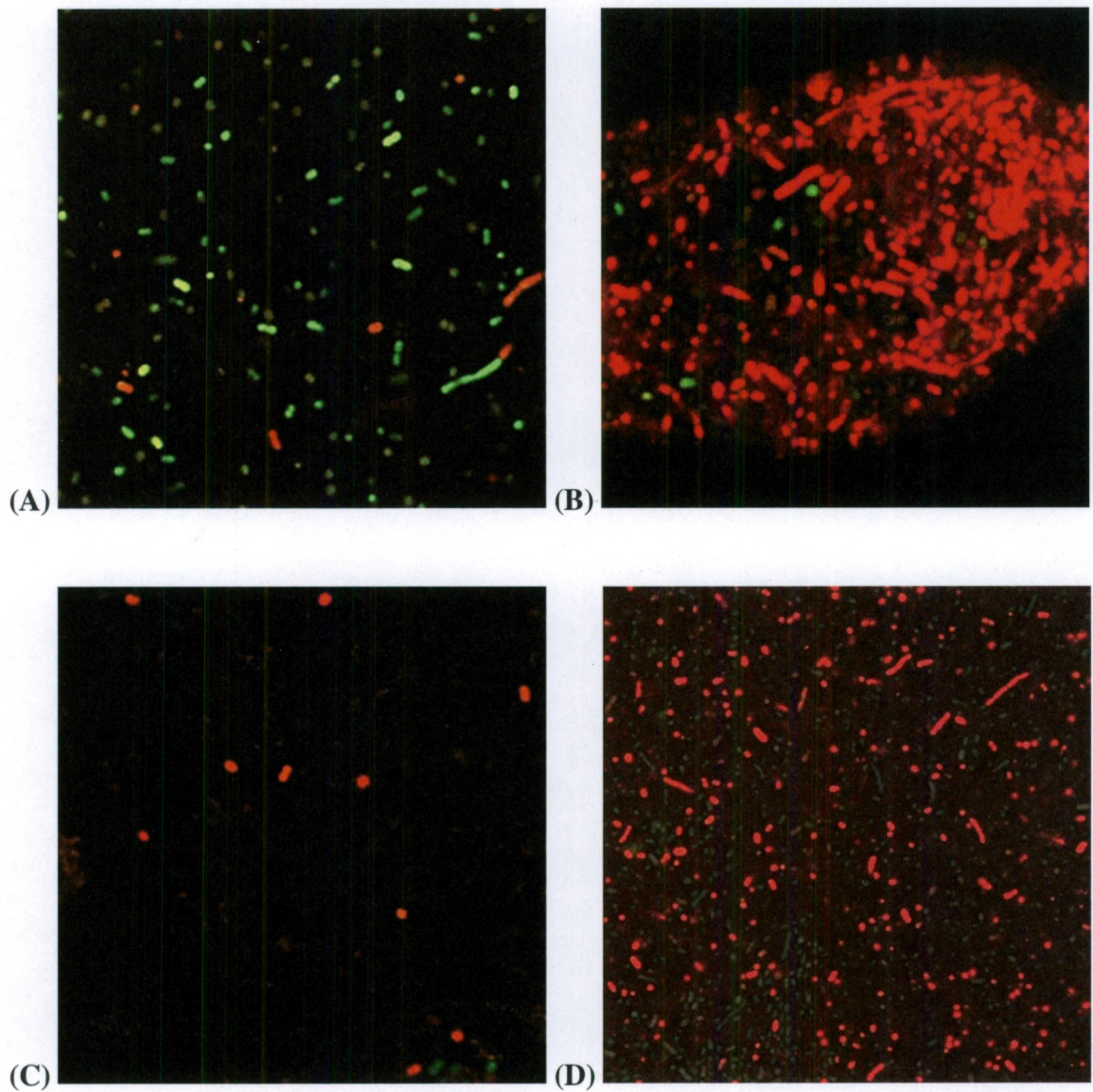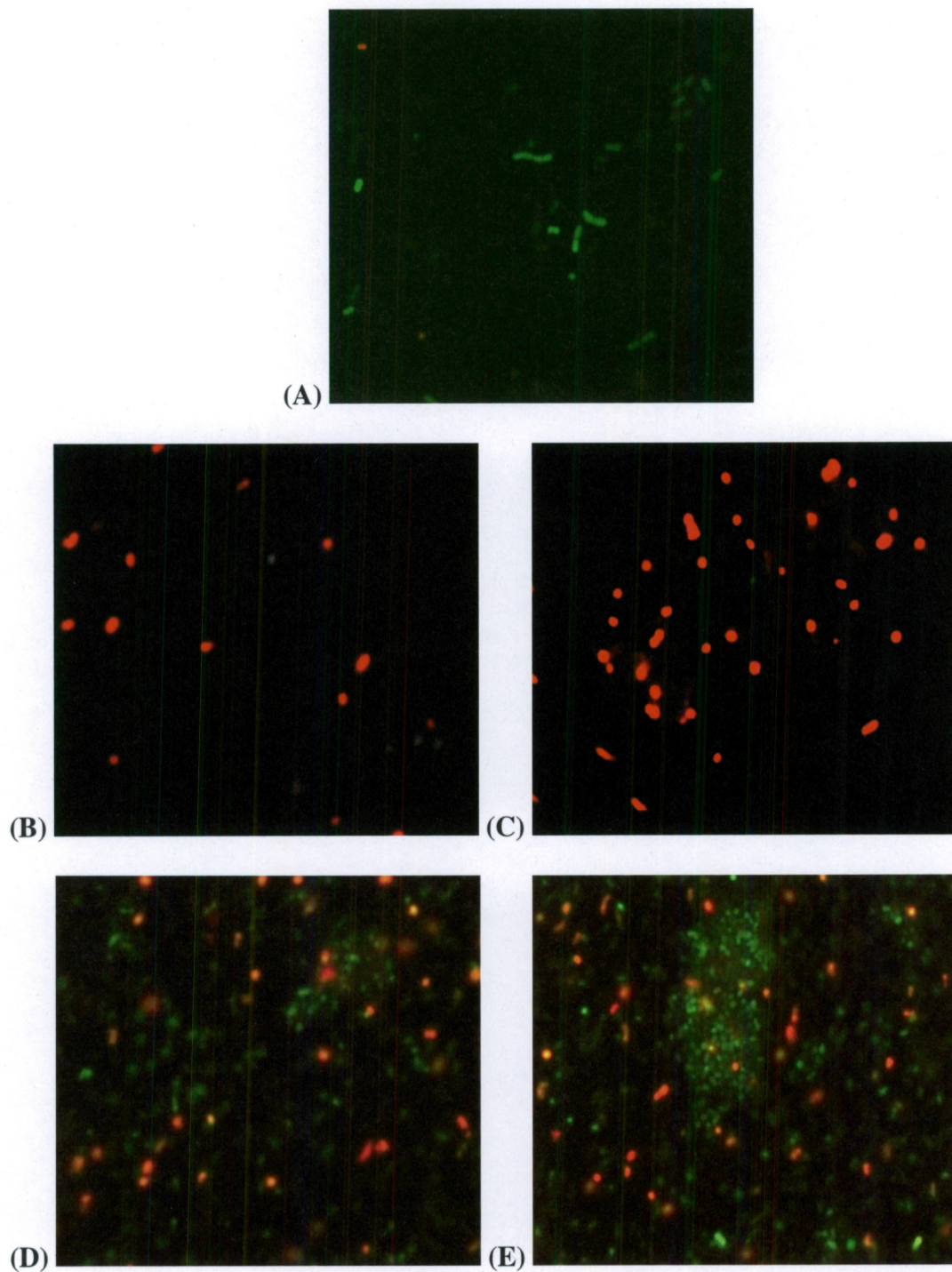
Figure 11. Live/ Dead qualitative analysis of cell viability. (A) The control group after 1 day. The green color represents live, viable cells. (B) *S. mutans* treated with EGCG-S for 6 days at 4°C. (C) *S. mutans* treated with EGCG-S for 6 days at 25°C. (D) *S. mutans* treated with EGCG-S for 6 days at 37°C.
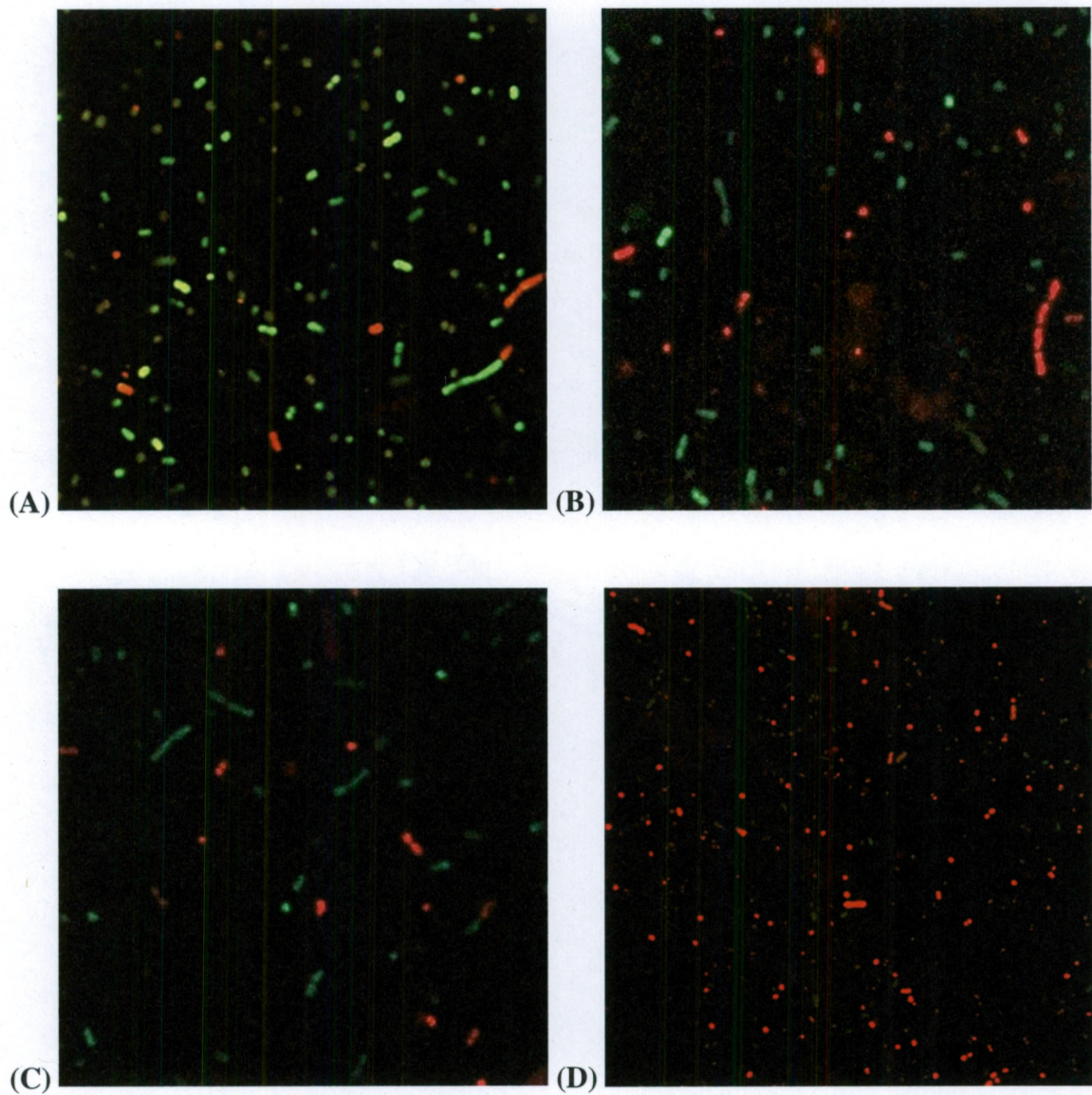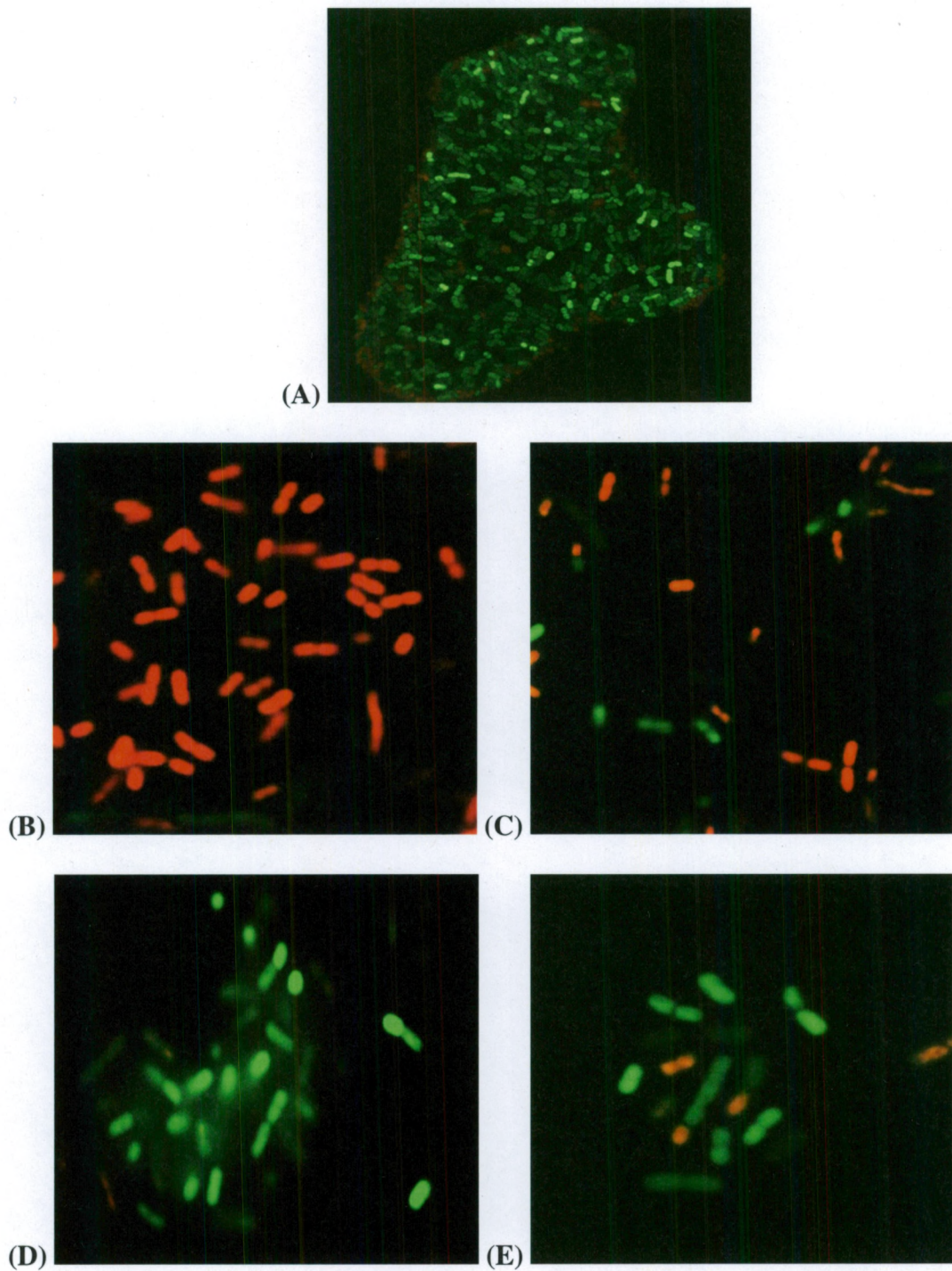
Figure 12. Live/ Dead qualitative analysis of cell viability. (A) The control group after 1 day. The green color represents live, viable cells. (B) *S. mutans* treated with EGCG for 6 days at 4°C. (C) *S. mutans* treated with EGCG for 6 days at 25°C. (D) *S. mutans* treated with EGCG for 6 days at 37°C.
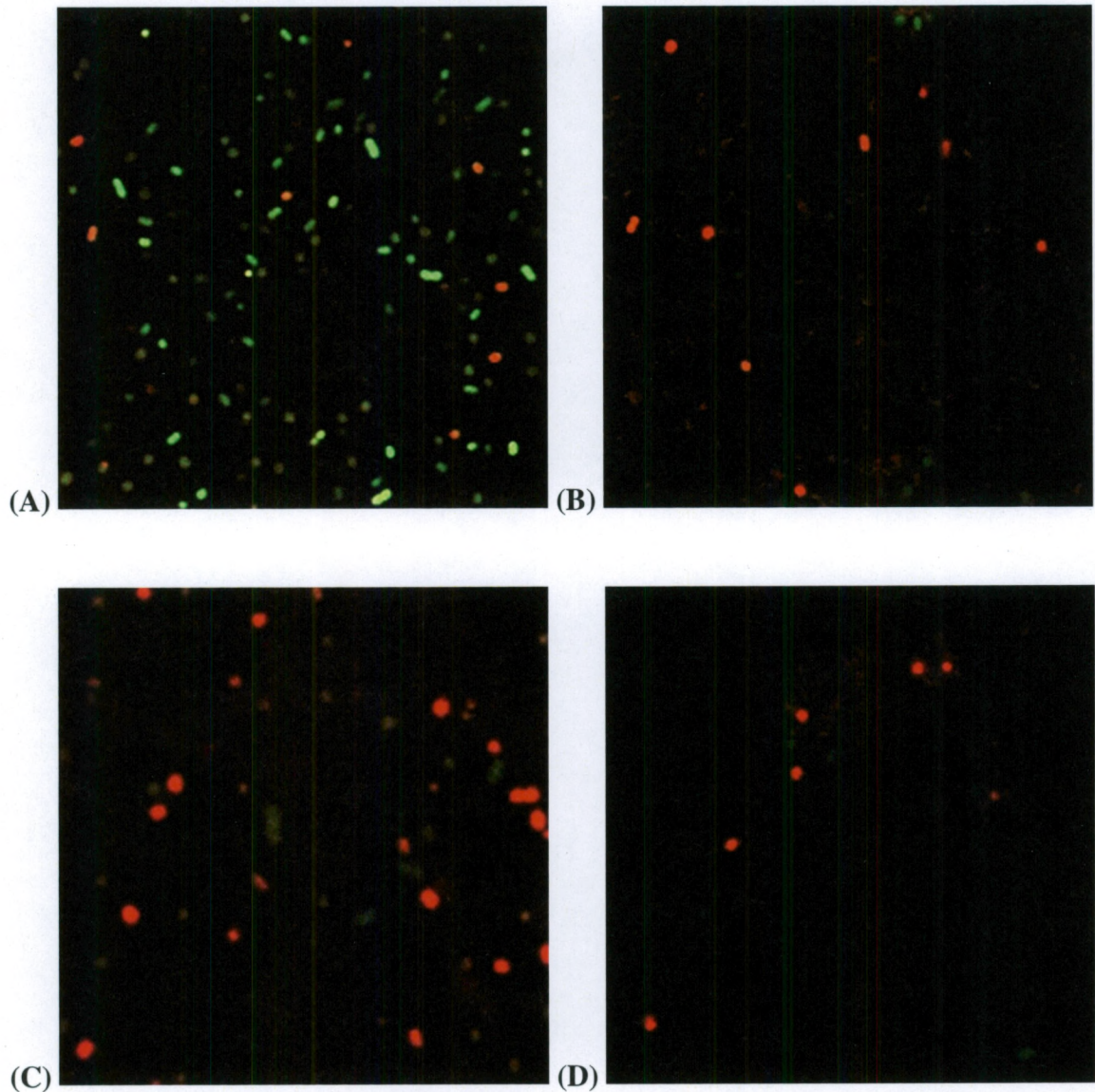
Figure 13. Live/ Dead qualitative analysis of cell viability. (A) The control group after 1 day. The green color represents live, viable cells. (B) *S. mutans* treated with EGCG-S for 12 days at 4°C. (C) *S. mutans* treated with EGCG-S for 12 days at 25°C. (D) *S. mutans* treated with EGCG-S for 12 days at 37°C.
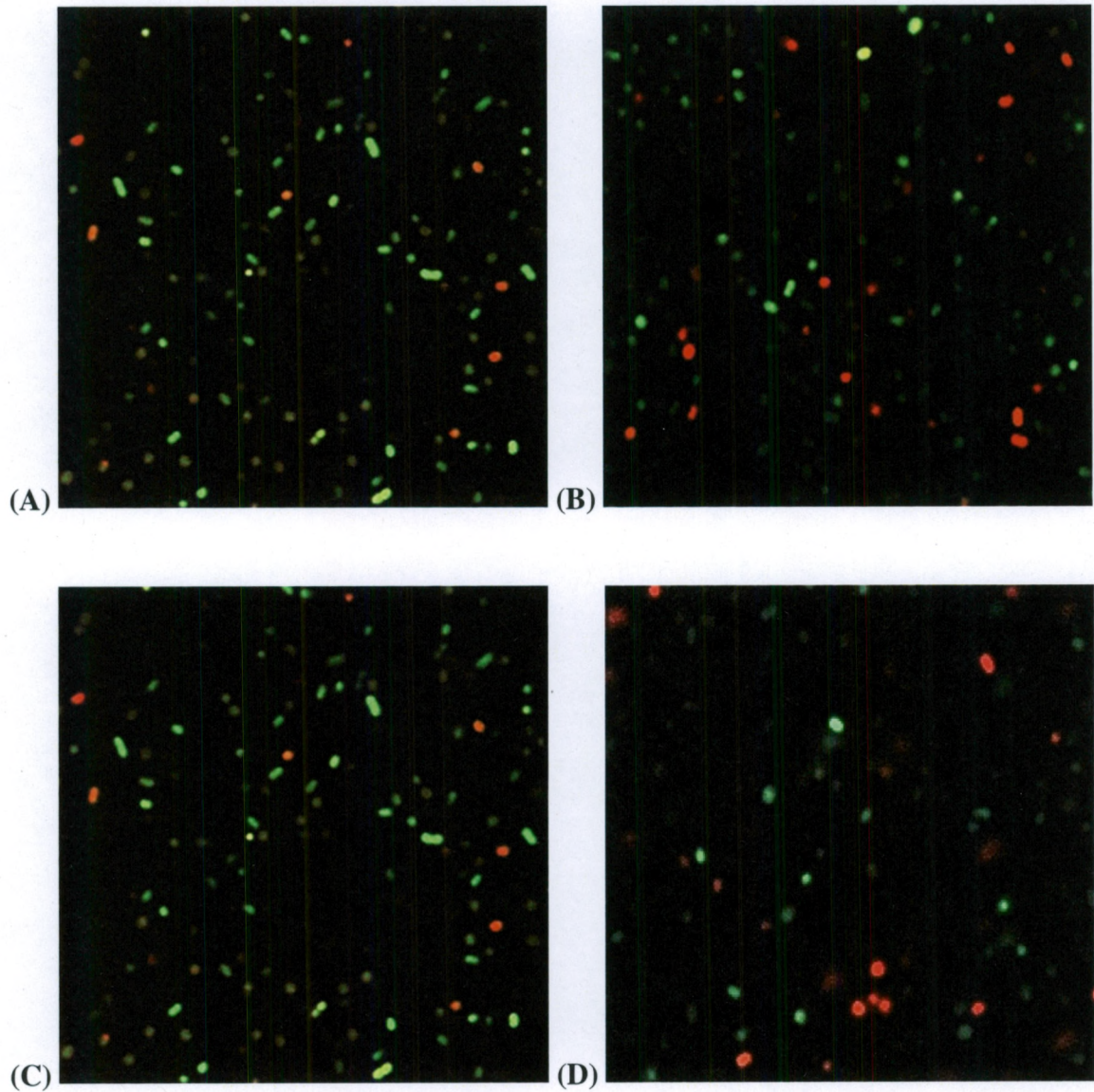
Figure 14. Live/ Dead qualitative analysis of cell viability. (A) The control group after 1 day. The green color represents live, viable cells. (B) *S. mutans* treated with EGCG for 12 days at 4°C. (C) *S. mutans* treated with EGCG for 12 days at 25°C. (D) *S. mutans* treated with EGCG for 12 days at 37°C.

