Green Tea Polyphenol EGCG-S as an Antimicrobial Agent

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Abstract

In recent years, a growing concern in the medical field has been the ability to treat infectious diseases. The microorganisms that cause these diseases have developed invasive mechanisms that elude possible treatments and increase their virulence. Some virulent factors that have been observed in microorganisms are biofilm and endospore formation. Development of new antimicrobial agents are needed to combat these microorganisms.

Green tea from the leaves of *Camellia sinensis*, has many health benefits, such as anti-bacterial, anti-spore, anti-cariogenic and anti-viral properties. Epigallocatechin-3-gallate (EGCG) is the most abundant catechin and contributes to these effects. Due to the instability of EGCG, a modified stable lipophilic based molecule Epigallocatechin Gallate-Stearate (EGCG-S) was used to determine the effectiveness as an antimicrobial agent on eight gram-positive bacteria, seven gram-negative bacteria, one acid-fast bacterium, one fungus. Colony forming unit (CFU) was used to determine the growth. LIVE/DEAD® Baclight™ Kit was used to observe the viability of the cells; Congo red assay was used to study the biofilm formation and Rapid Agar Plate Assay (RAPA) was used to study the application of EGCG-S as an antimicrobial agent. The results indicated that the minimum inhibitory concentration (MIC) for most bacteria is 250μg/mL of EGCG-S within 2 hours, except *K. pneumoniae* and *M. smegmatis*. MIC for biofilm formation is 500μg/ml EGCG-S for most bacteria. One percent (1%) and 5% sucrose concentrations can reduce the inhibitory effect of EGCG-S. Formulated prototype, ProtecTeaV is effective in treating *C. albicans* and three endospore forming *Bacillus* spp. Percentage of inhibition was above 95.97 within 60 seconds. This study suggested that EGCG-S can be a good potential antimicrobial agent.
Green Tea Polyphenol EGCG-S as an Antimicrobial Agent

By

Theresa Renee Aponte

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Thesis Committee:

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Green Tea Polyphenol EGCG-S as an Antimicrobial Agent

A THESIS

Submitted in partial fulfillment of the requirements
For the degree of Master of Science

By

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Montclair, NJ
May 2018
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Introduction

Since the 19th century, microorganisms have been identified as the causative agent of many diseases such as tuberculosis caused by *Mycobacterium tuberculosis*, cholera caused by *Vibrio cholerae*, and *Candida albicans* causing thrush of the oral and vaginal cavity (Aminov, 2010; Kadosh & Johnson, 2005; Lederberg, 2000). Penicillin was discovered in 1928 by Alexander Fleming and was seen as a breakthrough in treating bacterial infections (Aminov, 2010). Therapeutic agent, fluconazole was formulated and treats fungal infections (Kartsonis et al., 2003). Due to the excessive misuse of antimicrobial agents, microorganisms have begun to display resistance to certain antimicrobial agents (Aminov, 2010; CDC, 2015; Cohen, 1992). Antimicrobial resistance has been observed in penicillinase-producing *Staphylococcus aureus* and antifungal resistance to fluconazole has been observed in *Candida albicans* (Aminov, 2010; CDC, 2015; Hirasawa & Takada, 2004; Saga et al., 2009). Resistance to these antimicrobial agents has been attributed to a variety of mechanisms: antimicrobial selective pressures, incorporation of plasmid, and efflux pumps (Cohen, 1992; Davin-Regli & Pagès, 2015; Hirasawa & Takada, 2004; Zhang et al., 2015).

Pathogenesis and the invasive mechanisms

The prevalence of infections has decreased since the introduction of antimicrobial agents; however, microorganisms have developed and acquired virulent mechanisms that enable them to elude detection from the host immune system (Rohmer et al., 2011, Saga et al., 2009, Vila et al., 2017). This allows their survival and establishes a niche that causes persistent infections. Virulent factors are a growing concern in the medical field and have been associated with persistent bacterial and fungal infections, bacterial biofilm formation, and endospore formation. Bacteria have evolved and acquired genes that are used to establish the foundation biofilm formation that antibiotics are unable to penetrate (Donlan & Costerton, 2002; Meh & O’Toole, 2001). The virulence of *C. albicans* is established through the filamentous activity, which enhances size of the organism without replication (Vila et al., 2017). This activity has been a focus for development of new antifungal agents.
The vegetative cells of *Bacillus* and *Clostridium* spp. form endospores in hostile environments when nutrients are limited and sporulation begins (Drobniewski, 1993).