Figure 15. Live/ Dead qualitative analysis of cell viability. (A) The control group after 1 day. The green color represents live, viable cells. (B) *S. mutans* treated with EGCG-S for 3 months at 25°C. (C) *S. mutans* treated with EGCG-S for 3 months at 37°C. (D) *S. mutans* treated with EGCG for 3 months at 25°C. (E) *S. mutans* treated with EGCG-S for 3 months at 37°C.

Figure 16. Live/ Dead qualitative analysis of cell viability. (A) The control group after 1 day. The green color represents live, viable cells. (B) *S. mutans* treated with EGCG-S for 5 months at 25°C. (C) *S. mutans* treated with EGCG-S for 5 months at 37°C. (D) *S. mutans* treated with EGCG for 5 months at 25°C. (E) *S. mutans* treated with EGCG for 5 months at 37°C.

3. The Effect of Green Tea Polyphenols on *Streptococcus mutans*

a. Colony Forming Units

To test the effect of Green Tea Polyphenols on *S. mutans*, a Colony Forming Units (CFU) experiment was carried out. This Experiment quantitatively assesses the cell's ability to reproduce and form colonies under selected conditions. Two different types of Green Tea Polyphenols were used in this study, LTP and EGCG-S, as well as various concentrations (100, 200, and 250ug/mL) of each. The results indicated that the effect of both tea polyphenols is dose-dependent. As shown in Table 1 and Figure 17, EGCG-S reduced the amount of colonies drastically from 657 to 0 when the concentration was increased from 100 to 200 and 250ug/mL. LTP (Table 1 and Figure 18) at the concentration of 100ug/mL was 619 CFU; at the concentration of 200ug/mL the CFU was 22. The concentration of 250ug/mL LTP was able to completely inhibit colony formation. In this study, EGCG-S required 200ug/mL and LTP required 250ug/mL to completely inhibit colony formation.
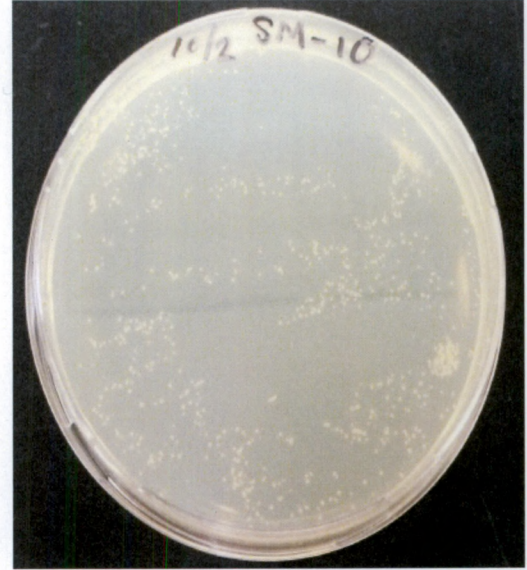
Colony Forming Units		
Treatment	Number of Colonies	% Inhibition
Control 10^{-8}	9600	
EGCG-S (100)10^{-8}	657	93.16%
EGCG-S (200)10^{-8}	0	100%
EGCG-S (250)10^{-8}	0	100%
LTP(100)10^{-8}	619	93.55%
LTP(200)10^{-8}	22	99.77%
LTP(250)10^{-8}	0	100%

Table 2. The CFU for each concentration of EGCG-S and LTP were counted and the percentage of inhibition was then calculated.
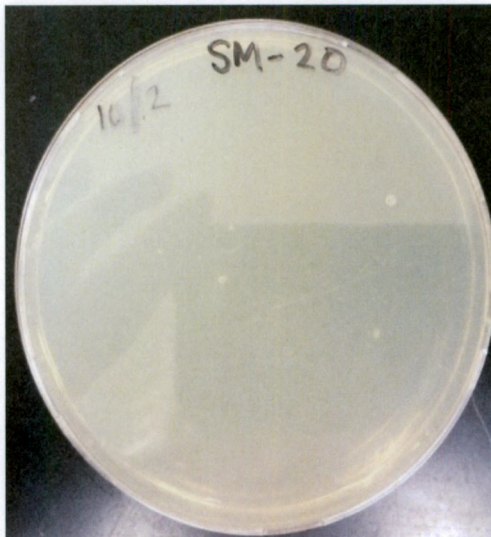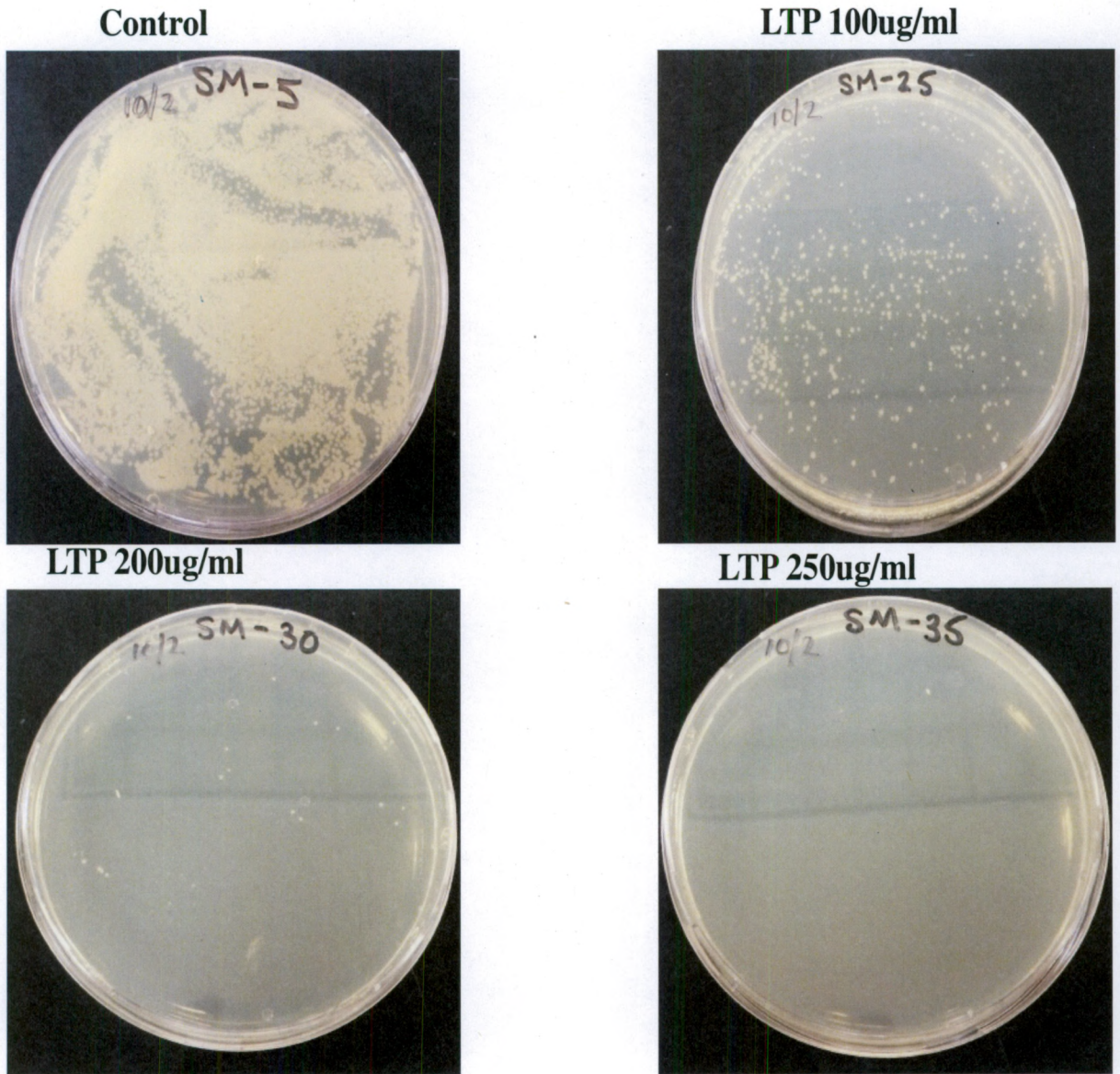
Control

EGCG-S 100ug/ml

EGCG-S 200ug/ml

EGCG-S 250ug/ml

Figure 17. Colony Forming Units experiment treated with various concentrations of EGCG-S. The results demonstrate a dose-dependent relationship between EGCG-S and the number of colonies formed.

Control

**LTP 100ug/ml**

LTP 200ug/ml

LTP 250ug/ml

Figure 18. Colony Forming Units experiment treated with various concentrations of LTP. The results demonstrate a dose-dependent relationship between LTP and the number of colonies formed.

b. Live/ Dead

Cell viability can be assessed through fluorescent microscopy via the Live and Dead BacLight ® Assay Kit. This experiment helps detect cell viability by the quality of the cell membrane. Figure 19 represent live and dead assays done on *S. mutans* alone and when treated with 250ug/mL of EGCG-S. For the treated sample, *S. mutans* was incubated with the treatment for 4 days at 37°C to allow biofilm formation to occur. Figure 19A represents the control of *S. mutans*, which resulted in high-density growth, all of which displayed the green fluorescence. This indicates that the entire control population was alive. Figure 19B shows *S. mutans* grown under similar conditions, with the addition of a 250ug/mL EGCG-S treatment. Post-treatment, the entire population of *S. mutans* was dead. Visualization of this was evident by the red fluorescent chains of cocci under the microscope. These results alone suggest that tea polyphenols may inhibit biofilm growth through the destruction of cell wall integrity. Further more, these results support the data obtained from CV study that EGCG-S is effective in inhibiting the growth of *S. mutans*.
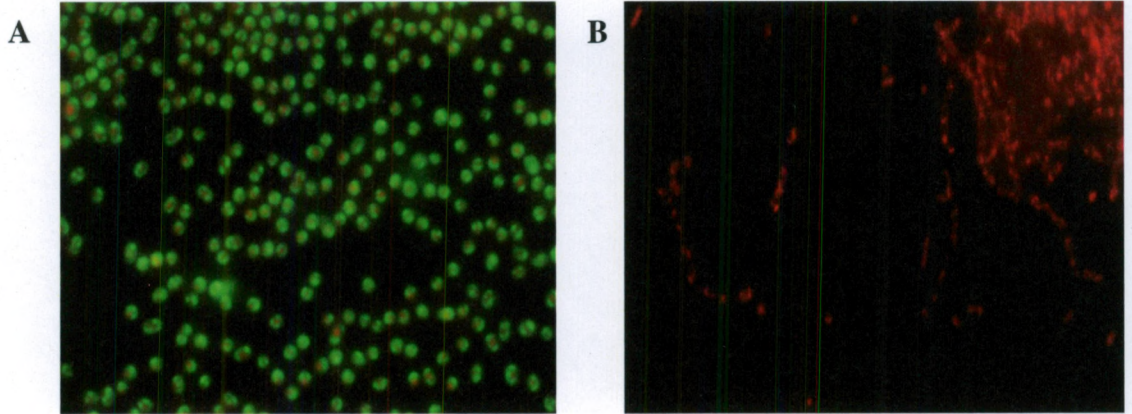
Figure 19. Live and Dead Assay of *Streptococcus mutans*. **(A)** The control figure represents cells with intact cell membranes, which are considered alive and fluoresce a green color. **(B)** After treatment with EGCG-S 250ug/mL, all cell membranes are damaged, thus cells are considered dead and fluoresce a red color. All results are viewed under 1000x magnification.
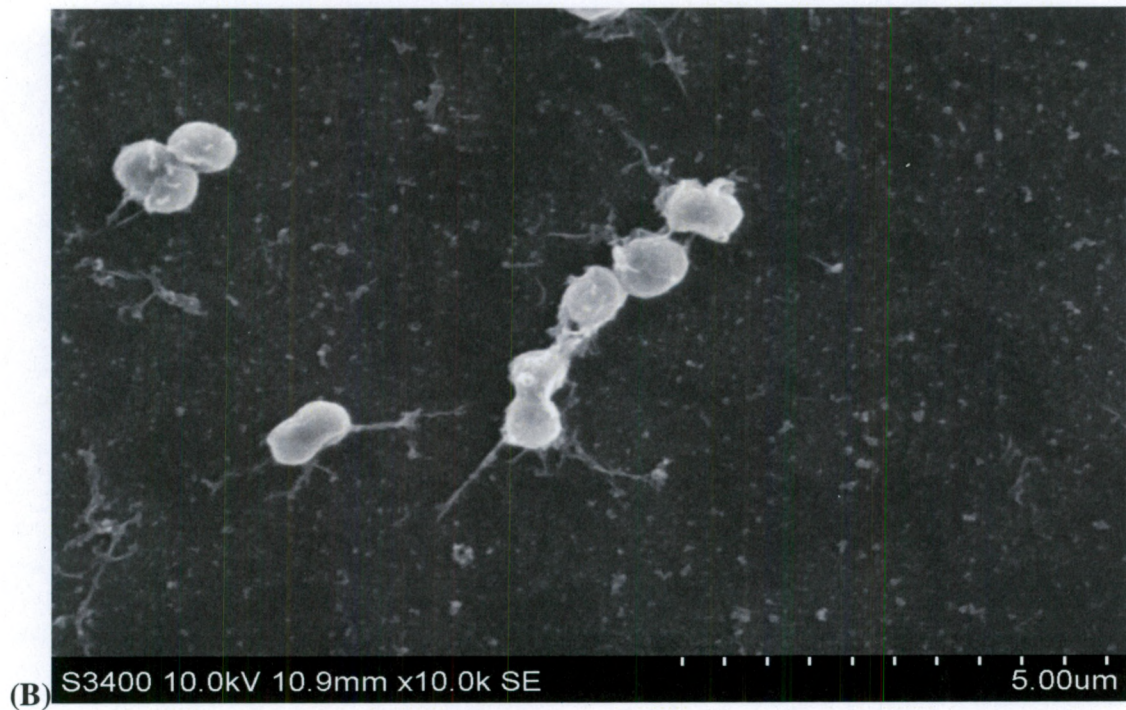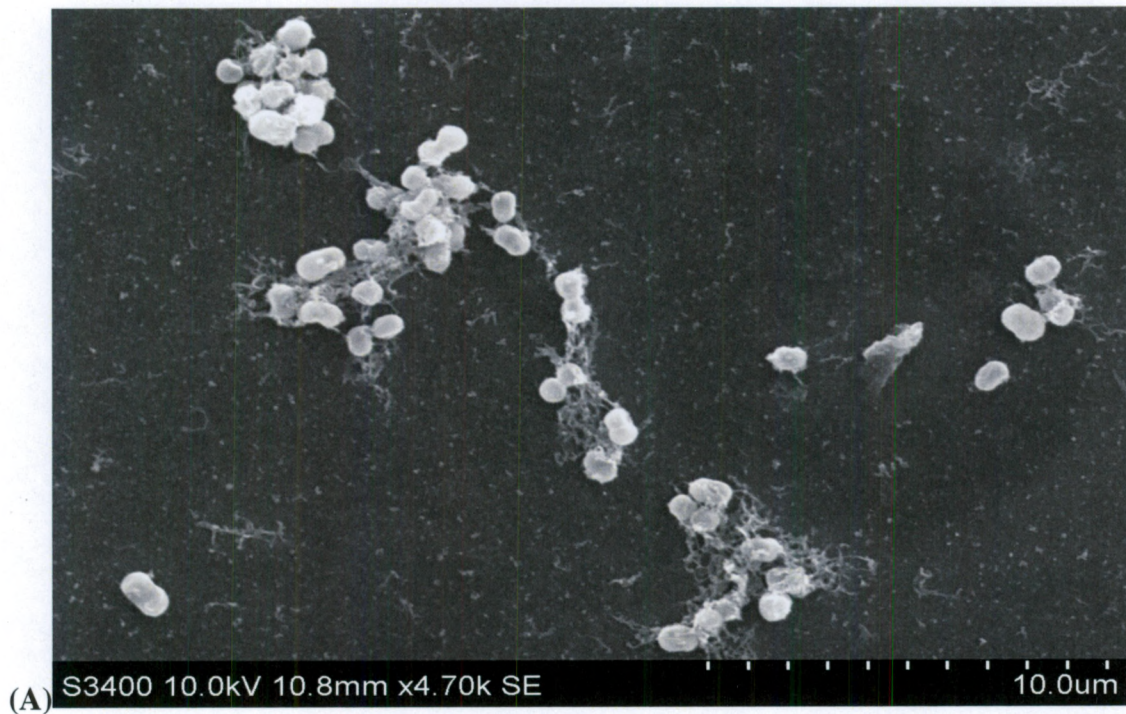
c. Scanning Electron Microscopy (SEM)

Microscopic observation via Scanning Electron Microscopy(SEM), was performed in the MMRL lab under the guidance of the MMRL Director, Dr. Laying Wu. While the Live and Dead assay allows one to see the viability of the cells through fluorescence, SEM allows scientists to examine the cell morphology and cell density at a greater magnification. Figures 20-21 display the SEM images for *S. mutans* alone and after treatment with 250ug/mL EGCG-S. Figure 20 was allowed to grow for 60 hours before microscopic observation. These two images confirm the morphology of *S. mutans* by displaying many chains of cocci shaped bacteria. In Figure 20B, the development of biofilm is evident by the massive grouping together of *S. mutans* in the middle of the image. These biofilm structures were found throughout the slide. After confirming both the morphology and presence of biofilm, Figure 21 demonstrates that when *S. mutans* is treated with 250ug/mL EGCG-S both the morphology and formation of biofilm is destroyed. In both treated images, the number of chains drastically decreased, the morphology of the cocci is lost, the cells appear to be losing its contents, and the formation of biofilm is not seen throughout the entire sample. These results confirm that EGCG-S may inhibit *S. mutans* growth through destruction of the cell wall. This also confirms that EGCG-S is a potential treatment for inhibiting the growth and development of biofilm in *S. mutans*.
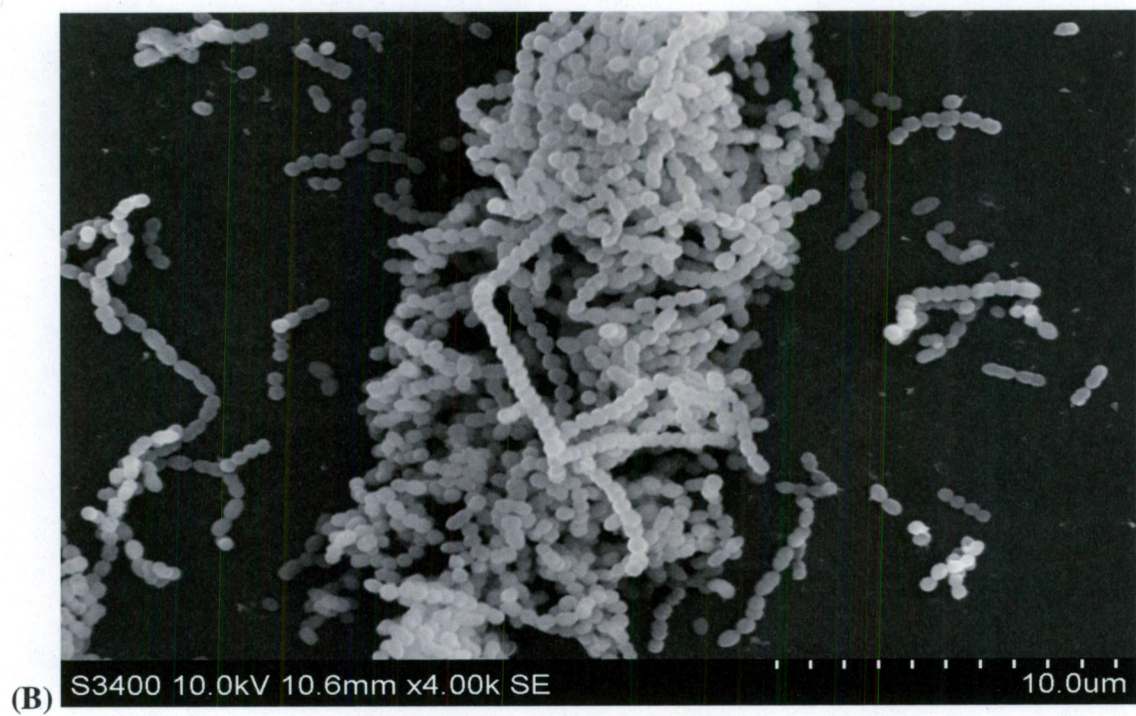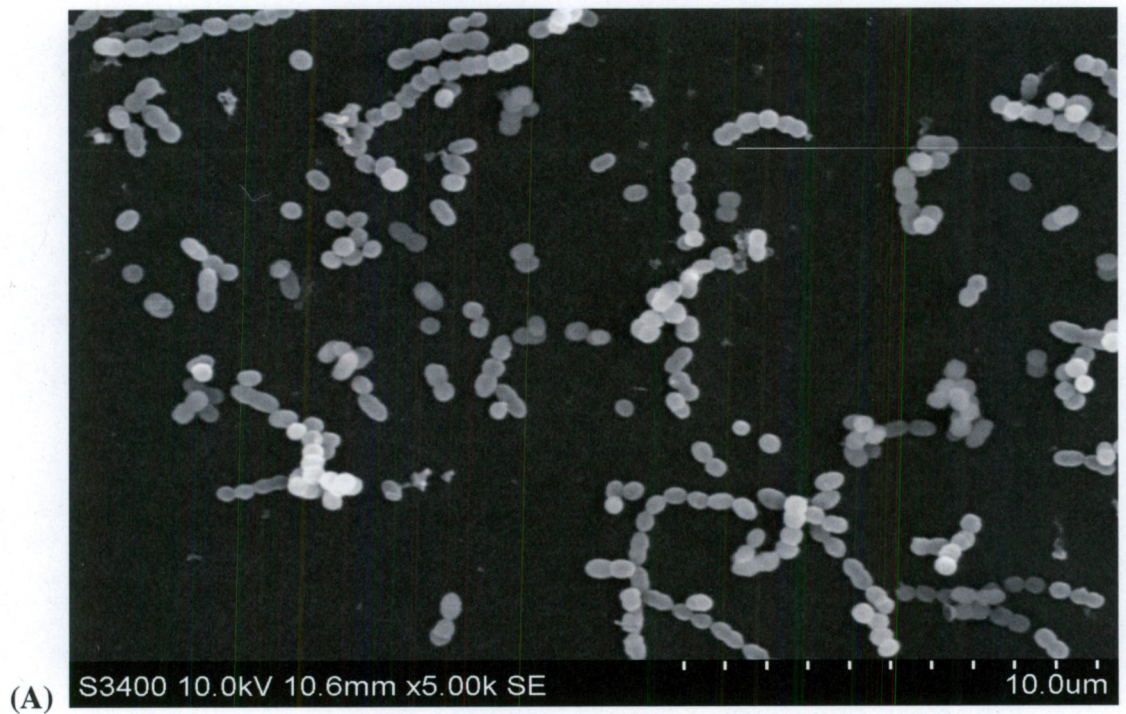
Figure 20. Scanning Electron Microscopy (SEM) of *Streptococcus mutans*. *S. mutans* were grown for 60 hours before viewing. **(A)** Confirms the morphology of *S. mutans* by being present in chains of cocci. **(B)** Represents *S. mutans* ability to form biofilm

(A) S3400 10.0kV 10.8mm x4.70k SE   10.0um

(B) S3400 10.0kV 10.9mm x10.0k SE   5.00um

Figure 21. Scanning Electron Microscopy (SEM) of *Streptococcus mutans*. *S. mutans* were grown for 60 hours before viewing. After being treated with EGCG-S 250ug/mL for 60 hours, destruction of the cell morphology and ability to form biofilm is evident at both magnifications in (A) and (B).

## 4. The Effect of Green Tea Polyphenols on the Cavity Causing Biofilm of *Streptococcus mutans*

### a. The Effect of Sugars on Biofilm Formation in *Streptococcus mutans*

To determine the condition under which biofilm is most adequately formed, different sugars with different concentrations were used in this study. Dextrose and sucrose were prepared in Tryptic Soy Broth from Difco[TM] with the following concentrations: 0.1%, 1%, and 5%, of each sugar in 6 well plates. In each well, a sterile cover glass was put in the center of the well that contained broth, the bacteria and a specific concentration of sugar. At 24 and 48 hours, the cover glasses were taken out, stained with Syto®9 dye, and observed under fluorescent microscope. The results are shown in Figures 22-26. The negative control (0% of sugar) picture shows that the bacteria are alive and growing, but only minimal biofilm formation is occurring. At 24 hours all samples demonstrated biofilm formation. Sucrose concentrations 0.1% and 1% demonstrated more biofilm that its counterpart dextrose, which is exhibited by the cloudiness of the image. However, at the 5% concentration dextrose demonstrated more biofilm formation than sucrose. To allow for further biofilm development to occur, the second set of samples was grown for 48 hours. Dextrose (0.1%) developed more biofilm when compared with 24 hours of incubation, resulting in more biofilm growth at 0.1% and 1% than the higher concentration of 5% dextrose.

To simulate a more realistic environment for the bacteria, another set of samples was grown with the addition of artificial saliva. Since sucrose developed

more biofilm in a number of concentrations, we chose to work with sucrose for the duration of the study. Samples were observed after 24 hours of incubation. For 0.1% and 1%, an abundance of biofilm was seen consistently throughout the sample. However, at 5%, only minimal biofilm formation was found.
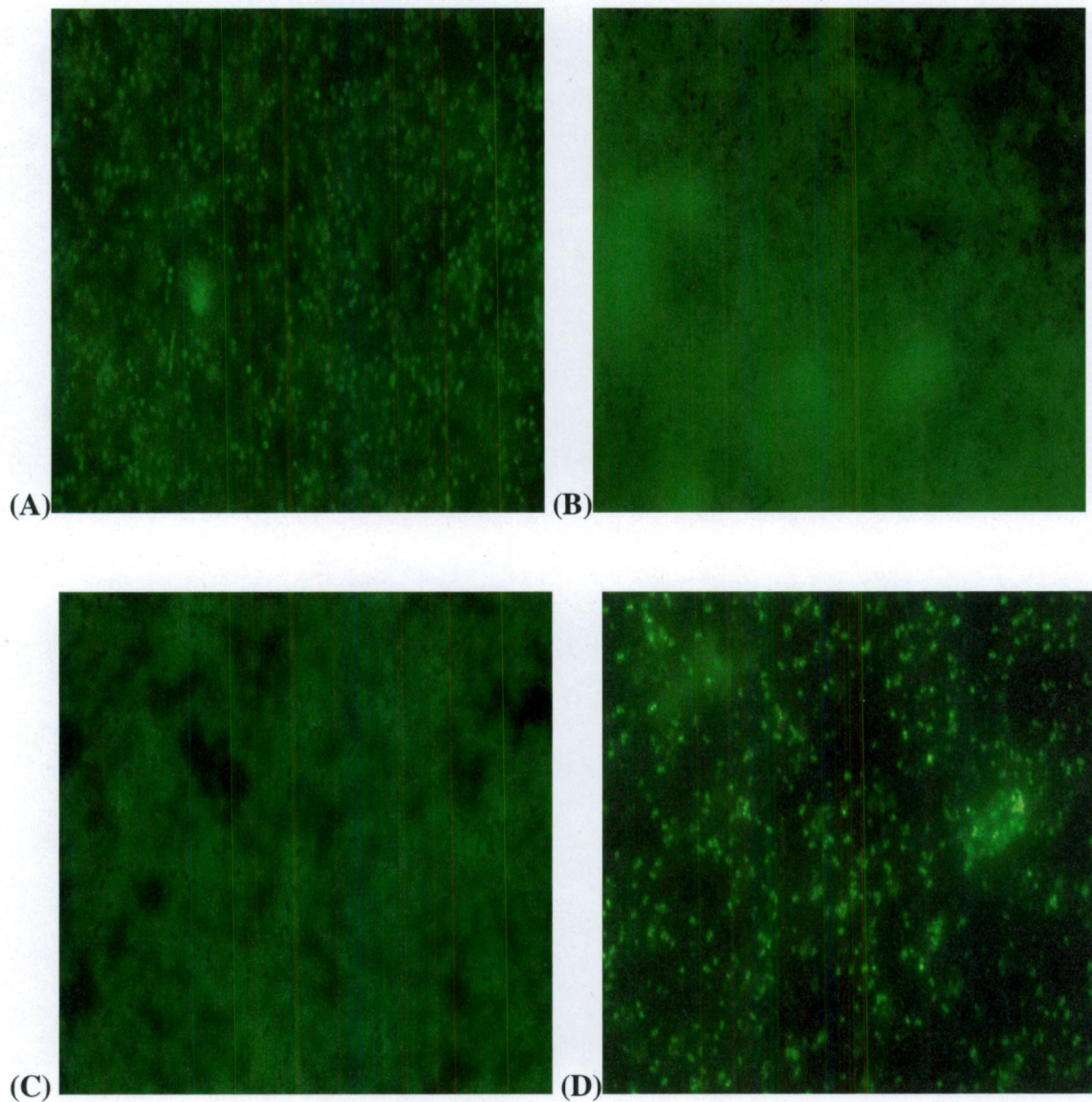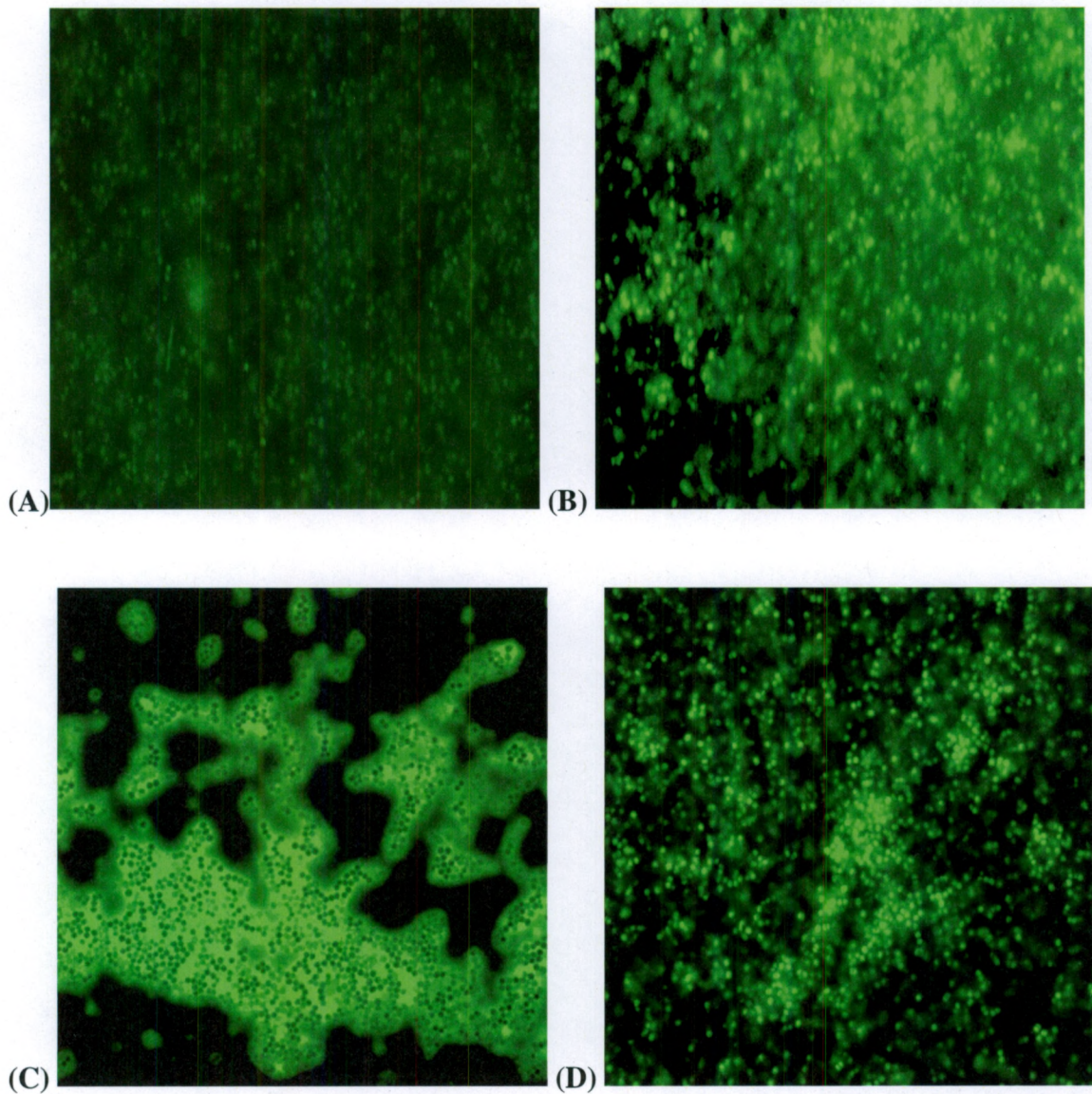
Figure 22. *Streptococcus mutans* was grown in the presence of varying concentrations of sucrose to determine which induced the greatest amount of biofilm formation after 24 hours of incubation at 37°C. Biofilm formation is indicated by colonies of *Streptococcus mutans* growing together in a matrix, which is shown by the cloudy structures around the group. **(A)** *S. mutans* positive control grown in the absence of sugar. **(B)** *S. mutans* grown in 0.1% sucrose. **(C)** *S. mutans* grown in 1% sucrose. **(D)** *S. mutans* grown in 5% sucrose.
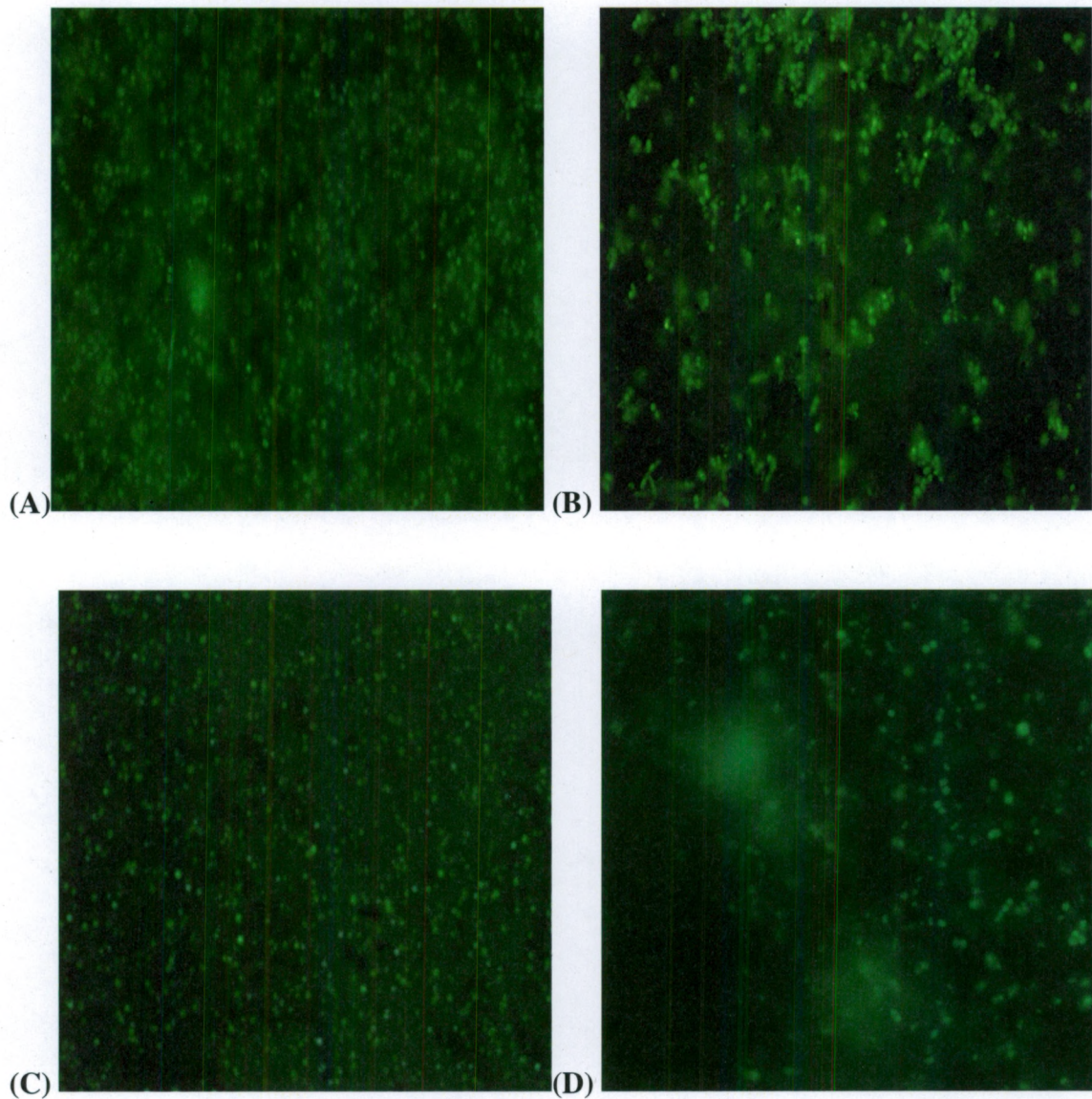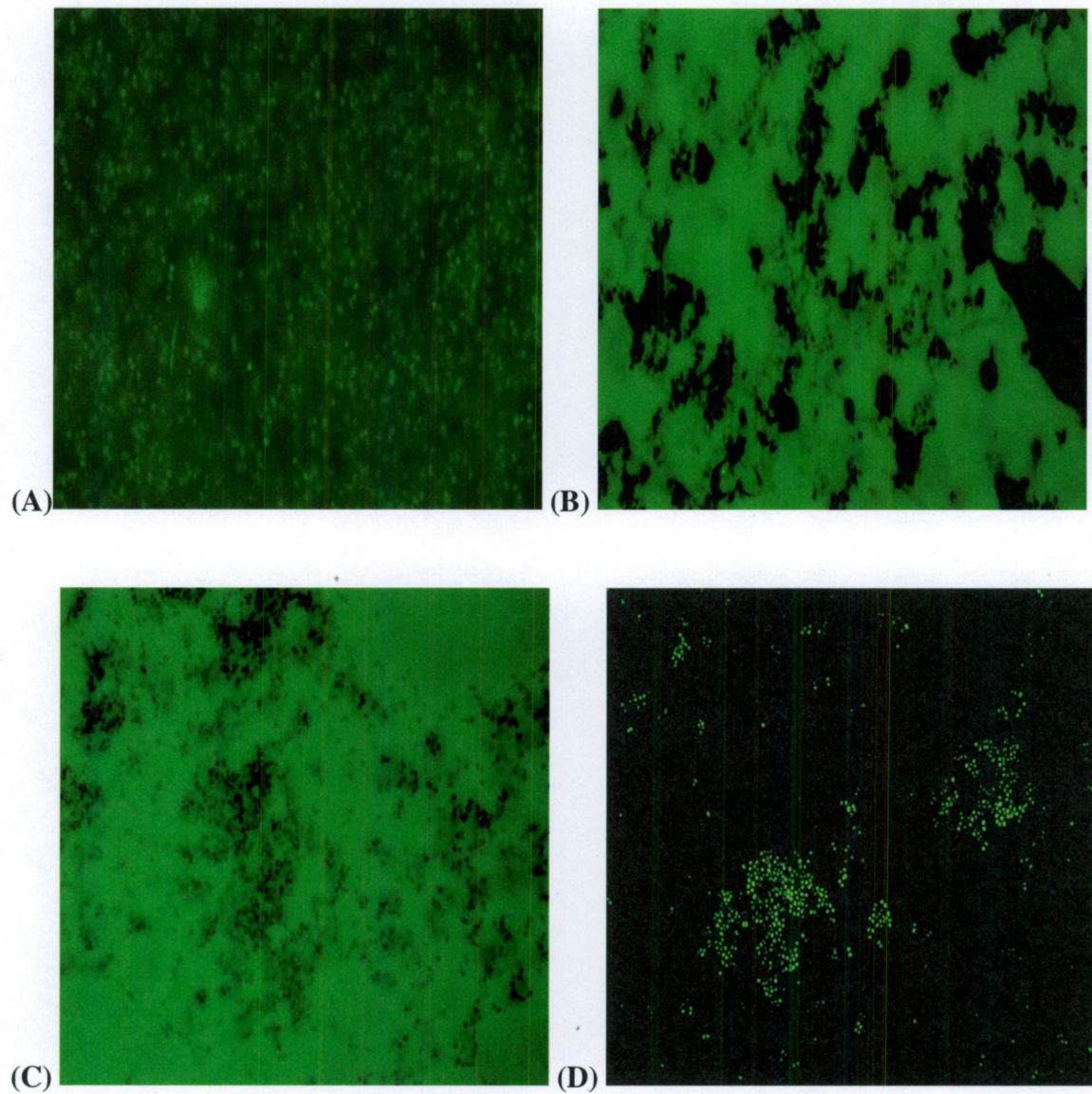
Figure 23. *Streptococcus mutans* was grown in the presence of varying concentrations of dextrose to determine which induced the greatest amount of biofilm formation after 24 hours of incubation at 37°C. Biofilm formation is indicated by colonies of *Streptococcus mutans* growing together in a matrix, which is shown by the cloudy structures around the group. **(A)** *S. mutans* positive control grown in the absence of sugar. **(B)** *S. mutans* grown in 0.1% dextrose. **(C)** *S. mutans* grown in 1% dextrose. **(D)** *S. mutans* grown in 5% dextrose.
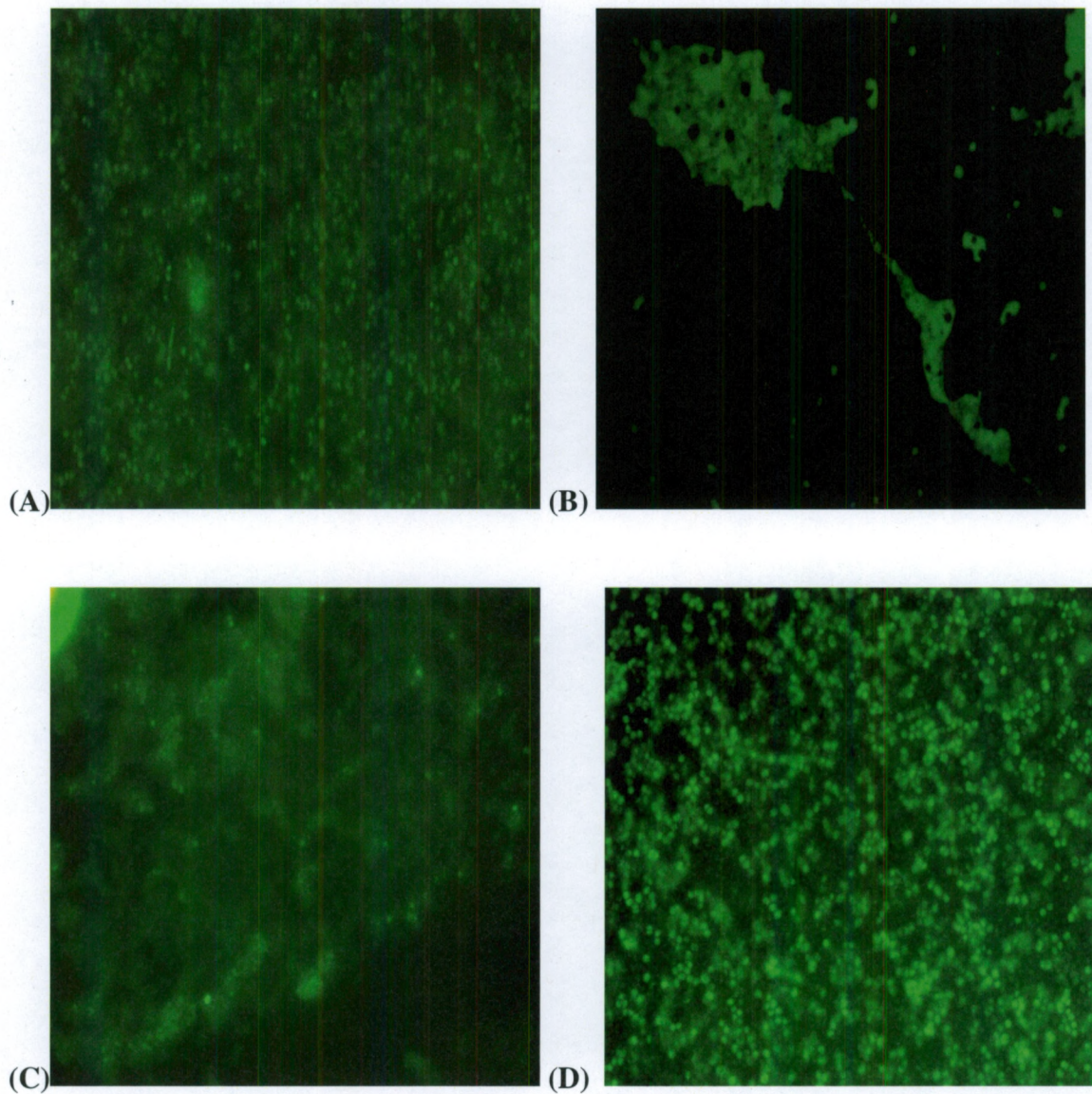
Figure 24. *Streptococcus mutans* was grown in the presence of varying concentrations of sucrose to determine which induced the greatest amount of biofilm formation after 48 hours of incubation at 37°C. Biofilm formation is indicated by colonies of *Streptococcus mutans* growing together in a matrix, which is shown by the cloudy structures around the group. **(A)** *S. mutans* positive control grown in the absence of sugar. **(B)** *S. mutans* grown in 0.1% sucrose. **(C)** *S. mutans* grown in 1% sucrose. **(D)** *S. mutans* grown in 5% sucrose.