**Biofilm Formation**

Biofilms are aggregations of microorganism that adhere to one another and surfaces. The biofilm provides structure and protection from the environment (Donlan & Costerton, 2002; Wingender et al, 2011). The formation of biofilm is seen in sewage plants, water systems and medical devices (Donlan & Costerton, 2002; Wingender et al, 2011). In clinical settings, biofilm formation has been observed in medical implants, dental caries, and intravascular catheters (Donlan & Costerton, 2002; Meh & O’Toole, 2001). The persistence of biofilm infections is enhanced by mechanisms that aid in the survival of these bacterial communities, such as extracellular polymeric substance (EPS) and the presence of glycocalyx which inhibits efficacy of antibiotics (Limoli et al., 2015). The EPS secreted by bacteria surrounds the cell with a layer of protection from adverse environments. Polysaccharides and proteins are used to enhance the structure of the biofilm. It has been observed that the morphological structure of microorganisms in biofilms is altered when compared to planktonic cells.

Bacterial biofilm formation is enhanced by the secreted signaling molecule, autoinducer, and is dependent on bacterial density (Huber et al., 2002; Schauder et al., 2001). When bacteria secrete this signaling molecule, it enables cell to cell communication known as Quorum Sensing (QS) (Huber et al., 2003; Lerat and Moran, 2004; Waters et al., 2008). Gene regulation increases the virulence factor of these organisms through the ability of QS. This allows the establishment of infection to remain concealed through gene regulation. The virulence of these microorganisms is elevated by addition of antibiotic resistant genes, which are able to be exchanged with other microorganisms (Ramage et al., 2002; Saga et al., 2009). Virulence of these microorganisms can be elevated by horizontal gene transfer or incorporation of plasmids (Davin-Regli & Pagès, 2015; Saga et al., 2009; Viveirose et al., 2007). The exchange of antibiotic genes from *Klebsiella pneumoniae* to *Enterobacter* spp. can be observed through horizontal gene transfer.

The formation of fungal biofilm has not been studied to the same extent as bacterial biofilm (Chandra et al., 2001). QS has also been observed in *C. albicans*. The ability for
C. albicans to form a biofilm is enhanced by filamentation, allowing growth in size rather than cell number (Ramage et al., 2017). Sucrose enhances the attachment and virulence of C. albicans with fibrillar.

**Endospore formation**

The formation of endospores enables Bacillus and Clostridium spp. to become dormant and resistant to heat, solvents and ultraviolet light. Bacillus spp. are responsible for food poisoning and spoilage. Heat shock proteins of Bacillus spp. spores are enhanced by increases in temperature. (Citation?)

**Mode of Treatments**

Once an antibiotic is able to attack a specific area of the bacterium, it will cause a disruption in an important function within the bacterium. There are two mechanisms by which antibiotics (ABs) affect bacteria: bactericide or bacteriostatic (Nemeth et al., 2014). The development of new antibiotics is taking pre-existing ABs and chemically modifying them, thus acquiring many different ABs within a particular class (CDC, 2015). The mechanism by which ABs work is by the production of inhibitory enzymes and the prevention of binding.

Fluconazole and Amphotericin B are the common treatments for fungal infections. It has been observed that C. albicans has presented resistance to both treatments (Ramage et al., 2002).

**Antimicrobial Resistance**

In recent years the excessive use of antibiotics has initiated a global health concern resulting in antibiotics resistance (CDC, 2015). The inappropriate use of antibiotics by individuals has played a role in the acquisition of antibiotic resistance by bacteria due to incomplete antibiotic regimen and persistence of patients’ requests for antibiotics (CDC, 2015; McDevitt et al., 2002; Saga et al., 2009). Many once commonly treated bacterial infections have become difficult to manage due to overuse as growth promoters for fish and livestock (CDC, 2015; Gilchrist et al., 2007). Livestock that are contaminated with ARB can be transferred to individuals during food preparation and consumption (CDC,
13

It has been noted that the use of antibiotics in livestock should be limited to incidents of infection within a group of livestock that are produced for consumption.

The detection of antibiotics present in the sediment and soil can be attributed to overflow of livestock waste and industrial waste from pharmaceutical companies (CDC, 2015; Kolpin et al., 2002; Wingerder et al., 2011). Antibiotics Resistant Bacteria (ARB) eventually reside in environmental water reservoirs such as sewage waste, surface water, and aquatic sediment in the vicinity of livestock and pharmaceutical companies (CDC, 2015; Kolpin et al., 2002; Wingerder et al., 2011). Significant levels of ARB are detected in the environment higher than initially predicted (Kolpin et al., 2002; Wingerder et al., 2011).

![Figure 1: Flow chart of Antibiotic Resistance (CDC, 2015)](image_url)

However, over the course of 70 years of antibiotic usage, it was observed that certain infections were not being eliminated by the antibiotics once commonly prescribed to treat them (Li et al., 2015). Recently, much effort has been made to produce stronger antibiotics that can combat ARB. Vancomycin (produced in 1972) was typically used as the final antibiotic for treatment of infections (McDevitt et al., 2002; Viveirose et al., 2007). ARB resistance to Vancomycin is observed in several species of bacteria. The rate of microorganisms acquiring antibiotic resistance can be observed within a year of
production. The amount of antibiotic production has decreased due to the acquired resistance to whole classes of antibiotics. It can be observed in Figure 2 that the development of antibiotics has decreased in the past 40 years (Aminov, 2010; CDC, 2015; Saga et al., 2009).