Figure 25. *Streptococcus mutans* was grown in the presence of varying concentrations of dextrose to determine which induced the greatest amount of biofilm formation after 48 hours of incubation at 37°C. Biofilm formation is indicated by colonies of *Streptococcus mutans* growing together in a matrix, which is shown by the cloudy structures around the group. **(A)** *S. mutans* positive control grown in the absence of sugar. **(B)** *S. mutans* grown in 0.1% dextrose. **(C)** *S. mutans* grown in 1% dextrose. **(D)** *S. mutans* grown in 5% dextrose.

Figure 26. *Streptococcus mutans* was grown in the presence of artificial saliva and varying concentrations of sucrose to determine which induced the greatest amount of biofilm formation after 24 hours of incubation at 37°C. Biofilm formation is indicated by colonies of *Streptococcus mutans* growing together in a matrix which is shown by the cloudy structures around the group.

b. Congo Red Agar Qualitative Analysis on Biofilm Formation of *S. mutans*

In order to study the biofilm forming properties of *S. mutans*, a quantitative method, Congo Red Assay was used. This is a quick and simple method to identify and determine biofilm formation in microorganisms (Schwartz Kelly, 2012). Overnight cultures of *S. mutans* were treated with different concentrations of LTP or EGCG-S (50, 100, 150 and 200 ug/mL) for different time periods (0.5, 1.0, 2.0 and 4.0 hours). After treatment, the samples were plated onto 24 well plates and incubated for 1 to 4 days at 37°C. The untreated cells were used as a control as shown in Figure 27A. After incubation, the plates were observed; positive biofilm formation showed a black color, while a red color indicated that there was no biofilm formation as shown in Figure 27B. The results of LTP and EGCG-S at 50 and 100 ug/mL treated for 0.5, 1, 2 and 4 hours are shown in Figure 29 and Figure 31 respectively. The results indicated that at 0.5 and 1 hour treatment, the biofilm formation was similar to the positive control and showed black color; at 2 and 4 hours treatment with both concentrations, the biofilm formation was significantly reduced.

LTP and EGCG-S at concnetrations of 150 and 200 ug/mL were used to further evaluate the inhibitory effect of tea polyphenols on the biofilm formation in *S. mutans* and the results are shown in Figure 33. This study clearly indicated that treating cells with either LTP or EGCG-S for 2 hours and 4 hours was able to completely inhibit biofilm formation. The summary results of these studies are shown in Figure 34 and Figure 35. This study suggested that LTP and EGCG-S at 200 ug/mL for 2 hours treatment can completely inhibit the formation of biofilm

in *S. mutans*. In summary, the Congo Read assay provided the information for the exposure time and concentrations of LTP and EGCG-S for further quantitative study.
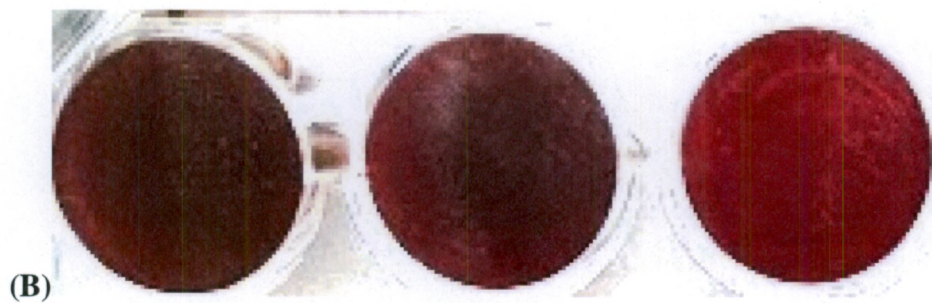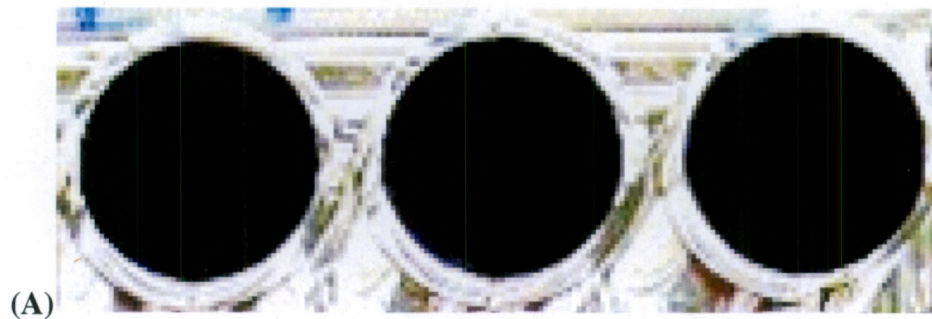
Figure 27. (A) Represents the positive control of untreated *S. mutans* cells. The black color indicates that biofilm has been formed. **(B)** Represents the negative control, which indicates no biofilm formation has occurred.

| 0.5 hrs 100 ug/mL LTP	0.5 hrs 100 ug/ mL LTP	0.5 hrs 100 ug/ mL LTP	0.5 hrs 50 ug/ mL LTP	0.5 hrs 50 ug/ mL LTP	0.5 hrs 50 ug/ mL LTP
1.0 hrs 100 ug/ mL LTP	1.0 hrs 100 ug/mL LTP	1.0 hrs 100 ug/ mL LTP	1.0 hrs 50 ug/ mL LTP	1.0 hrs 50 ug/ mL LTP	1.0 hrs 50 ug/ mL LTP
2.0 hrs 100 ug/ mL LTP	2.0 hrs 100 ug/ mL LTP	2.0 hrs 100 ug/ mL LTP	2.0 hrs 50 ug/ mL LTP	2.0 hrs 50 ug/mL LTP	2.0 hrs 50 ug/ mL LTP
4.0 hrs 100 ug/ mL LTP	4.0 hrs 100 ug/ mL LTP	4.0 hrs 100 ug/ mL LTP	4.0 hrs 50 ug/ mL LTP	4.0 hrs 50 ug/ mL LTP	4.0 hrs 50 ug/ mL LTP

Figure 28. The template for the Congo Red assay for LTP concentrations 50 and 100ug/mL
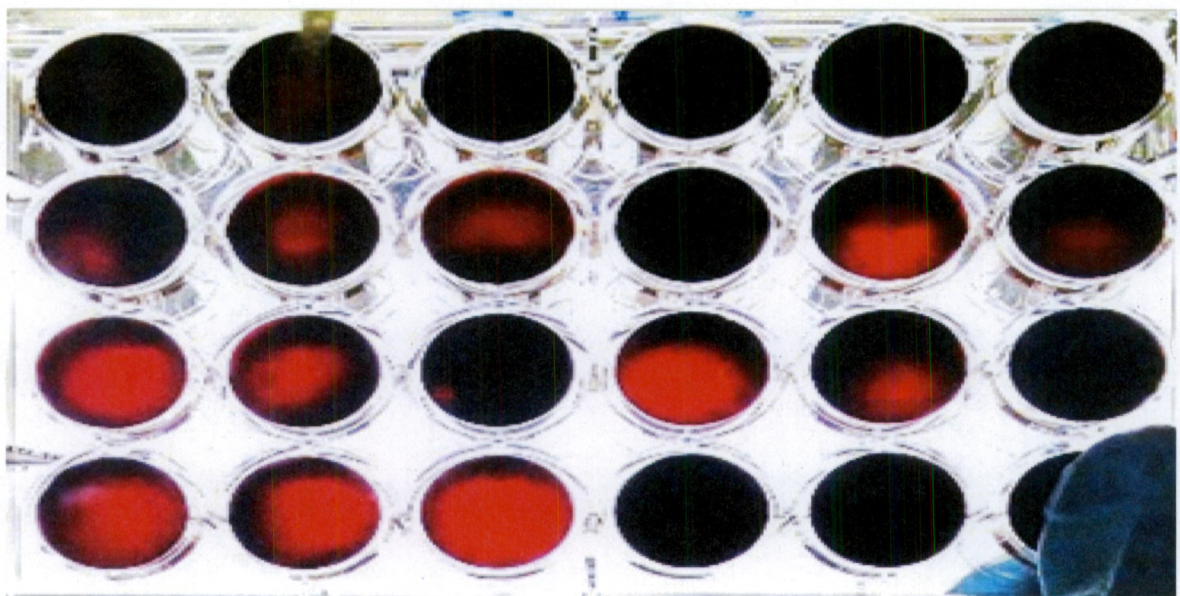
Figure 29. The Congo Red results for LTP 50 and 100ug/mL following the template in Figure 28.

| 0.5 hrs 100 ug/ mL EGCG-S	0.5 hrs 100 ug/ mL EGCG-S	0.5 hrs 100 ug/ mL EGCG-S 0	0.5 hrs 50 ug/ mL EGCG-S	0.5 hrs 50 ug/ mL EGCG-S	0.5 hrs 50 ug/ mL EGCG-S
1.0 hrs 100 ug/ mL EGCG-S	1.0 hrs 100 ug/ mL EGCG-S	1.0 hrs 100 ug/ mL EGCG-S 0	1.0 hrs 50 ug/ mL EGCG-S	1.0 hrs 50 ug/ mL EGCG-S	1.0 hrs 50 ug/ mL EGCG-S 0
2.0 hrs 100 ug/ mL EGCG-S	2.0 hrs 100 ug/ mL EGCG-S	2.0 hrs 100 ug/ mL EGCG-S 0	2.0 hrs 50 ug/ mL EGCG-S	2.0 hrs 50 ug/ mL EGCG-S	2.0 hrs 50 ug/ mL EGCG-S 0
4.0 hrs 100 ug/ mL EGCG-S	4.0 hrs 100 ug/ mL EGCG-S	4.0 hrs 100 ug/ mL EGCG-S 0	4.0 hrs 50 ug/ mL EGCG-S	4.0 hrs 50 ug/ mL EGCG-S	4.0 hrs 50 ug/ mL EGCG-S 0

Figure 30. The template for the Congo Red assay for EGCG-S concentrations 50 and 100ug/ mL
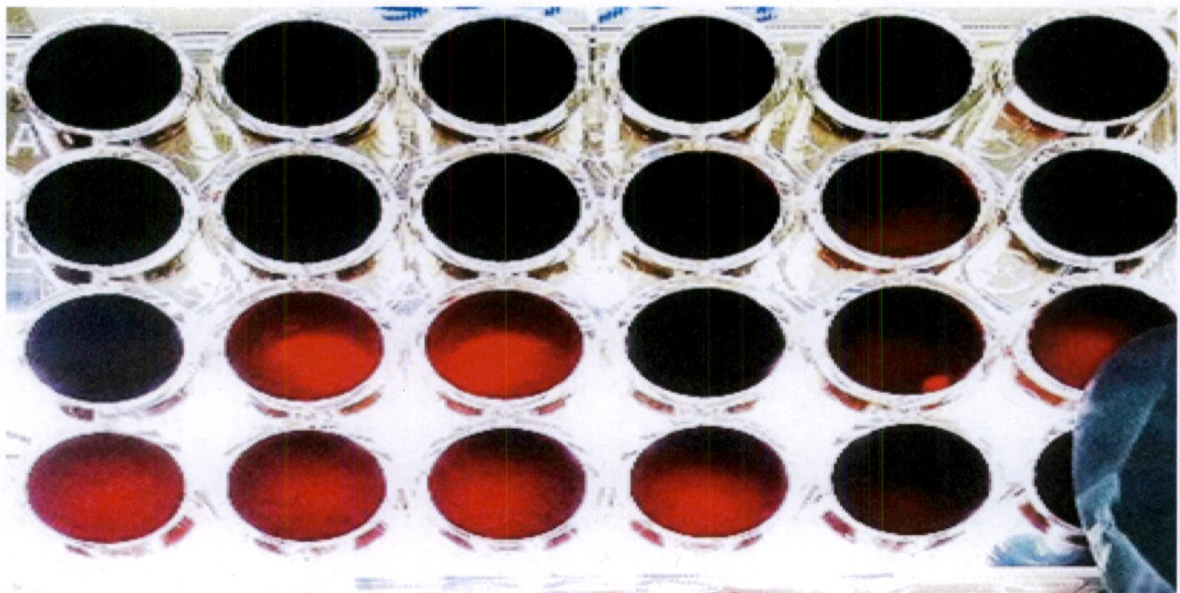
Figure 31. The Congo Red results for EGCG-S 50 and 100ug/ mL following the template in Figure 30.

2.0 hrs 150 ug/ mL LTP	2.0 hrs 150 ug/ mL LTP	2.0 hrs 150 ug/ mL LTP	Positive Control	Positive Control	Positive Control
4.0 hrs 150 ug/ mL LTP	4.0 hrs 150 ug/ mL LTP	4.0 hrs 150 ug/ mL LTP	2.0 hrs 150 ug/ mL EGCG-S	2.0 hrs 150 ug/ mL EGCG-S	2.0 hrs 150 ug/ mL EGCG-S 0
2.0 hrs 200 ug/ mL LTP	2.0 hrs 200 ug/ mL LTP	2.0 hrs 200 ug/ mL LTP 0	2.0 hrs 200 ug/ mL EGCG-S	4.0 hrs 150 ug/ mL EGCG-S	4.0 hrs 150 ug/ mL EGCG-S 0
4.0 hrs 200 ug/ mL LTP	4.0 hrs 200 ug/ mL LTP	4.0 hrs 200 ug/ mL LTP	2.0 hrs 200 ug/ mL EGCG-S	4.0 hrs 200 ug/ mL EGCG-S	4.0 hrs 200 ug/ mL EGCG-S 0

Figure 32. The template for the Congo Red assay for EGCG-S concentrations 150 and 200ug/ mL
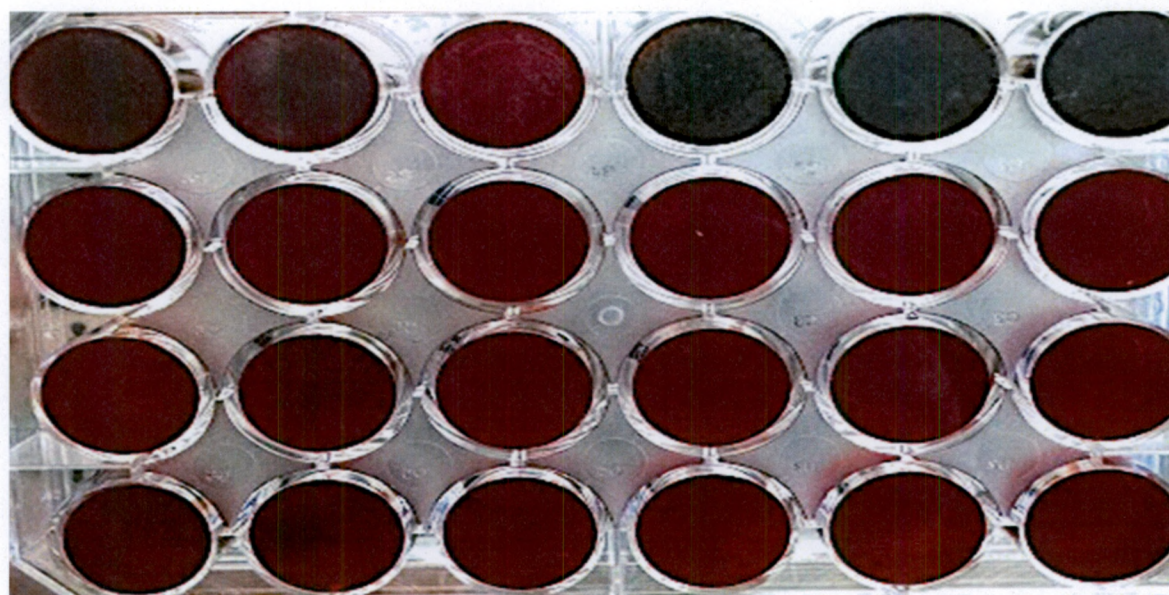
Figure 33. The Congo Red results for LTP 50 and 100ug/ mL following the template in Figure 32.