Figure 2. Decreased development of antibiotics (CDC, 2015)

**Antimicrobial Resistance Mechanisms**

A variety of physical and genetic mechanisms have been linked to antibiotic resistance in bacteria (Saga et al., 2009; Viveirose et al., 2007). Efflux pumps and membrane permeability are physiological mechanisms that are attributed to antibiotic resistance (Viveirose et al., 2007). Efflux pumps have been able to control the direction of the flow of antibiotics back out of the cell (Figure 3) (Giedraitiene et al., 2011; Li et al., 2015; Viveiros et al., 2007).

Mobile genetic elements (MGE), plasmids and horizontal gene transfer (HGT) are genetic mechanisms that are associated with bacteria acquiring antibiotic resistant genes (ARGs) (Davin-Regli & Pagès, 2015, Saga et al., 2009; Viveirose et al., 2007). Horizontal gene transfer is the process whereby bacteria are able to pick up pieces of DNA or plasmids that are around them and incorporate them into their own genome (Giedraitiene et al., 2011).
Selective pressures in the presence of antibiotics have forced bacteria to acquire significant resistance, thus directing research towards alternative treatments. *Camellia sinensis*, more commonly known as green tea, has many health benefits (Steinmann et al., 2013). Green tea contains polyphenols which are composed of catechins. Catechins are components of green tea which have demonstrated antibacterial, antifungal, antitoxic and antiviral effects (Nakayama et al., 2015; Steinmann et al., 2013). Epigallocatechin-gallate (EGCG) is the most abundant catechin in green tea (Du et al., 2012; Zaveri, 2006). EGCG has a greater effect on gram-positive bacteria than gram-negative bacteria. It has been observed that green tea products that contain high levels of carbohydrates have decreased spoilage because of the presence of polyphenols within the product (Sakanaka et al., 2000).
The incorporation of EGCG at increasing concentrations has been shown to decrease bacterial numbers (Sakanaka et al., 2000). The effects of Green Tea Polyphenol (GTP) were shown to inhibit bacteria and spore formation even at elevated levels of sugar (Sakanaka et al., 2000). The use of EGCG can have an effect on the biochemical activity within the bacterial cell. Research has shown that when *P. aeruginosa* and *E. coli* were exposed to EGCG, grooves can be seen within the lipid bilayer (Cui et al., 2012). EGCG is dose and concentration dependent (Zaveri, 2006). Polyphenols have the ability to interfere with biofilm formation and QS (Du et al., 2012; Steinmann et al., 2013). EGCG demonstrates the ability to reduce microbial biofilm formation (Du et al., 2012; Steinmann et al., 2013). The possible mechanisms of EGCG on bacteria are summarized in Table 1 and the possible mechanisms on fungi are listed in Table 2.

<table>
<thead>
<tr>
<th>Target of EGCG on Bacteria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibit of multi-drug resistance of P-glycoprotein</td>
<td>Roccaro et al., 2004.</td>
</tr>
<tr>
<td>Hydrogen Peroxide Production</td>
<td>Arakawa et al., 2004; Cui et al., 2012.</td>
</tr>
<tr>
<td>Binding to cell wall peptidoglycan (Gram Positive)</td>
<td>Roccaro et al., 2004.</td>
</tr>
<tr>
<td>Decrease efflux efficiency and blockage of efflux pump passage: Tetracycline</td>
<td>Roccaro et al., 2004.</td>
</tr>
<tr>
<td>Inhibit fatty-acid type II synthesis (β-ketoacyl-ACP reductase (FabG) and the <em>trans</em>-2-enoyl-ACP reductase (FabI)) - reduction of elongation</td>
<td>Zhang &amp; Rock, 2004.</td>
</tr>
<tr>
<td>Inhibits Co-factor of enzymes: - EGCG interferes with activity by binding to the free enzyme and preventing the binding of NADH</td>
<td>Zhang &amp; Rock, 2004.</td>
</tr>
</tbody>
</table>
- Nucleotide cofactor binding site, and with FabG, EGCG has the additional property of binding to the enzyme-cofactor complex.

| Cytoplasmic enzyme dihydrofolate reductase | Steinmann et al., 2013. |

Table 1. Target of EGCG on Bacteria

<table>
<thead>
<tr>
<th>EGCG Target- Mode of Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disruption in folic acid synthesis</td>
<td>Navarro et al., 2006</td>
</tr>
<tr>
<td>Cytoplasmic enzyme dihydrofolate reductase</td>
<td>Steinmann et al., 2013.</td>
</tr>
</tbody>
</table>

Table 2. Target of EGCG on Fungi

**Epigallocatechin- Gallate-Stearate**

Epigallocatechin-Gallate-Stearate (EGCG-S) (Patent#20120171423) is a stable, lipophilic form of EGCG. EGCG-S was used to determine the minimum inhibitory concentration (MIC) to inhibit bacterial growth and biofilm formation in various sucrose concentrations.