	Time (hrs)	50ug/mL	100ug/mL	150ug/mL	200ug/mL
EGCG-S	2				
	4				
LTP	2				
	4				

Figure 34. The summary of the Congo Red Assay results that qualitatively analyzes the inhibition of biofilm in *S. mutans* by tea polyphenols at various concentrations and time intervals.
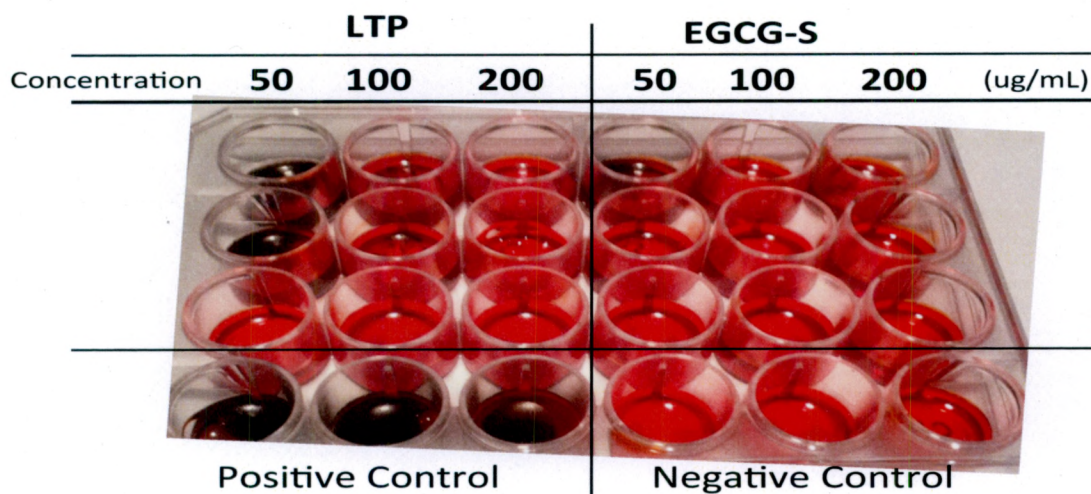
Figure 35. An anterior view of the 24 well plate used in the Congo Red Assay. The black color in the positive control indicates that biofilm was formed. The negative control's red color represents the absence of biofilm.

c. Quantitative Analysis Using Crystal Violet Assay

The crystal violet assay was used to quantitatively determine how much biofilm was inhibited by the different treatments of tea polyphenols. The Congo red experiment was used first to qualitatively confirm the presence of biofilm, but often it does not reflect the actual amount of biofilm present. The crystal violet assay eliminates this uncertainty through quantitative analysis. Examples of a positive and negative control for the crystal violet assay are shown in Figure 36. The results below include *S. mutans* incubated with 2 different green tea polyphenols, LTP and EGCG-S. The concentrations for both green tea polyphenols tested in this experiment were 25ug/ mL, 50ug/ mL, 100ug/ mL, 200ug/ mL, and 250ug/ mL. The results in Tables 3-4 and Figure 37 indicate that when *S. mutans* was treated with either green tea polyphenol tested in this experiment, at all concentrations, a percent inhibition between 90.7-100% was reached. While this experiment indicates that lower concentrations of green tea polyphenols are also effective, it also demonstrates that the highest concentration tested, 250ug/ mL, gave the best results for both polyphenols (Figure 37). EGCG-S 250ug/ mL had 100% inhibition, while LTP had 99.4%. Because of the impressive results at 250ug/ mL, this concentration was used for all other experiments.
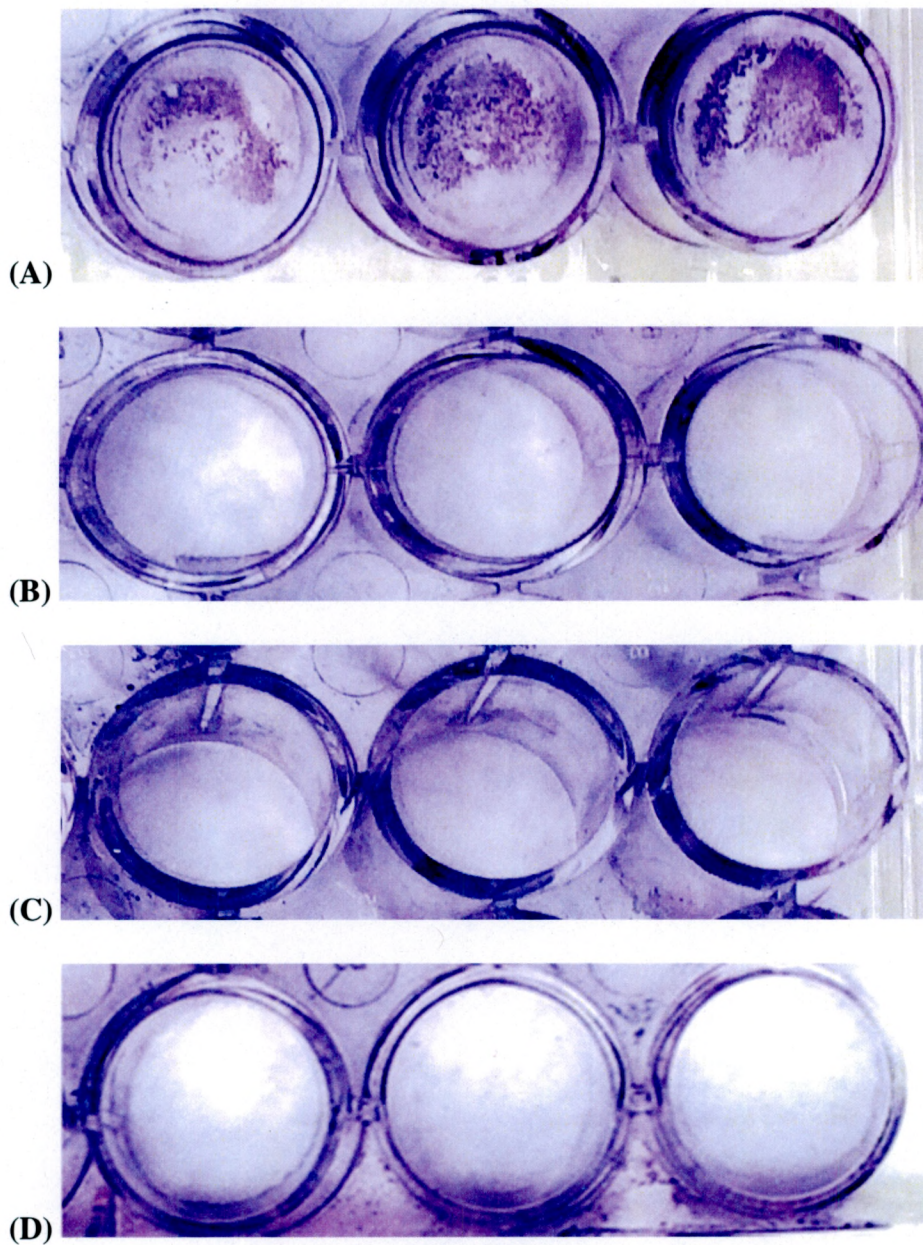
Figure 36. Crystal Violet Assay: (A) The positive control displays the development of biofilm by *Streptococcus mutans* on the bottom of a 24 well plate. (B) The negative control shows what the bottom of the plate looks like when exposed to nutrient broth without any bacteria present. When treated with EGCG-S 250ug/ mL (C), no biofilm is present, while LTP250ug/mL(D) produced minimal biofilm.

Treatment with EGCG-S		
Concentration	% Inhibition	Standard Deviation
50	98.52315854	0.9772
100	100	0.084787971
200	92.749	0.029
250	100	0.047444705

Table 3. Crystal Violet Assay analysis on the effect of various concentrations of EGCG-S on biofilm inhibition in *Streptococcus mutans*. Results were averages of triplicate measures.

Treatment with LTP		
Concentration	% Inhibition	Standard Deviation
100	97.16811845	1.233
200	90.717	0.034268547
250	99.4198	0.104241706

Table 4. Crystal Violet Assay analysis on the effect of various concentrations of LTP on biofilm inhibition in *Streptococcus mutans*. Results were averages of triplicate measures.
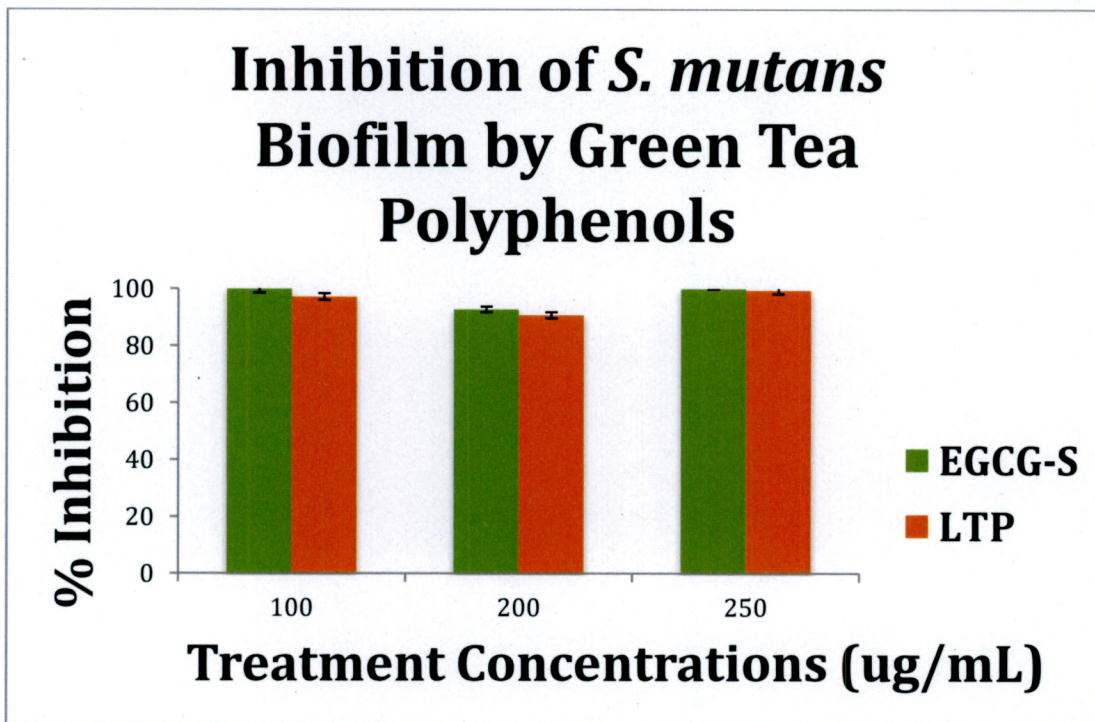
Figure 37. Crystal Violet Assay graphical analysis from tables 3 and 4. Both tea polyphenols demonstrate an excellent inhibitory effect by having over 90% inhibition at every concentration of tea polyphenol.

5. Possible Synergistic Effect of Green Tea Polyphenols with Oral Care Products

a. Disk Diffusion

Before beginning a synergistic study, the effectiveness of current oral care products was tested via the disk diffusion experiment. Five common over the counter mouthwashes and one prescription mouthwash were utilized for this experiment and the next three. Table 5 lists the mouthwash brands used. Agar plates were continuously streaked with *S. mutans* and then a disk soaked in a single mouthwash was placed on top of the bacteria. After the 24 hour incubation period, the edge of the disk to the edge of the clearing zone was measured in millimeters (mm) as defined as Zone of inhibition (ZOI) (Lee et al., 2015). Because the clearing zones are not always perfectly circular, the average of four measurements per disk was utilized as the final clearing zone.

After observing the effectiveness of the mouthwashes alone, the next step was to add in green tea polyphenols to the mouthwashes to see if either polyphenol enhances the mouthwash's ability to prevent bacterial growth. Table 6 lists the clearing zone in mm for the mouthwash alone, mouthwash enhanced with 250ug/ mL EGCG-S, and mouthwash enhanced with 250ug/ mL LTP. These measurements were taken on plates as pictured in Figures 38-39. From these values, the percent of increased inhibition was calculated. For all of the mouthwash brands, except scope, 250 ug/ mL EGCG-S was more effective than 250 ug/ mL LTP. Listerine demonstrated the most need for antibacterial additives since Listerine alone displayed a very minimal clearing zone of 0.255mm. When enhanced with EGCG-S and LTP, the percent inhibition jumped to 96% and 95%

respectively. Cepacol and crest provided similar results in that the treatment with EGCG-S was 64% and 69% respectively. When the same mouthwashes were treated with LTP, the percent inhibitions decreased to 11% and 20% respectively. The prescription mouthwash had the second highest clearing zone alone, and when enhanced with EGCG-S or LTP its percent inhibition increased to 80% and 68% respectively. Both green tea polyphenols were effective in the prescription brand, so further testing needs to be done to distinguish which one would be more beneficial to this particular mouthwash. Colgate mouthwash had the highest clearing zone value of all the mouthwashes before adding any polyphenols. The percent inhibitions were not significant at 40% for EGCG-S and only 5% for LTP. In this case, Colgate mouthwash may already have a lot of antibacterial properties than the addition of the polyphenols was not that helpful.

Mouthwash Brand	Flavor	Active Ingredients	Inactive Ingredients
Listerine (Antiseptic)	Cool Mint	Eucalyptol 0.092%, Menthol 0.042%, Methyl salicylate 0.060%, Thymol 0.064%	Water, alcohol (21.6%), sorbitol solution Flavoring, poloxamer 407, benzoic acid, sodium saccharin, sodium benzoate, FD&C Green No. 3
Cepacol (Antibacterial)	Cinnamon, eucalyptol and mint flavor	Cetylpyridinium chloride 0.05% (Ceepryn ®)	Purified water, alcohol 14% v/v, glycerin, sodium phosphate dibasic, eucalyptus oil, polysorbate 80, methyl salicylate, cinnamon oil, peppermint oil, saccharin sodium, sodium phosphate monobasic anhydrous, menthol, edetate disodium, FD&C Yellow #5
Scope Classic	Original Mint	Cetylpyridinium Chloride	Water , Alcohol , Glycerin , Flavor , Polysorbate 80 , Sodium Saccharin , Sodium Benzoate , Benzoic Acid , Blue 1 , Yellow 5
Crest	Refreshing Clean Mint	Cetylpyridinium chloride 0.07% (Ceepryn ®)	water, glycerin, flavor, poloxamer 407, methylparaben, sodium saccharin, propylparaben, blue 1
Chlorohexidine Gluconate 0.12% Oral Rinse	Spearmint	Chlorohexidine Gluconate (1,1'-hexamethylene bis [5-(p-chlorophenyl) biguanide]di-D-gluconate)	water, 11 .6% alcohol, glycerin, PEG-40 sorbitan diisostearate, flavor, sodium saccharin, and FD&C Blue No. 1
Colgate Total Gum Health	Clean Mint	Cetylpyridinium chloride 0.075% (Ceepryn ®)	Ater, glycerin, propylene glycol, sorbitol, poloxamer 407, flavor, potassium sorbate, citric acid, sodium saccharin, sucralose, FD&C green no. 3

Table 5. List of all mouthwashes used including their flavors and active/inactive ingredients
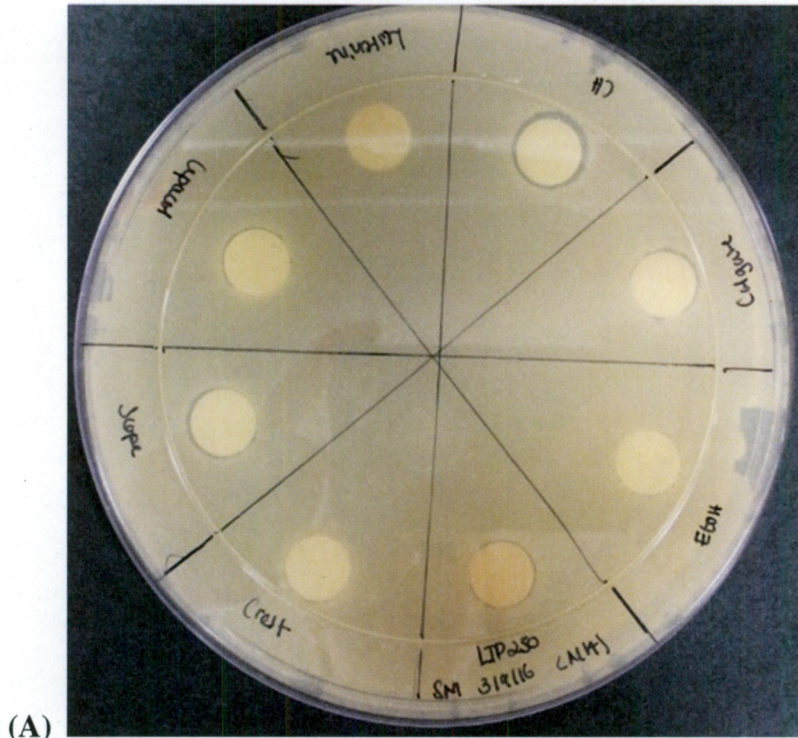
(A)
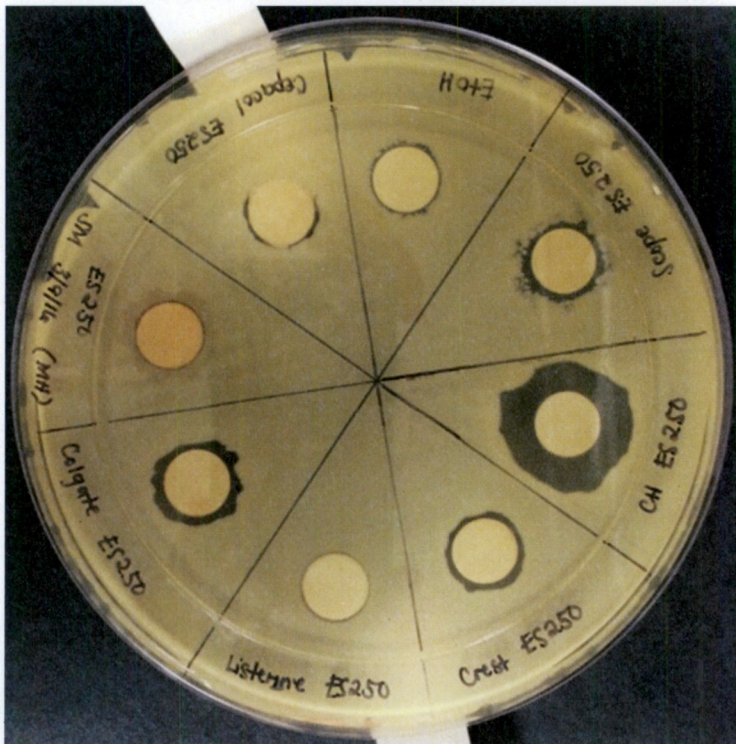
(B)

Figure 38. Disk Diffusion Experiment was carried out on Muller-Hinton agar plates streaked with *Streptococcus mutans* shown above. Disks were soaked in various mouthwash treatments and placed on top of the bacteria plate. After a 24 hour incubation period at 37°C, the zone of inhibition was measured. Figure (A) shows the ineffectiveness of disks soaked in common mouthwashes alone. Figure (B) shows an increase in the zone of inhibition when EGCG-S 250ug/mL is added to the common mouthwashes.
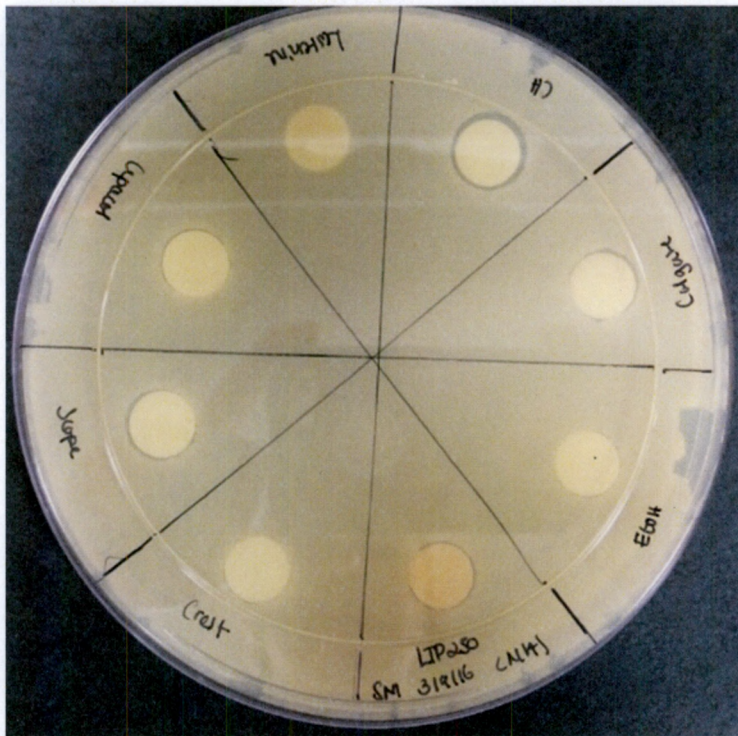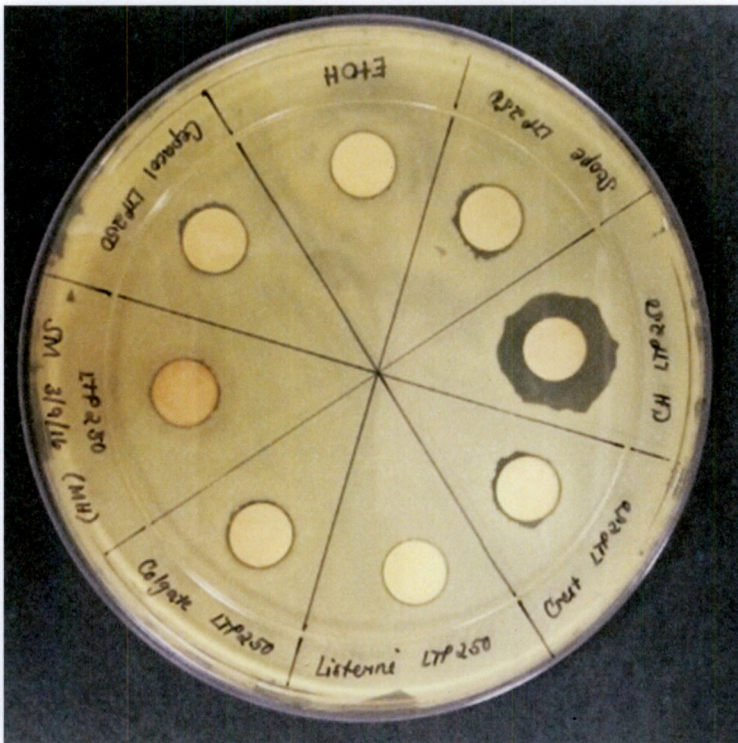
Figure 39. Figure (A) shows the ineffectiveness of disks soaked in common mouthwashes alone. Figure (B) shows a slight increase in the zone of inhibition when LTP 250ug/mL is added to the common mouthwashes.

ES 250 Combinations	Mouthwash alone	Combination	% Increased Inhibition	LTP 250 Combinations	Mouthwash alone	Combination	% Increased Inhibition
Listerine	0.255	6.75	96%	Listerine	0.255	5.25	95%
Cepacol	1	2.75	64%	Cepacol	1	1.125	11%
Scope	1.5	2	25%	Scope	1.5	4.125	64%
Crest	1	3.25	69%	Crest	1	1.25	20%
CH	1.75	8.75	80%	CH	1.75	5.5	68%
Colgate	2.25	3.75	40%	Colgate	2.25	2.375	5%
ES 250	2.25			LTP 250	0.1		

Table 6. The measurements for Zone of Inhibition (ZOI) taken during the disk diffusion experiment in millimeters (mm). From these measurements the percentage of increased inhibition was calculated.
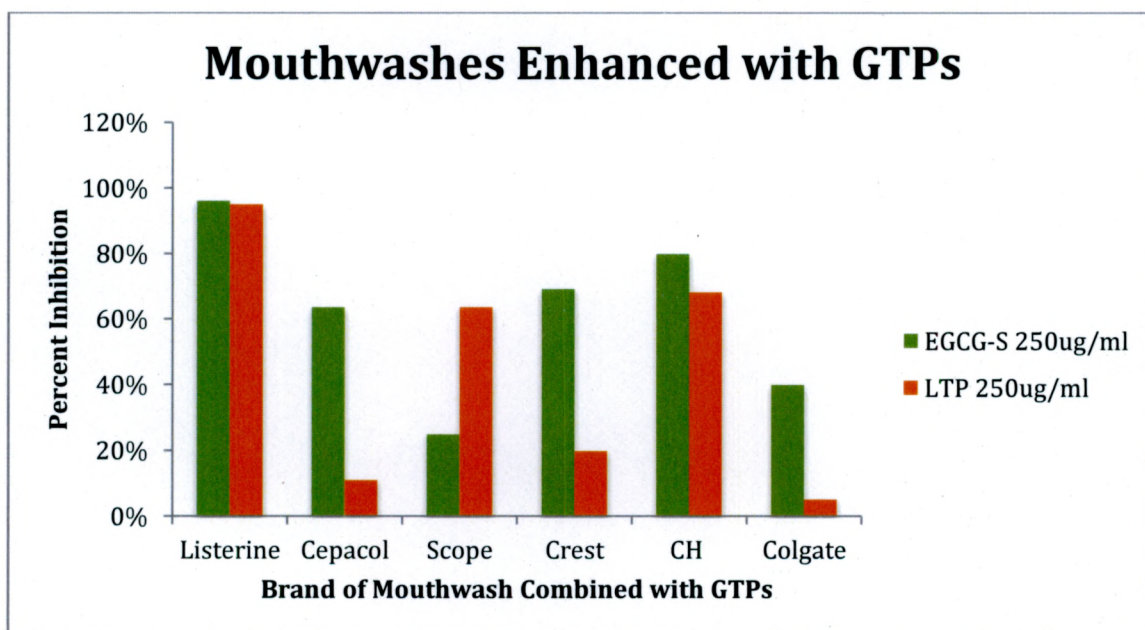
Figure 40. From the measurements shown in Table 6, the percent inhibitions are graphically represented in the figure above. EGCG-S 250ug/mL is more effective than LTP 250ug/mL when combined with all common mouthwashes, except scope.

b. Study the effect of mouthwash and tea polyphenols alone or in combination on the growth of *S. mutans*

Monitoring the effect of the combination of mouthwash and tea on the growth of *S. mutans* was done by using a 96 well plate assay. The growth was monitored over 16 hours and readings were taken at an optical density of 650nm every hour for the first 10 hours and a final reading at the 16th hour. This study was done in triplicate and the results are shown in mean and SD in Figure 41-42.

Listerine (Figure 41) mouthwash alone was not effective long term. At 15 hours, its OD_{650} reading is almost as high as the control. Both EGCG-S and LTP alone were effective in reducing the amount of bacterial growth consistently over the 15 hour period, however the combination of EGCG-S and Listerine was the most effective. The LTP and Listerine combination was not effective. It is shown that initially this treatment increased the amount of growth for the first 6 hours before any reduction was seen as seen in Figure 41.

Cepacol mouthwash alone displayed the worst effectiveness (Figure 41) of all the mouthwashes tested in this experiment by having a much higher OD_{650} reading than the control. Similar to Listerine, the most effective treatment involving Cepacol was when it was in combination with EGCG-S. Both polyphenols alone also posted similar results to the Cepacol and EGCG-S combination. The LTP and Cepacol combination was also analogous to that of Listerine by increasing growth for the first six hours before a reduction was seen.

All treatments for Scope mouthwash (Figure 41) worked similarly for the first 8 hours, except for the LTP and Scope combination, which resulted in higher

OD_{650} readings. EGCG-S, LTP, and EGCG-S and Scope combination had the most effective results for the 15-hour experiment. Scope alone had the highest OD_{650} reading at the end of the 15 hours, however for the first 8 hours it was posting similar readings as all of the effective polyphenol treatments.

For Crest mouthwash (Figure 42), the most effective treatments were the polyphenols alone, followed by the combination of EGCG-S and Crest. Crest alone was effective after 6 hours, but still not as efficient as the polyphenols or the EGCG-S and Crest combination. The LTP and Crest combination was not successful at all until hour 9, but the final OD_{650} reading at the 15th hour increased almost as high as the control.

Chlorohexidine Gluconate, the only prescription mouthwash tested, was very effective on its own and had the lowest OD_{650} reading at the final reading, as seen in Figure 42. This was the only mouthwash when combined with EGCG-S was not more effective than the mouthwash alone. However, EGCG-S and LTP alone were more efficient than Chlorohexidine Gluconate alone for the first 8 hours. Similar to the other types of mouthwashes, the LTP and Chlorohexidine Gluconate combination was the least effective.

As seen in Figure 42, all alternative treatments to Colgate mouthwash were more effective than Colgate itself. EGCG-S, LTP, and the EGCG-S and Colgate combination all had similar OD_{650} readings and are the most efficient in reducing bacterial growth. The LTP and Colgate combination worked better than Colgate alone, but was not as successful as either polyphenol or the EGCG-S Colgate combination.

In summary, the prescription mouthwash, Chlorohexidine Gluconate, alone was able to completely inhibit bacterial growth. The next best mouthwashes alone were Scope and Colgate which were able to severely inhibit the growth of the bacteria. Some growth was observed in the Crest treated bacteria. Listerine was not able to efficiently inhibit the growth of bacteria. Approximately 50% of growth was observed when compared with the control. Cepacol have no effect on the growth of *S. mutans*.

In the combination study, EGCG-S was able to completely inhibit the growth of the bacteria in combination with Listerine, Cepacol, Colgate, and Scope. LTP worked very well with Colgate, Scope and Cepacol, but does not have any synergistic effect on Chlorohexidine Gluconate or Crest. LTP worked to a certain extent in combination with Listerine.

This study suggests that EGCG-S is a better candidate as a synergistic agent for mouthwashes.
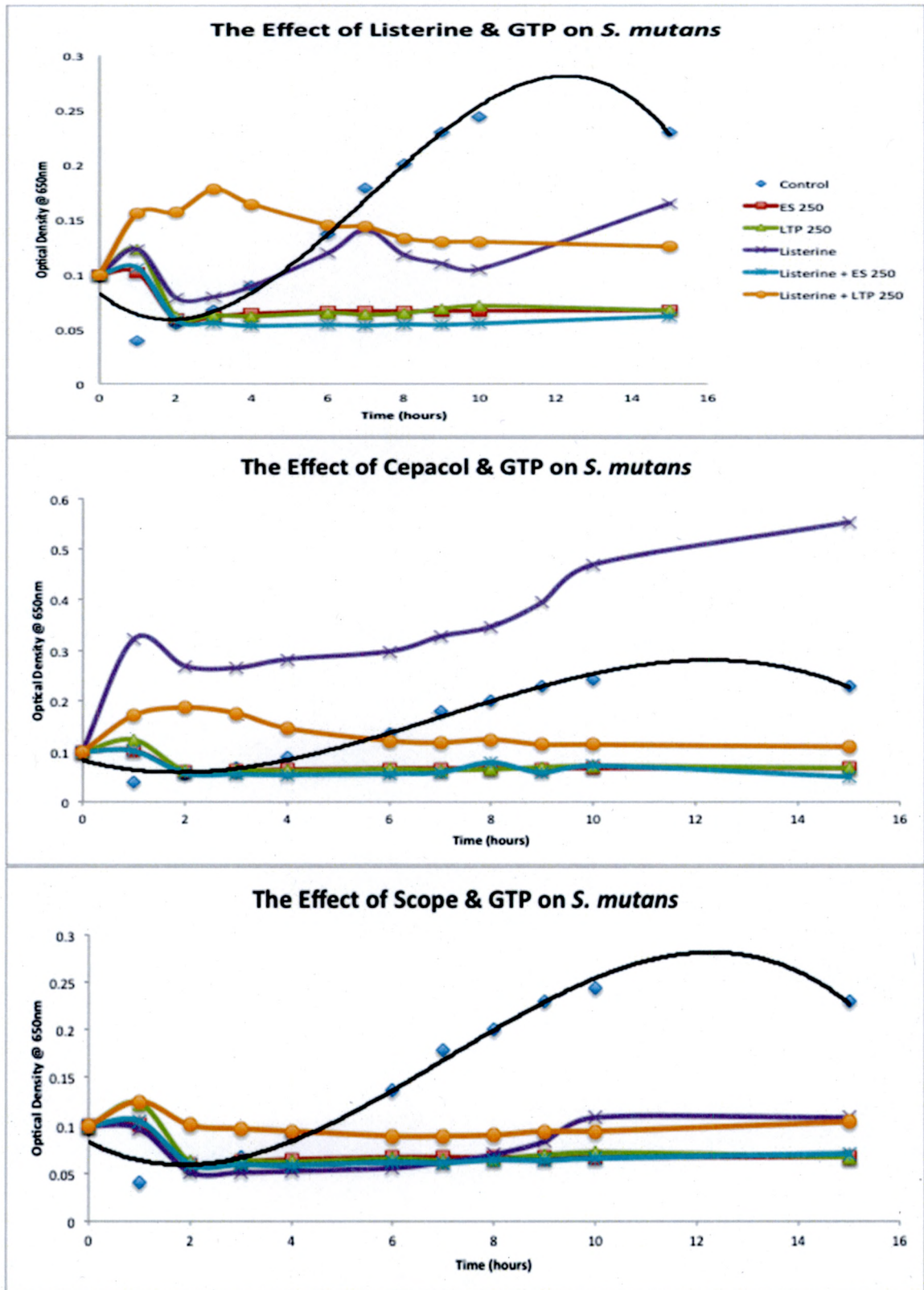
Figure 41. A 96 well micro-titer plate assay demonstrating the effectiveness of various mouthwash treatments against *S. mutans*.
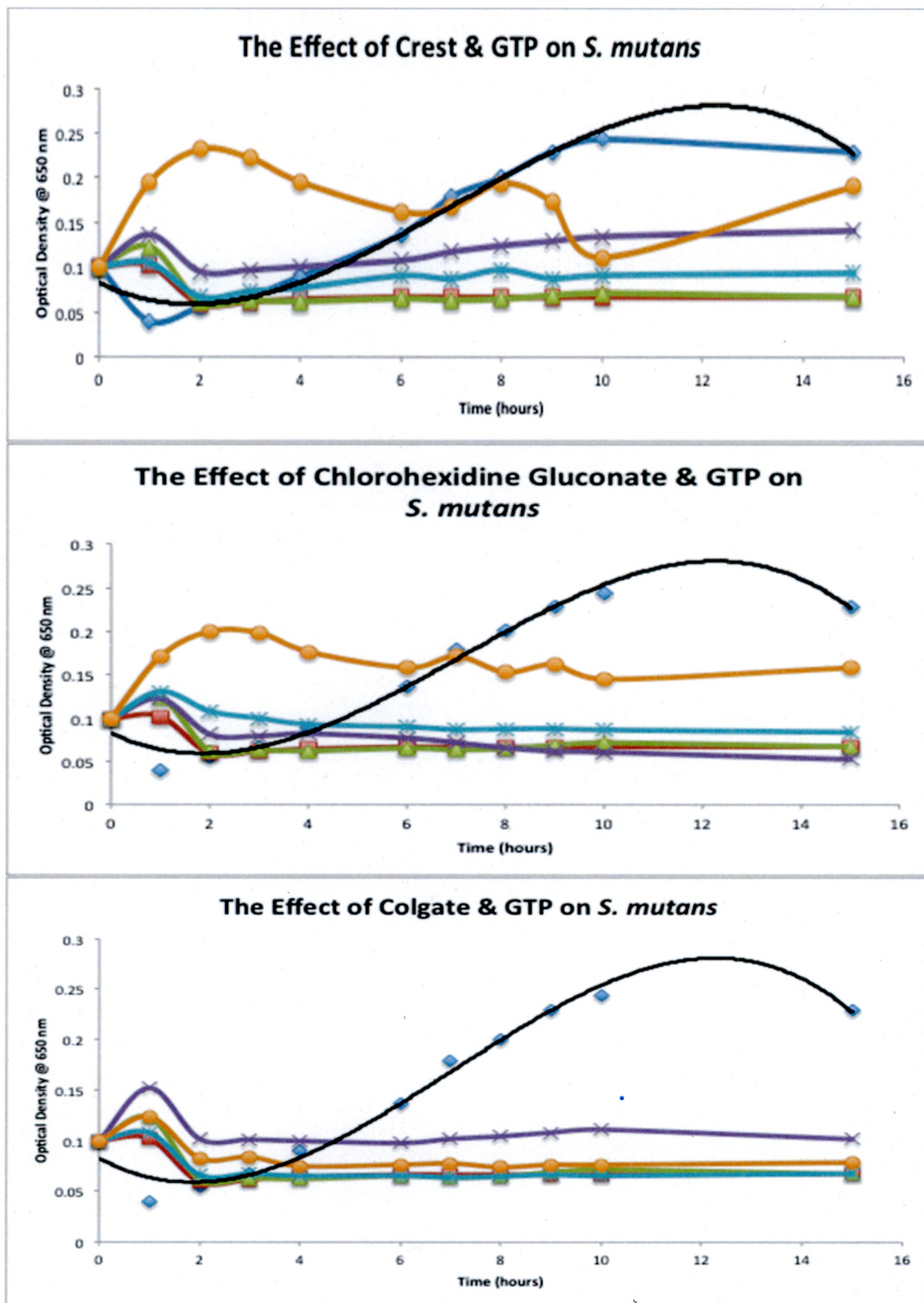
Figure 42. A 96 well micro-titer plate assay demonstrating the effectiveness of various mouthwash treatments against *S. mutans*.

c. Time Course Study of Mouthwash and Tea Polyphenols alone or in combination

To determine when each treatment has the highest reduction of bacterial growth, a Time Kill study was carried out using CFU. Bacteria were grown overnight, and then reduced to an OD_{650} of 0.1. Different treatments were added to the microcentrifuge tubes and samples were taken out and plated at the following time intervals: 5(s), 10(s), 15(s), 30(s), 1(min), 5(min), 1(hr), and 24(hr). Plates were incubated at 37°C for 24 hours. After the incubation period, colonies forming units were counted to determine which treatment method worked best, the mouthwash alone or the mouthwash in combination with EGCG-S 250ug/mL. EGCG-S 250ug/mL alone was efficient in reducing all colony forming units at every time interval tested. As shown in Tables 7-8, Listerine and Cepacol both demonstrated that the addition of EGCG-S helped in reducing the number of colonies formed. The EGCG-S and Scope mixture reduced the CFU to 0 at all time intervals as well, but Scope alone was also fairly effective with only three and two CFU at 15(s) and 30(s) respectively, before going to 0 CFU for all time periods after. Chlorohexidine Gluconate was similar to scope in that at 5(s) and 15(s) it had six and four colonies, respectively, but when in combination with EGCG-S no colonies were formed at any time interval. In agreement with the 96 well micro-titer plate assay, the EGCG-S and Crest combination may not be the most effective treatment because colonies were forming and disappearing inconsistently throughout the experiment. Colgate alone was efficient for 30(s), but at 1 minute (the suggested amount of time you use mouthwash) eight colonies

were formed. When Colgate was combined with EGCG-S, only one colony was formed at 15(s), while no other colonies grew at any other time interval, which suggests that the combination of Colgate and EGCG-S is more effective than Colgate alone.

Time Kill Study on *S. mutans*								
Mouthwash	5s	10s	15s	30s	1 min	5 min	1hr	24hr
S. mutans 10^{-8}	9×10^9	9.6×10^9	1.09×10^{10}	2×10^{10}	2.53×10^{10}	-	3.76×10^{10}	-
EGCG-S 250ug/mL	0	0	0	0	0	0	0	0
Listerine	26	43	5	1	3	0	0	0
Listerine + EGCG-S	1	9	0	1	0	0	0	0
Cepacol	-	-	41	25	14	0	0	0
Cepacol+ EGCG-S	-	-	0	0	0	0	0	0
Scope	-	-	3	2	0	0	0	0
Scope+EGCG-S	-	-	0	0	0	0	0	0
Crest	1	1	0	0	7	0	0	0
Crest+ EGCG-S	1	0	0	0	1	0	0	0
Chlorohexidine Gluconate	6	0	4	0	0	0	0	0
Chlorohexidine Gluconate + EGCG-S	0	0	0	0	0	0	0	0
Colgate	0	0	0	8	0	4	0	0
Colgate + EGCG-S	0	0	1	0	0	0	0	0

Table 7. Time Kill Experiment assesses the amount of Colony Forming Units (CFUs) when exposed to various mouthwash treatments at different time periods.

Time it Takes Treatment to Reach 100% Inhibition	
Mouthwash	**Time**
EGCG-S 250ug/mL	<5s
Listerine	5min
Listerine + EGCG-S	1min
Cepacol	5min
Cepacol+ EGCG-S	15s
Scope	1min
Scope+EGCG-S	15s
Crest	5min
Crest+ EGCG-S	5min
Chlorohexidine Gluconate	30s
Chlorohexidine Gluconate + EGCG-S	5s
Colgate	1hr
Colgate + EGCG-S	30s

Table 8. The amount of time it takes for mouthwash treatments to be completely effective. When combined with EGCG-S, the time it takes for a mouthwash to be effective was significantly reduced in almost all cases.
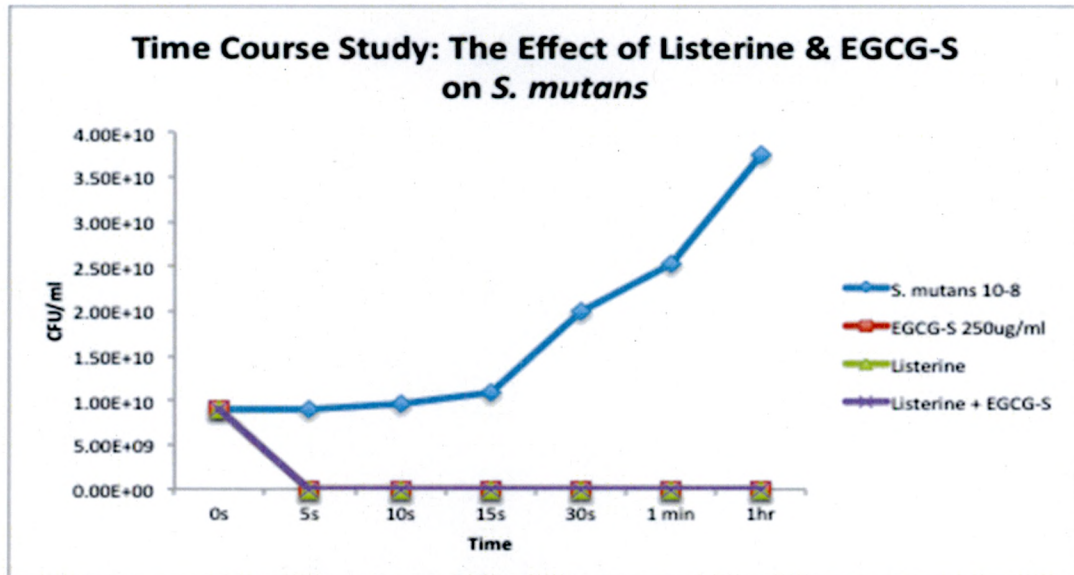
Figure 43. Time course study demonstrating the amount of time it takes EGCG-S, Listerine and the combination of EGCG-S and Listerine to completely inhibit the growth of *S. mutans*.
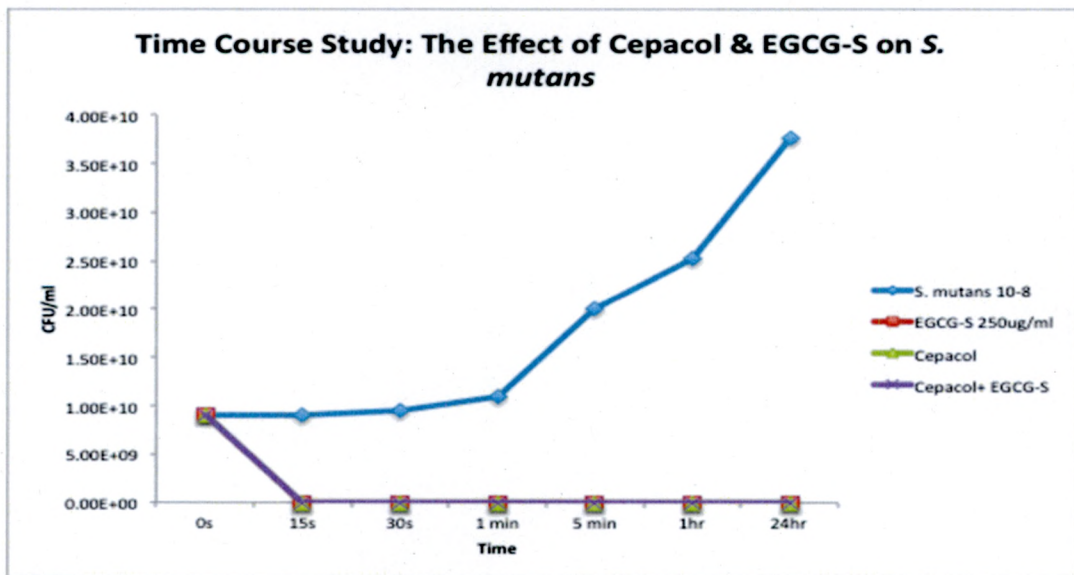
Figure 44. Time course study demonstrating the amount of time it takes EGCG-S, Cepacol and the combination of EGCG-S and Cepacol to completely inhibit the growth of *S. mutans*.