![Figure 5. The Molecular Structure of EGCG-S (Hsu, 2012)](image-url)
**Bacterial Profiling**

In this study, a diverse selection of microorganisms were used to determine the effectiveness of EGCG-S as an antimicrobial agent. Gram-positive and gram-negative bacteria, as well as an acid-fast bacterium, a fungus, and endospores produced by *Bacillus* spp. were studied in the presence of EGCG-S.

The gram-positive bacteria used are divided into two groups: bacillus and cocci. *Bacillus* spp. used are *Bacillus cereus* (*B. cereus*), *Bacillus megaterium* (*B. megaterium*), and *Bacillus subtilis* (*B. subtilis*). These three species were also used in the spore study due to the formation of spores in hostile environments. *Bacillus* spp. is known to cause food poisoning and spoilage (Drobniewski, 1993; Errington, 2003). The gram-positive cocci bacteria used are: *Enterococcus faecalis* (*E. faecalis*), *Micrococcus luteus* (*M. luteus*), *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Streptococcus mutans* (*S. mutans*). Gram-positive cocci bacteria are the cause of diseases such as boils and cellulitis (*S. aureus*) (Lowy, 1999). *S. mutans* is known caused of oral diseases and formation of dental cavities (Xu et al., 2012).

The gram-negative bacteria used are as follows: *Enterobacter aerogenes* (*E. aerogenes*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus vulgaris* (*P. vulgaris*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Pseudomonas fluorescens* (*P. fluorescens*), and *Serratia marcescens* (*S. marcescens*).

An acid-fast bacterium, *Mycobacterium smegmatis* (*M. smegmatis*), which has characteristics similar to the organism responsible for tuberculosis was selected due to cell wall composition (McDevitt et al, 2002).

*Candida albicans* (*C. albicans*) was chosen as representative fungal microorganism. It is a unicellular yeast responsible for Candidiasis, causing cavities and periodontitis (Ramage et al., 2002).
**Objectives of this study**

These studies demonstrate the potential use of EGCG-S as an alternate therapy to excessive antibiotic agents:

1) Profiling the inhibitory effect of EGCG-S against 17 microorganisms (eight Gram-positive, seven Gram-negative, one Acid-fast, and one Fungus)

2) Profiling the effectiveness of EGCG-S on biofilm formation and pre-formed biofilm in 16 bacterial spp.

3) Evaluation of EGCG-S as antibacterial and antifungal agent
   a) Pre- and Post-Application using EGCG-S
   b) Application of Prototype ProtecTeaV
Materials and Methods

1. Pre/Post-experiment Set-up

The Biosafety Level 2 (BSL-2) hood was wiped down with 10% bleach and subjected to ultraviolet light for 20 minutes before and after experiment.

2. Media Preparation

There are five types of media used in this research: Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), Congo Red (CR), Potato Dextrose Agar (PDA), and Modified Nutrient Agar (MNA). All the media were prepared according to manufacturer’s protocols. After agar media was poured into plates, they were left under the hood to solidify overnight. The next day, the solidified agar plates were placed back into the plastic sleeve, taped and labeled with appropriate media type and stored into a 4°C cold room for prospective experiments. Only Congo Red medium was used within 24 hrs after preparation.

a. Tryptic Soy Agar Preparation

TSA from Carolina was prepared using 30 grams of TSA powder media and 1L of deionized H₂O. The medium was autoclaved for 30 minutes at 121°C. The TSA medium was poured into mono plates, X plates, or Y plates, (Figure 6) depending on experimental design.

![Figure 6. Depiction of petri dishes used based on experimental design.](image-url)
b. Tryptic Soy Broth Preparation
TSB from Carolina was prepared using 30 grams of TSB powder media and 1L of deionized H₂O. Sucrose (Spectrum) containing media were prepared with a final concentration of 0.1%, 0.5%, 1%, and 5%, respectively, in Falcon flasks and autoclaved for 30 minutes at 121°C and then stored at 4°C until needed.

c. Congo Red (CR) Media Preparation
CR agar medium was prepared in two separate preparations. 37 g/L of Brain heart infusion broth from Sigma-Aldrich, 10 g/L of Bacteriological agar from Scholar Scientific was added to 1L of deionized water. In a separate test tube, 0.8g/L of Congo Red indicator was autoclaved. Different sucrose concentrations of 0.1%, 0.5%, 1% and 5% were also prepared in sucrose containing BHI plates. All solutions were autoclaved at 121°C for 30 minutes. Once completed, the Congo Red solution and agar were cooled down to 55°C, evenly mixed and poured into X-plates (Gamborg & Phillips, 1995).

d. Potato Dextrose Agar (PDA) Preparation
PDA (HIMEDIA) was prepared with 39 grams of PDA powder media in 1L of deionized H₂O, autoclaved for 30 minutes at 121°C. Once media cooled down to 55°C, it was poured into sterile mono plates.

e. Modified Nutrient Agar (Spore Plates)
Nutrient Agar (NA) was adapted to prepare Modified Nutrient Agar (MNA). MNA was prepared with 23 grams of NA media (Difco™) in 1L of deionized H₂O and combined with 0.06 g/L of Magnesium sulfate and 0.25 g/L of Monopotassium phosphate. The medium was autoclaved for 30 minutes at 121°C and allowed to cool down, then poured into sterile X-plates (Gamborg & Phillips, 1995).

3. Culturing
a. Bacterial Cultures
Sixteen microorganisms were grown on TSA at 37°C for 24 hours, except for *M. luteus*, *P. fluorescens*, and *S. marcescens*, which were grown at 25°C.
Gram-positive bacteria used in this research:
*Bacillus cereus* (*B. cereus*), *Bacillus megaterium* (*B. megaterium*), *Bacillus subtilis* (*B. subtilis*), *Enterococcus faecalis* (*E. faecalis*), *Micrococcus luteus* (*M. luteus*), *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Streptococcus mutans* (*S. mutans*).

Gram-negative bacteria used in this research:
*Enterobacter aerogenes* (*E. aerogenes*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus vulgaris* (*P. vulgaris*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Pseudomonas fluorescens* (*P. fluorescens*), and *Serratia marcescens* (*S. marcescens*).

Acid-fast bacteria used in this research:
*Mycobacterium smegmatis* (*M. smegmatis*).

After overnight growth of these bacteria, cultures were wrapped in parafilm and stored at 4°C for future use. Bacterial purity was checked periodically by using simple or Gram stain to confirm purity. In cases of contamination, a discontinuous streaking method was used to isolate colonies to start a new stock. The stocks of all bacteria were prepared and stored in -80°C freezer. Eppendorf tubes containing 600μL of overnight cultures with 400μL of 40% glycerol were prepared for deep freezer. (Prakash, Nimonkar, & Shouche, 2012).

**b. Harvesting and Purification of Endospores**

Modified Nutrient Agar (MNA) was used to grow endospore forming microorganisms, *B. cereus*, *B. megaterium*, and *B. subtilis*, for 10 days at 37°C. After 10 days, *Bacillus* spp. spore production was confirmed with simple stain. Spores from *Bacillus* spp. were collected in sterile DI H₂O and the spores were centrifuged at 10,000rpm for 10 mins; this process was performed three times. Spore suspension was then stored at 4°C for future use.
c. *Candida albicans*

A continuous streak of *C. albicans* was plated on PDA and incubated at 37°C overnight. A simple stain was conducted to determine purity. After overnight growth, *C. albicans* was wrapped in parafilm and stored at 4°C for future use. Continuous steak plate was periodically prepared every two weeks to sustain *C. albicans*.

4. Overnight Cultures

Prior to each experiment, overnight culture of each bacterium was prepared in TSB and grown at optimal temperature with constant shaking at 250rpm.

5. Staining Techniques

a. Simple Stain

The protocol for simple stain used in this study is described in the research lab manual (Lee et al., 2015). Methylene blue was used for simple stains to determine purity and morphology of all organisms in this study.

b. Gram Stain

The Gram stain was used to differentiate between Gram-positive and Gram-negative bacteria, as well as to determine the morphology of the bacteria. The Gram stain protocol was performed as described in Microbiology Laboratory Manual (Lee et al., 2015).

c. Schaeffer-Fulton Spore Stain

Schaeffer-Fulton spore staining (Endospore stain) method was used to stain the spores formed by specific bacteria. The detailed protocol for Schaeffer-Fulton spore staining was described in Laboratory manual (Lee et al., 2015). This differential staining technique stained spores green and vegetative cells red.

d. Ziehl-Neelsen Stain

The Ziehl-Neelsen Stain (Acid-fast stain) was used to identify *Mycobacterium* spp. *Mycobacterium* spp. retained red/pink color and non-acid-fast bacterium stained blue
(Russell, 2001; Lee et al., 2015). The detailed protocol is described in Microbiology laboratory manual (Lee et al., 2015).

6. Preparation of Green Tea Polyphenols

Green Tea Polyphenol EGCG-S (U.S. Patent #: 20120171423) was purchased from Camellix LLC, Augusta, GA. EGCG-S stocks were prepared in concentrations of 10x of 100 μg/mL, 10x of 250μg/mL and 10x of 500μg/mL.