d. Live/ Dead

Cell viability of *Streptococcus mutans* was assessed the same way as previously stated through the Live and Dead BacLight ® Assay Kit. The bacteria were pelleted down and resuspended in either a mouthwash or a combination of EGCG-S and mouthwash for 1 minute before being placed onto a cover glass and exposed to both the Syto®9 and propidium iodide dyes. After the samples dried, they were observed under a fluorescent microscope to examine cell viability. The results are shown in Figures 45-50. The control group fluoresces green, representing that all the cells are alive. As previously shown, when the cells are treated with EGCG-S 250ug/mL all of the cells turn red, demonstrating that the cell membrane has been destroyed, thus the cells are considered dead. Listerine mouthwash alone shows mostly green cells, with a few dead cells. When Listerine is combined with EGCG-S 250ug/mL, the number of dead cells increases, while the number of alive cells drops. This agrees with our previous experiments in that an EGCG-S and Listerine combination is more effective than Listerine alone. Cepacol mouthwash presents a lot of alive cells, with very few dead cells. When EGCG-S 250ug/mL is added to Cepacol, all of the cells present are dead. Scope mouthwash alone shows a large amount of alive cells, with almost no dead cells present. The combination of EGCG-S and Scope still displays a large amount of alive cells, however there is a lot more dead cells present. Surprisingly, Crest mouthwash alone did not show any dead cells at all. With the combination of EGCG-S and Crest, all cells present fluoresced red, indicating all the cells have died. The prescription mouthwash, Chlorohexidine Gluconate, fluoresced a

seemingly equal ratio of alive to dead cells, while when combined with EGCG-S, more dead cells were visible than alive. Finally, Colgate mouthwash alone had very few dead cells, but when combined with EGCG-S all visible cells were dead.
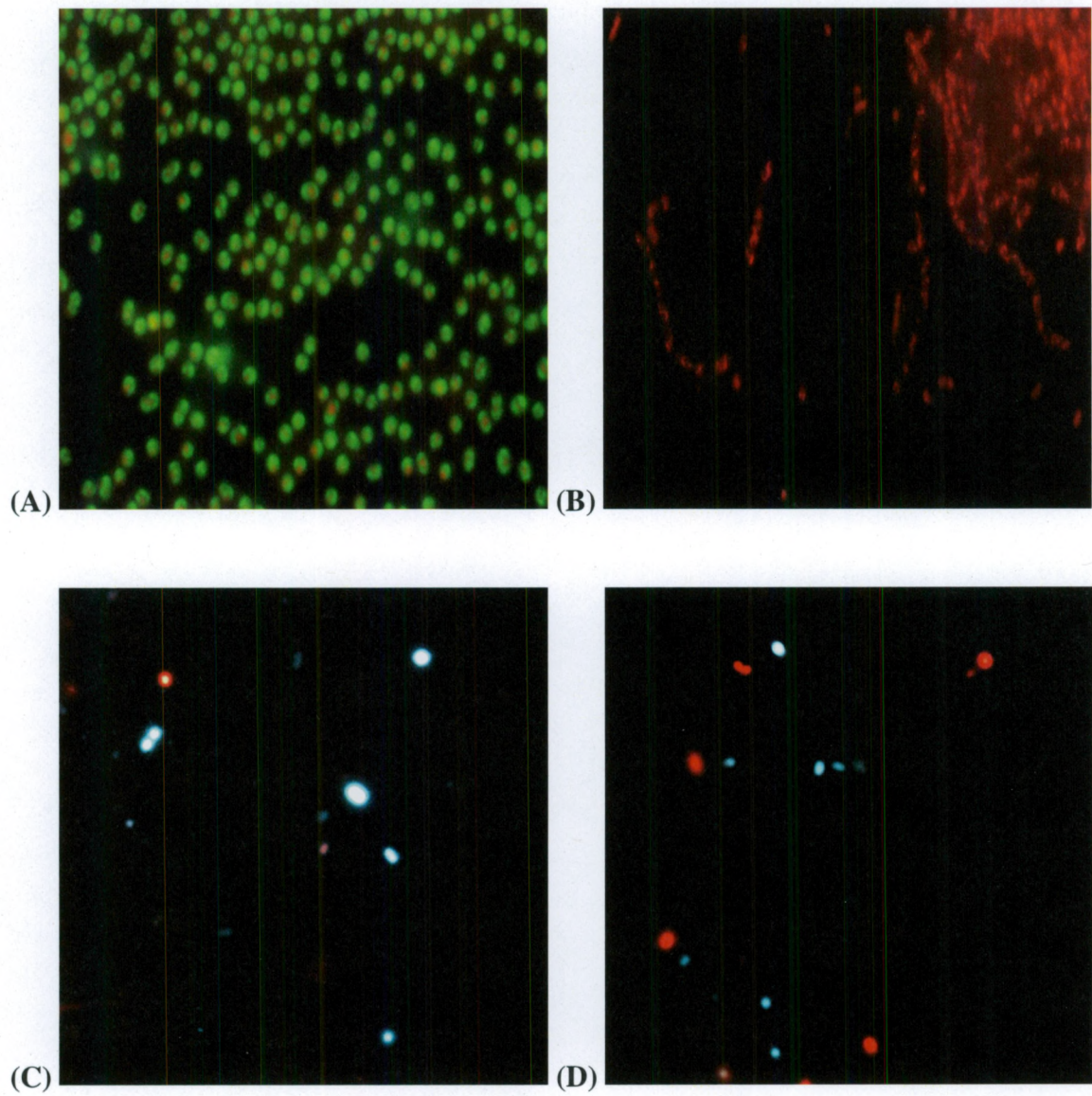
Figure 45. Live/ Dead qualitative analysis. (A) The untreated control group. (B) *S. mutans* treated with EGCG-S 250ug/mL. (C) *S. mutans* treated with Listerine mouthwash. (D) *S. mutans* treated with a combination of Listerine and EGCG-S 250ug/mL.
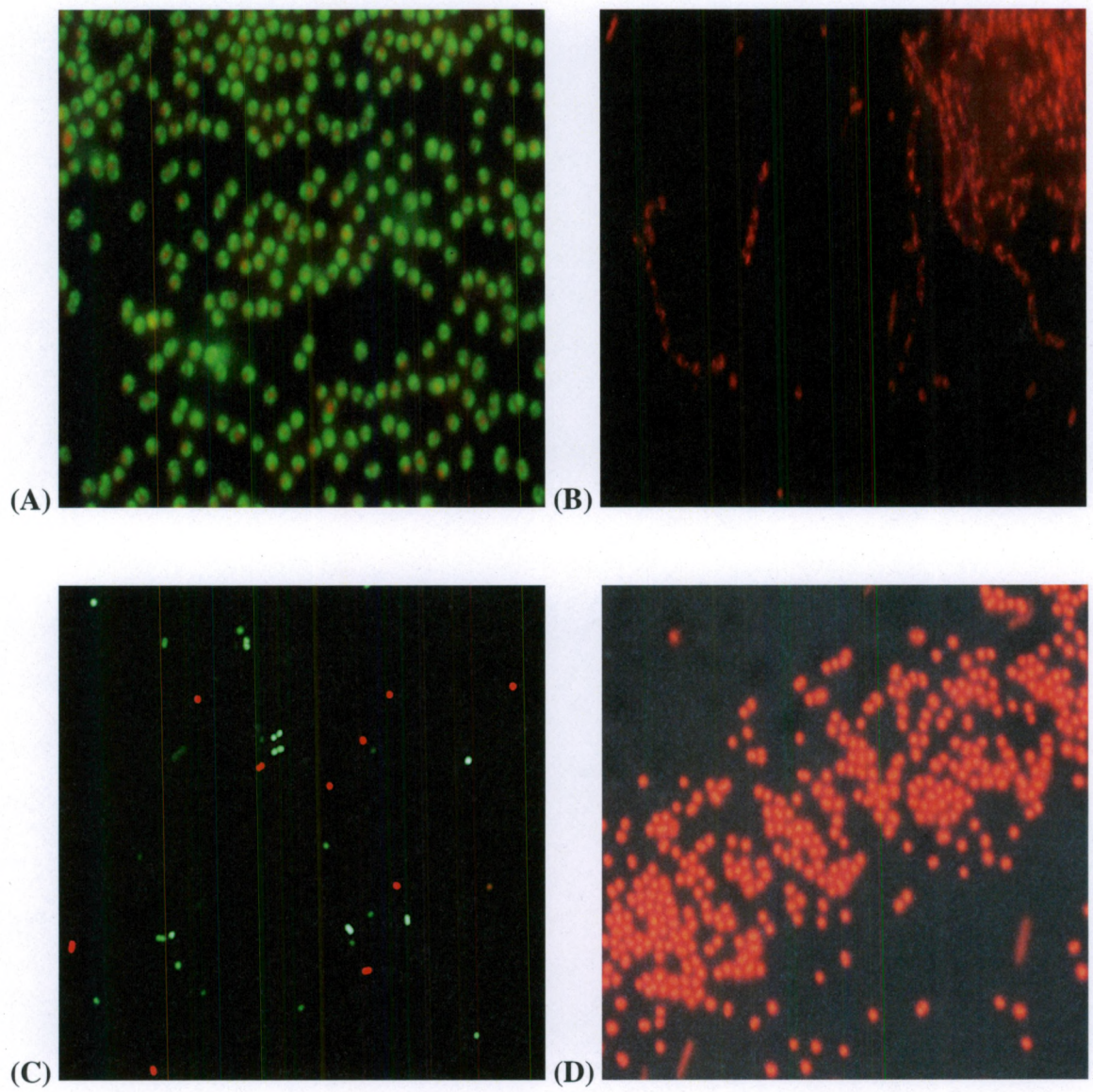
Figure 46. Live/ Dead qualitative analysis. (A) The untreated control group. (B) *S. mutans* treated with EGCG-S 250ug/mL. (C) *S. mutans* treated with Cepacol mouthwash. (D) *S. mutans* treated with a combination of Cepacol and EGCG-S 250ug/mL.
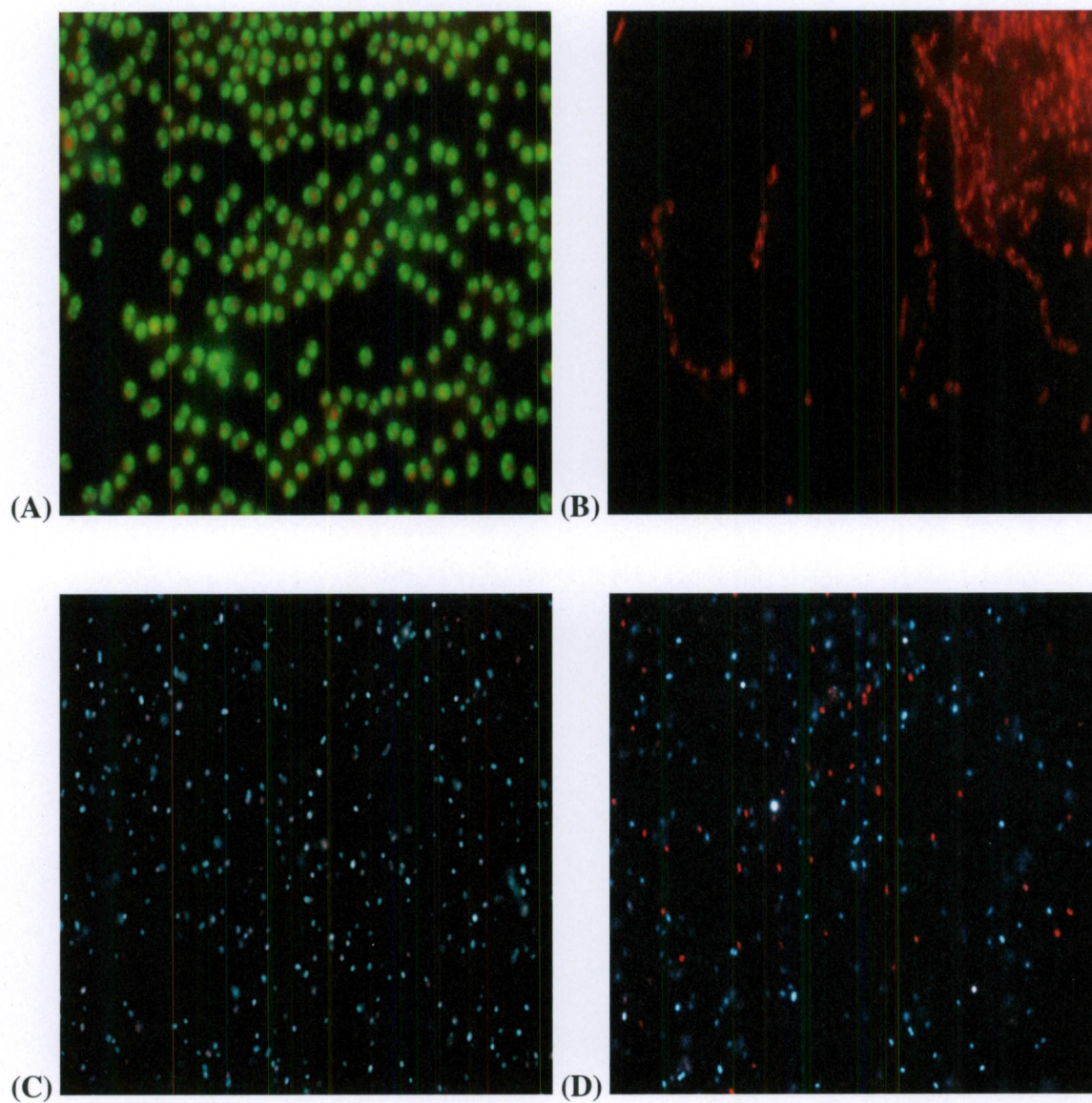
Figure 47. Live/ Dead qualitative analysis. (A) The untreated control group. (B) *S. mutans* treated with EGCG-S 250ug/mL. (C) *S. mutans* treated with Scope mouthwash. (D) *S. mutans* treated with a combination of Scope and EGCG-S 250ug/mL.
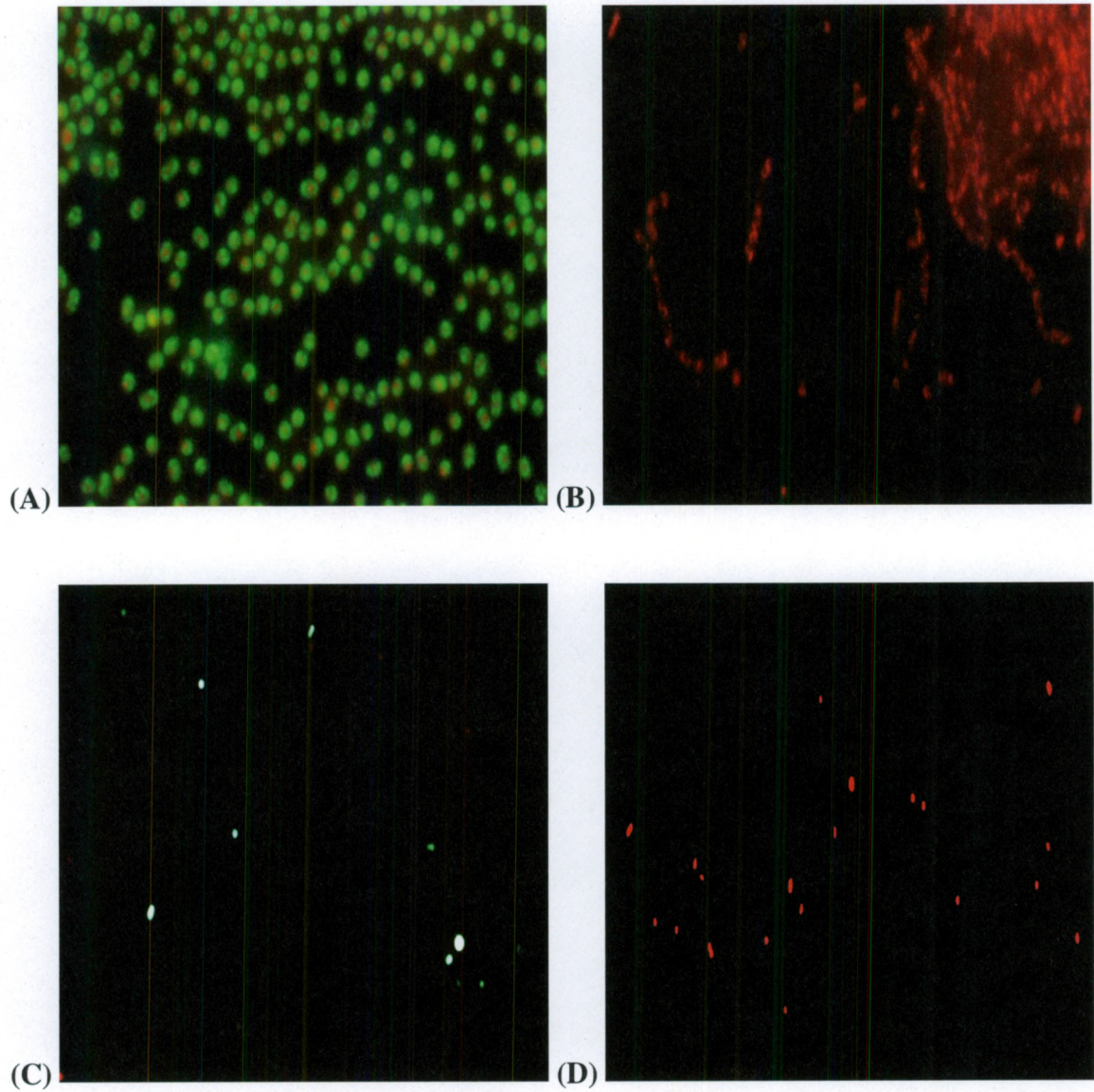
Figure 48. Live/ Dead qualitative analysis. (A) The untreated control group. (B) *S. mutans* treated with EGCG-S 250ug/mL. (C) *S. mutans* treated with Crest mouthwash. (D) *S. mutans* treated with a combination of Crest and EGCG-S 250ug/mL.
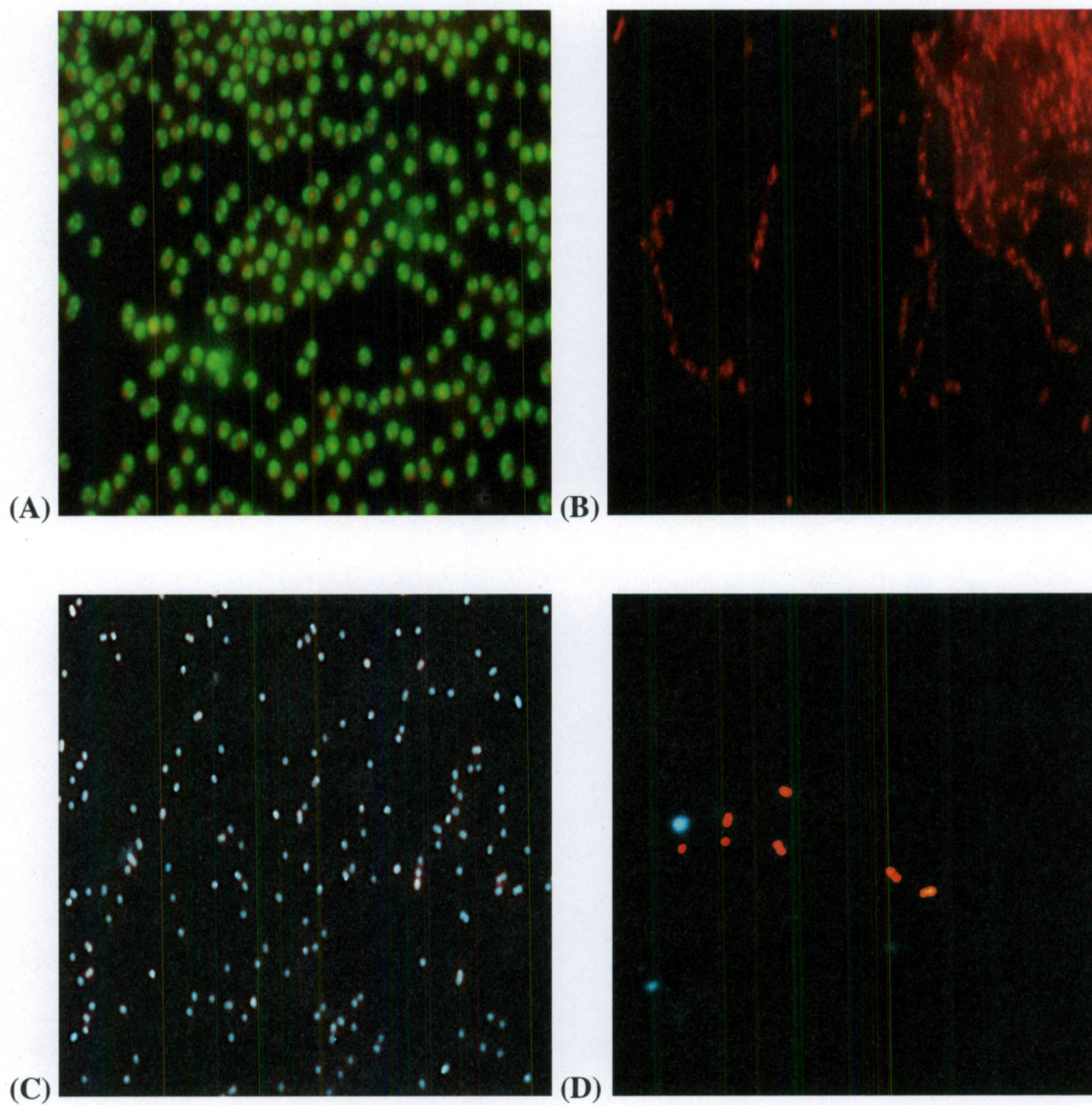
Figure 49. Live/ Dead qualitative analysis. (A) The untreated control group. (B) *S. mutans* treated with EGCG-S 250ug/mL. (C) *S. mutans* treated with Chlorohexidine Gluconate mouthwash. (D) *S. mutans* treated with a combination of Chlorohexidine Gluconate and EGCG-S 250ug/mL.
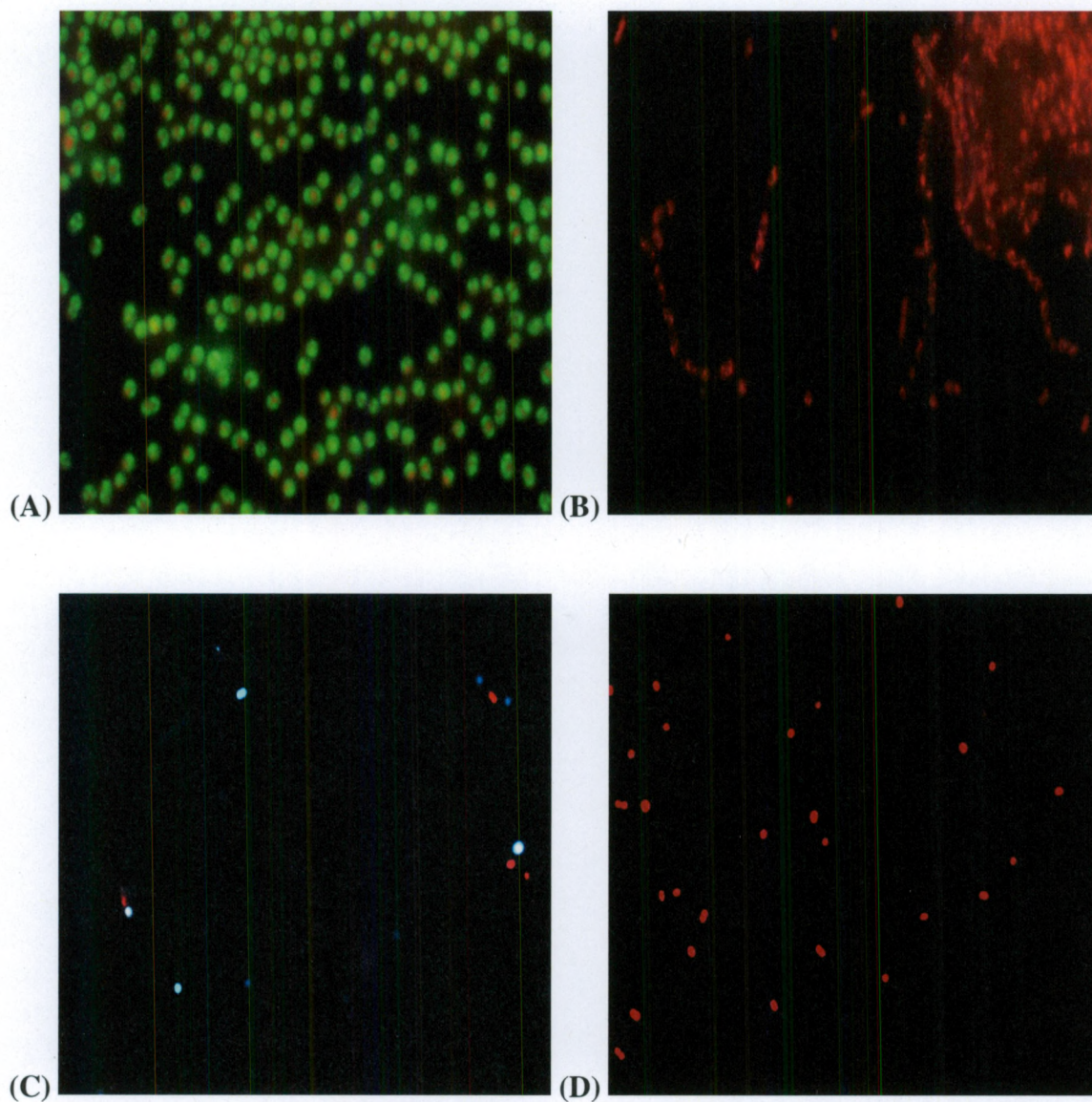
Figure 50. Live/ Dead qualitative analysis. (A) The untreated control group. (B) *S. mutans* treated with EGCG-S 250ug/mL. (C) *S. mutans* treated with Colgate. (D) *S. mutans* treated with a combination of Colgate and EGCG-S 250ug/mL.

6. The Effect of EGCG-S on *Streptococcus mutans* grown on dental materials

To determine if EGCG-S was effective in preventing bacterial growth on the surfaces of various dental materials, three common materials used in dental procedures were chosen and grown in the presence of either *S. mutans* alone or in combination with EGCG-S and *S. mutans*. An example of *S. mutans* biofilm on the surface of dental materials is shown in Figure 51. On Bruxzir®, a solid zirconia material, *S. mutans* was able to attach to the surface in the control (Figure 52A and C) and when treated with EGCG-S 250ug/mL (Figure 52B and D). When treated with EGCG-S 250ug/mL cell density is drastically reduced and the streptococcus chains become longer. When grown on an EVA mouthguard, *S. mutans* was growing in chains in the control (Figure 53A and C), while in Figure 53B and D their size, shape and possibly their cell membrane is being compromised. In Figures 54A and 54B, *S. mutans* was not able to attach and grow on the surface of PMMA. This suggests that PMMA may be the best material of the three tested to use in dental procedures.

S3400 10.0kV 9.8mm x6.50k SE   5.00um
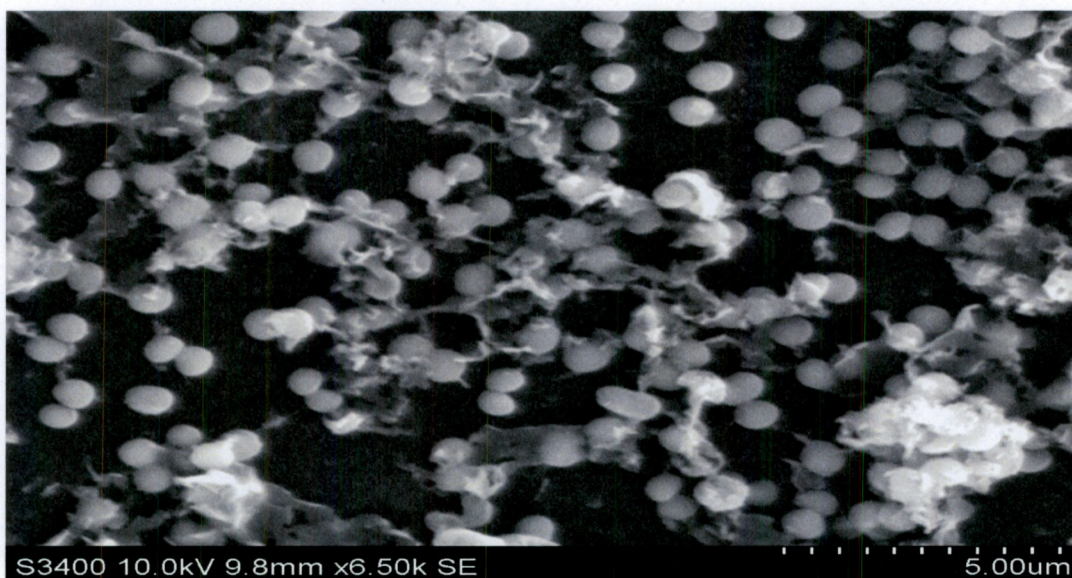
Figure 51. An example of Biofilm growing on the surface of a dental material.

98
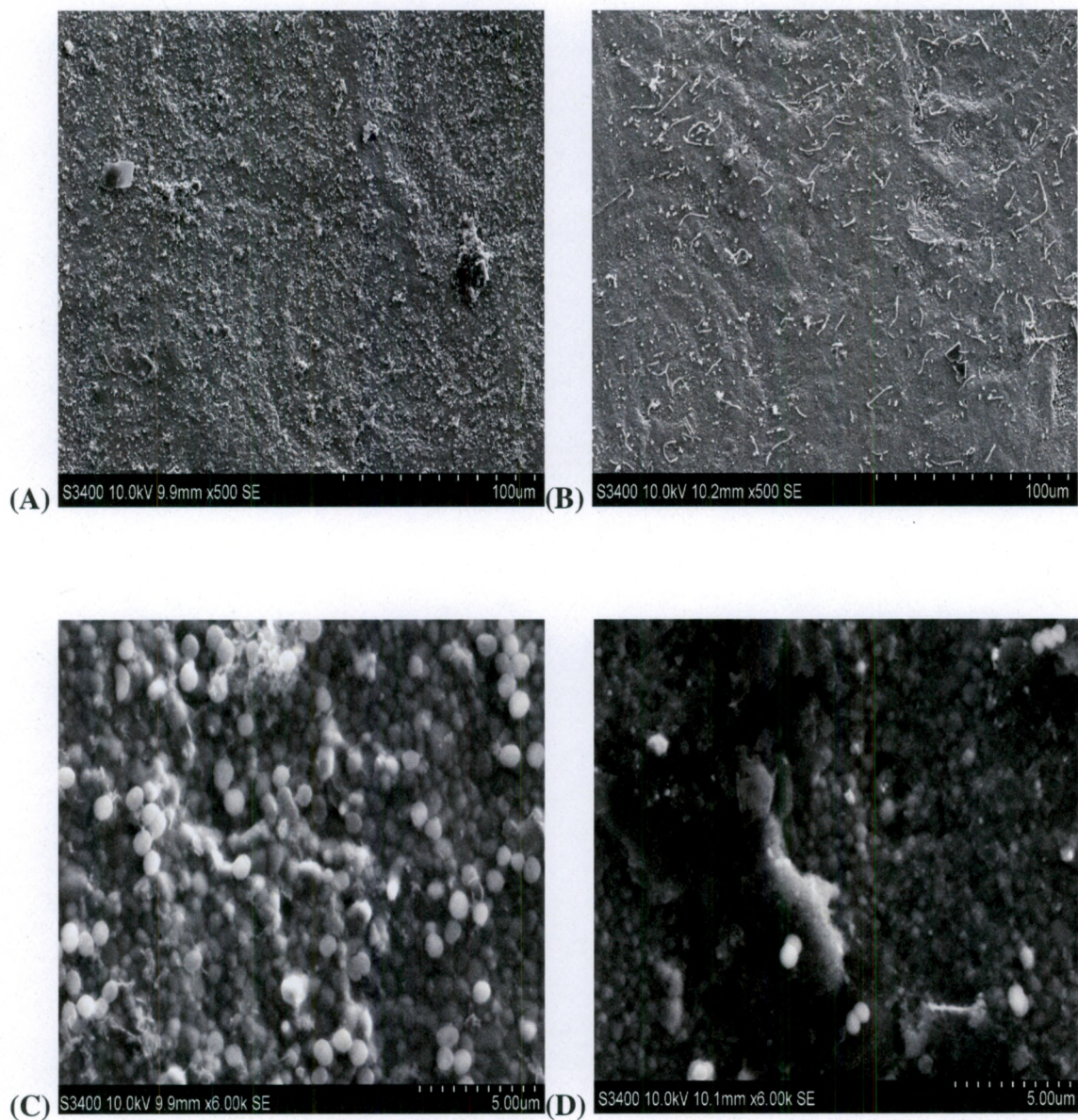
Figure 52. *S. mutans* was grown on BruxZir® for 4 days in the presence of 1% sucrose. (A) Confirms that chains of *S. mutans* were formed on the surface. (B) Suggests that EGCG-S 250ug/ mL was able to reduce the cell density and streptococcus chains get longer. (C) A higher power magnification view confirms that chains of cocci are growing on the surface and forming biofilm. (D) Shows a significant reduction in the amount of *S. mutans* present on the surface and the cells become smaller and irregular once treated.
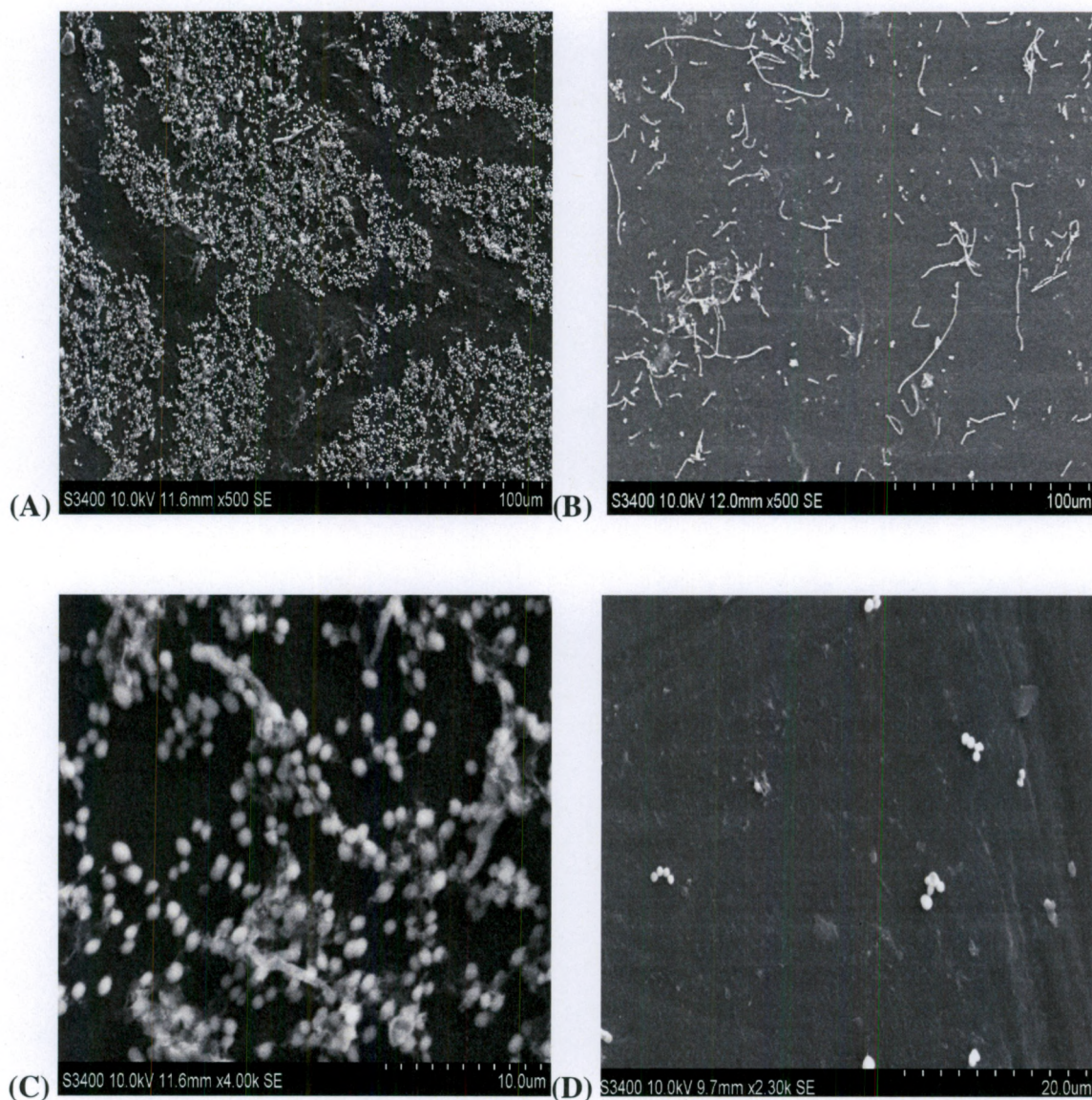
Figure 53. *S. mutans* was grown on a Shock Doctor mouthguard ® made primarily of EVA for 4 days in the presence of 1% sucrose. (A) Confirms that *S. mutans* grew on the surface and formed bioifilm. (B) Suggests that when treated with EGCG-S 250ug/mL *S. mutans* growth was reduced drastically and the chains get longer. (C) A higher magnification image confirms the growth of biofilm. (D) Confirms a significant reduction in *S. mutans* cell density and no biofilm formed when treated with EGCG-S 250ug/mL.
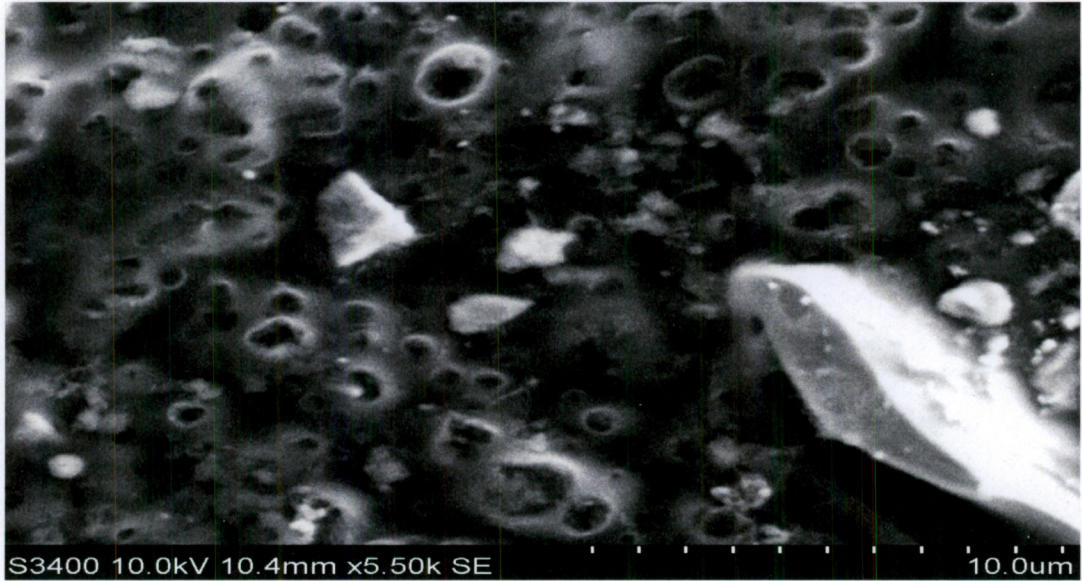
(A) S3400 10.0kV 10.4mm x5.50k SE   10.0um
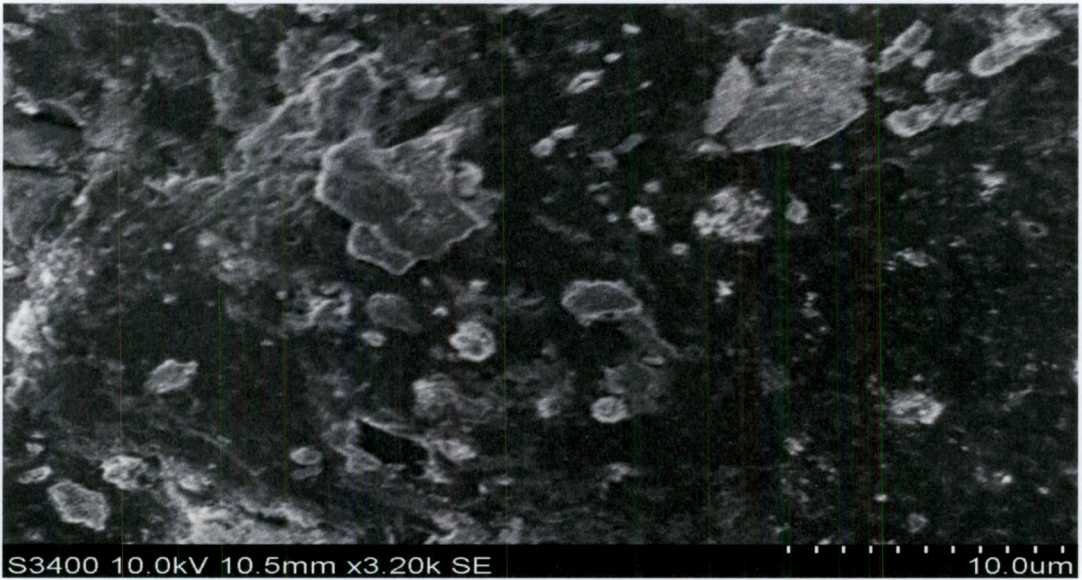
(B) S3400 10.0kV 10.5mm x3.20k SE   10.0um

Figure 54. *S. mutans* was grown on PMMA for 4 days in the presence of 1% sucrose. *S. mutans* was not found to grow in either the control (A) sample or in the presence of EGCG-S 250ug/mL (B).

Conclusions:

Overall, green tea polyphenols have a dose dependent effect on *Streptococcus mutans*. As seen in the stability study, EGCG-S stored at 25°C had the greatest effect on *S. mutans* over a period of 5 months. EGCG-S was able to inhibit growth of *S. mutans* 92% at 5 months, while its competitor EGCG was only able to inhibit 27% at the same temperature. This makes EGCG-S the better antimicrobial agent and can have a longer shelf life. EGCG-S was able to reduce the growth of *S. mutans* in a dose dependent manner with the greatest result being at the concentration of 250ug/mL as shown by a 100% inhibition in the CFU. The Live/Dead ® Assay and SEM images confirm that EGCG-S 250ug/mL was able to destroy cell viability, reduce cell density, and cause *S. mutans* to lose its shape.

In order for tea polyphenols to be an effective treatment in reducing dental caries, it needs to be effective in reducing a primary factor in caries development, biofilm. First the environment which best induces biofilm was determined to be 0.1% and 1% sucrose or dextrose at 24 hours. This result was confirmed when tested in a more life-like environment with the addition of saliva. The Congo Red agar experiment qualitatively demonstrated the dose-dependent relationship of EGCG-S and LTP's effect on *S. mutans* EGCG-S was more effective overall than LTP and was most effective at the concentration of 200ug/mL by having no biofilm growth present after 2 and 4 days. The crystal violet assay quantitatively confirms the results of the Congo Red agar experiment, in that EGCG-S was more effective than LTP and had a 100% inhibition rate at the concentration of 250ug/mL. These biofilm studies suggest that EGCG-S is successful in

reducing biofilm growth in *S. mutans* and could potentially be a great addition to oral care products.

The disk diffusion experiment tested the effect of mouthwashes alone, tea polyphenols alone and the combination of mouthwashes and tea polyphenols. The results suggested that when tea polyphenols are added to common brand mouthwashes the percent inhibition is increased. EGCG-S 250ug/mL enhanced all brands tested, except for scope, which was more successful in combination with LTP. Next a 96 well micro-titer plate assay was preformed to see the effect of the combination mouthwashes in comparison to the mouthwash and tea polyphenols over a period of 15 hours. The combinations of EGCG-S 250ug/mL with Listerine, Cepacol, Colgate and Scope were able to completely inhibit the growth of *S. mutans*. The combinations of LTP 250ug/mL and Colgate, Scope, and Cepacol were very effective, while the combination of LTP 250ug/mL and Chlorohexidine Gluconate or Crest had no effect at all on *S. mutans*. Overall EGCG-S 250ug/mL is a better potential synergistic additive to mouthwashes. The Time Course Study and Live/ Dead ® further confirm that EGCG-S 250ug/mL is effective when added to common brand mouthwashes by reducing the time it takes to reach 100% inhibition of *S. mutans* growth and compromising cell viability. With all of the experiments completed, EGCG-S makes for an excellent product to reduce *S. mutans* growth and biofilm and is a potential synergistic additive to mouthwashes.

Future Studies:

Further experiments are necessary to study the molecular analysis on the gene expression of *S. mutans* when treated with tea polyphenols. Additional molecular analysis should be done when *S. mutans* is treated with combinations of tea polyphenols and mouthwashes to further determine the best combination to reduce *S. mutans* biofilm. These studies should test the gtf gene, which is primarily responsible for the development of biofilm.

While EGCG-S is effective in compromising the growth and production of biofilm, the mechanism in which it is done is still unknown. Understanding the mechanism in which EGCG-S breaks down biofilm will not only provide help in perfecting treatments for dental caries, but will help in many other medical research areas such as prosthetic joint infections and antibiotic resistance.

This study observed only one type of oral care product. Future studies should determine the effectiveness of EGCG-S in combination with other oral care products such as toothpaste and denture cleansers. If effective, it can offer customers a more natural substitute than their typical product.

Since bacteria are rarely found in isolation, further studies need to be completed when *S. mutans* is in combination with other bacteria found in the oral cavity. Oral care products not only target cavity causing bacteria, but bacteria that cause infections of the gum and bad breath. A study of these groups bacteria alone and in combination would enhance EGCG-S's potential as a synergist in oral care products.

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