7. Growth Curve of Microorganisms

   a. Standard Growth Curve of 17 Microorganisms

      Overnight cultures were prepared and diluted using TSB with an Optimal Density (OD) of 0.1 at 600 nm. A 96 well plate was used to prepare 17 microorganisms and was placed in a shaking incubator at 125 rpm at optimal temperature. Multi-plate reader, TECAN InfiniteM200 Pro, was used to monitor the growth at 600 nm every hour for 11 hours with a final reading taken at 24 hours. The growth curve was plotted using the mean of three replicates with the Standard Deviation (SD) and plotted with OD (y-axis) vs. Time (x-axis). The generation time was calculated using the equation:

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

      to determine the doubling time required for each organism.

   b. The Effect of Sucrose on Growth of 17 Microorganisms

      Seventeen microorganisms were grown in TSB with 0%, 0.1%, 0.5%, 1% and 5% sucrose concentrations with an initial OD_{600} of 0.1 and were monitored for 24 hours. Growth curve for each microorganism with each sucrose concentration was generated. The growth was monitored as described in 6.a. The growth curves for sucrose concentrations 0%, 0.1%, 0.5%, 1%, and 5% were plotted using triplicate means with the Standard Deviation (SD). The generation time was calculated using the equation from 6.a. to determine doubling time for each microorganism in different sucrose concentrations.
8. The Effect of EGCG-S on 17 Microorganisms

Overnight cultures of 17 microorganisms were used to study the effectiveness of EGCG-S at 0% and 0.1% sucrose. Serial dilutions were carried out to obtain a countable range between 30 and 300. The flow chart for serial dilutions are illustrated in Figure 7.

Figure 7. Serial dilution follow chart to obtain countable range of microorganisms.

Figure 8. Serial dilution flow chart to obtain countable range of EGCG-S treated microorganisms.

After 2 and 24 hrs, samples for control and treated were diluted to $10^6$ and $10^3$, respectively. 25μL of each diluted sample was plated on a Y-plate and incubated at optimal temperature for 24 hours (Dhankhar et. Al, 2012). After incubation, the colony count was collected from triplicates control and treated microorganisms, respectively. The percent of inhibition was calculated for each organism to determine the effectiveness of EGCG-S.
% Inhibition = \frac{(CFU of Control – CFU of Treatment)}{CFU of Control} \times 100\%

**a. The Effect of EGCG-S on the Growth of 17 Microorganisms**

Overnight cultures of all microorganisms were prepared and diluted to an OD$_{600\text{nm}}$ at 0.1. Samples were aliquoted into Eppendorf tubes with 100μL of sample and centrifuged at 13,000 rpm for 1 minute. Control samples and treated samples were resuspended in 50μL and 45μL of TSB, respectively. The addition of 5μL of 10x of 250 EGCG-S for a total volume of 50μL in the treated sample. Triplicates were performed for each microorganism on TSA Y-plates. Control and treated samples were diluted to $10^{-6}$ and $10^{-3}$, respectively. Further dilution was required for certain bacteria. The plates were incubated 37°C for 24 hrs and colonies were counted to determine colony forming unit CFU/mL.

**b. The Effect of EGCG-S on growth in 0.1% Sucrose of 17 Microorganisms using Colony Forming Unit (CFU)**

The protocol from 7.a. was followed for all 17 microorganisms. Control samples and treated samples were resuspended in 50μL and 45μL of TSB with 0.1% sucrose, respectively. A total volume of 50μL was obtained with the addition of 5μL of 10x of 250 EGCG-S. The method was described in 7.a.

**9. Effect of Green Tea Polyphenol EGCG-S on Biofilm**

**a. Congo Red Assay on Biofilm Formation**

Overnight bacterial cultures were aliquoted into Eppendorf tubes: 50 μL, 49 μL, 47.5 μL, and 45 μL, respectively. The control samples had 50μL each. Concentrations of 1, 2.5 and 5 μg/mL of EGCG-S were added, respectively, to each Eppendorf tube, for a total volume of 50 μL. Samples were incubated at room temperature for 24 hrs. After 24hrs, X plates were used and each quarter of the plate was inoculated with 10 μL of control, 100, 250 and 500 μg/mL treated samples, respectively. Plates were incubated at optimal temperature and observed at 24 and 48 hrs to evaluate biofilm formation. Dark precipitation indicates biofilm formation.
b. Congo Red Assay on Biofilm Formation with Sucrose

Experiment was set up as described in 8a. Congo Red plates containing 0%, 0.1%, 0.5%, 1% and 5% sucrose were plated with 10 μL of each sample. Each quarter of the plate contained a control, treatment with 100, 250 and 500 μg/mL respectively. Plates were incubated at optimal temperature and observed at 24 and 48 hrs to observe biofilm formation at the various sucrose concentrations mentioned above.

c. Congo Red Assay on Pre-Formed Biofilm

The bacterial were grown at CR plate for 48 hrs to obtain biofilm. Congo Red plates were placed on inverted microscope to evaluate pre-formed biofilm and then treated with EGCG-S on Staphylococcus aureus. 50 μL of 500μg/mL was added to the pre-formed biofilm. The samples from both pre-formed biofilm and reversal treatment were viewed and photographed using inverted and fluorescence microscopes.

10. Microscopic observation

a. Fluorescence Microscopy using BacLight™ Live and Dead Assay

Ten μL of bacterial sample was smeared onto a plain slide on a labeled slide tray. The tray was kept in dark and 10 μL of Syto®9 dye from the LIVE/DEAD® Baclight™ Kit was added to each slide with a cover slip placed on top. Slide tray was covered with aluminum foil and left at room temperature for 20 minutes. Each slide was viewed under 1000X total magnification using a ZEISS fluorescence microscope.

b. Inverted Microscopy

Congo Red plates were placed on Jenco™ USA inverted microscope to evaluate biofilm formation and treatment of pre-formed biofilm. Images were taken under 40X magnification to determine density of biofilm before and after the treatment.

11. Pre and Post Application of EGCG-S Using Rapid Agar Plate Assay (RAPA)

a. Pre-Application of EGCG-S

Twenty-five (25) uL of 10x of 250 μg/mL of EGCG-S was applied to each section of TSA Y-plate 1 hour prior to application of the microorganism. All microorganisms were
diluted to an OD$_{600nm}$ reading of 1.0 and diluted to $10^{-6}$ using sterile DI H$_2$O. 25 μL of diluted microorganism samples were spread onto control and treated plates, respectively. All plates were incubated at optimal temperature overnight. CFU was obtained and percent of inhibition was calculated.

**b. Post-Application of EGCG-S**

25μL of all microorganism dilutions of $10^{-6}$ from 10a. were plated on TSA Y-plates and air dried for five minutes. 25μL of 10x of 250μg/mL of EGCG-S were applied onto section of the Y-plate. All plates were incubated at optimal temperature overnight and CFU was obtained, % of inhibition was calculated.

**12. Evaluation of formulated tea antimicrobial product**

Formulated ProtecTeaV 0 (Pro 0) and ProtecTeaV 0.1 (Pro 0.1) (Figure 9) was provided by Dr. Hsu to be used to determine the effectiveness of these products on *C. albicans* and *Bacillus* spp. spores. Formulations consist of ethyl alcohol (Pro 0) and ethyl alcohol with EGCG-S (Pro 0.1) respectively.

![Figure 9. ProtecTeaV](image)
a. *Candida albicans* Suspension Study

50μL of undiluted overnight culture of *C. albicans* was aliquoted into Eppendorf tubes and 450μL of Pro 0 and Pro 0.1 were added, respectively. 100μL of each treatment was plated on PDA. *C. albicans* was diluted to $10^{-4}$ and 100μL was plated on TSA mono plates. Plates were incubated for at 37°C. After 24 hrs, colonies were counted and percentage of inhibition was determined.

b. Spore Study

Spores, harvested as described in 3.a., were diluted to 0.1 at OD$_{600nm}$. 100μL of each *Bacillus* spp. spores were added to an Eppendorf tubes and centrifuged at 13,000 rpm for 60 seconds. The supernatant was removed and 100μL of Pro 0 and Pro 0.1, respectively, was added for 30 and 60 seconds. Each treatment sample was diluted to $10^{-3}$ and $10^{-4}$ and 25μL of each dilution was added to an X-plate. After incubation at 37°C for 24hrs, colony count was obtained for each dilution to determine the percentage of inhibition.
Results & Discussion

1. Characterization of 17 Microorganisms

All Microorganisms were divided into four groups: Gram-positive Bacillus and Cocci, Gram-negative, Acid-fast Bacterium and Fungi.

a. Gram-positive Bacteria
   i. Gram-positive Bacillus

   Three Gram-positive Bacillus spp. were gram stained and spore stained for their bacterial and endospore properties. *B. cereus*, *B. megaterium*, and *B. subtilis* were all grown at 37°C. Bacillus spp. can alternate between vegetative and endospore state, depending on their surrounding environment. In the vegetative state, Bacillus spp. were stained using the gram stain (Fig. 10 A- C). Represented by purple rods, indicating Gram-positive bacillus. Bacillus spp. are endospore formers and can form spores in hostile environments. A spore stain was performed to illustrate the endospores in these species (Fig. 10 A’- C’).
ii. Gram-positive Cocci

Five Gram-positive bacteria, *E. faecalis, M. luteus, S. aureus, S. epidermidis,* and *S. mutans,* were analyzed: All the bacteria were grown at 37°C, except for *M. luteus,* which was grown at 25°C. A Gram stain was performed on all five bacteria and were observed to

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**Figure 10.** Gram stain of A. *B. cereus,* B. *B. megaterium,* and C. *B. subtilis* at 1000x. Spore stain of A’. *B. cereus,* B’. *B. megaterium,* and C’. *B. subtilis* at 1000x.
be purple spherical, indicating that they are all gram-positive cocci bacteria, as shown in Fig. 11.

<table>
<thead>
<tr>
<th>Enterococcus faecalis</th>
<th>Micrococcus luteus</th>
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<tr>
<td>A.</td>
<td>B.</td>
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</table>

<table>
<thead>
<tr>
<th>Staphylococcus aureus</th>
<th>Staphylococcus epidermidis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.</td>
<td>D.</td>
</tr>
</tbody>
</table>
b. Gram-negative Bacteria

Seven Gram-negative bacteria were used in this study. *E. aerogenes, E. coli, K. pneumoniae, P. vulgaris, P. aeruginosa, P. fluorescens,* and *S. marcescens* were grown at 37°C, except for *P. fluorescens* and *S. marcescens,* which were grown at 25°C. A Gram stain was performed on all seven bacteria and each bacterium was observed to be pink rods, indicating gram-negative bacillus as shown in Fig. 12.
<table>
<thead>
<tr>
<th><strong>Enterobacter aerogenes</strong></th>
<th><strong>Escherichia coli</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>B.</td>
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<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Klebsiella pneumoniae</strong></th>
<th><strong>Proteus vulgaris</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>C.</td>
<td>D.</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>
**Pseudomonas aeruginosa** | **Pseudomonas fluorescens**
---|---
E. | F.  

![](image)

**Serratia marcescens**

G.  

![](image)

Figure 12. Gram stain of A. *E. aerogenes*, B. *E. coli*, C. *K. pneumoniae*, D. *P. vulgaris*, E. *P. aeruginosa*, F. *P. fluorescens*, and S. *marcescens* at 1000x.

c. Acid-fast bacterium

*M. smegmatis* was grown at 37°C for this study. They have an unusual lipid layer on the cell surface and are difficult to stain by Gram stain technique. Acid-fast stain was
designed specifically for *Mycobacterium* spp. and was used for this microorganism as shown in Fig. 13. The pink rods represent Acid-fast positive.

**Mycobacterium smegmatis**

![Mycobacterium smegmatis](image)

Figure 13: Acid-Fast Stain of *M. smegmatis* at 1000x.

d. **Fungi**

*C. albicans*, an unicellular eukaryotic fungus, was grown at 25°C and stained with methylene blue as shown in Fig. 14. The morphology is very similar to yeast and is spherical.
2. Standard Growth Curves of 17 Microorganisms

A standard growth curve was generated for each organism at its optical growth temperature. The initial optical density (OD) of each culture was 0.1 at 600\text{nm} in a 96-well plate containing TSB. The growth was monitored every hour for 11 hrs and a final reading was performed at 24 hrs. Each standard growth curve exhibited a lag phase, exponential (log) phase, and stationary phase. The generation time of each bacterium was determined by the log phase of growth. During the exponential phase of growth for each microorganism the generation time was calculated using the formula:

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

This formula generates the doubling time required for each microorganism’s growth at optimal temperature. At the end of 24 hrs, the OD was obtained to determine the density of the cells at the end of monitoring time.

a. Gram-positive Bacteria

i. Gram-positive Bacillus

The standard growth curves for three Bacillus spp. were monitored and shown in Fig. 15A-C. The growth curves are similar. All Bacillus spp. were observed to be entering
exponential phase at 4 hrs. At exponential growth, the cells have constant generation time: *B. cereus* was 43.56 mins, *B. megaterium* was 65.39 mins, and *B. subtilis* was 77.50 mins. *B. cereus*, *B. megaterium*, and *B. subtilis* reached the stationary phase at 6, 10 and 8, respectively. Since these microorganisms are endospore formers, the monitoring was continued up to 24 hrs.
Figure 15. Standard Growth Curves of three gram-positive bacillus cultures were performed in triplicates with the mean and standard deviation (SD) represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.

**ii. Gram-positive Cocci**

The standard growth curves for *E. faecalis, M. luteus, S. aureus, S. epidermidis* and *S. mutans* were generated as shown in Fig. 16. *E. faecalis, S. aureus, and S. mutans* were observed to have lag phase for about 2 hrs. *M. luteus* and *S. epidermidis* had lag phase for 3 and 4 hrs, respectively. The stationary phases of *E. faecalis, M. luteus*, and *S. aureus* were achieved at hour 8. Both *S. epidermidis* and *S. mutans* were observed to enter stationary phase at hour 10. The generation time for each bacterium were as follows: for *E. faecalis* was 84.41 mins, *M. luteus* was 73.15 mins, *S. aureus* was 94.71 mins, *S. epidermidis* was 56.87 mins, and *S. mutans* was 51.56 mins.

![Standard Growth Curve](image)
Figure 16. Standard Growth Curves of five gram-positive cocci cultures were performed in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs and a final reading taken at 24 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600nm.
b. Gram-negative Bacteria

The standard growth curves for *E. aerogenes*, *E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *P. fluorescens*, and *S. marcescens* were generated as shown in Fig. 17. Similar initiation of exponential phase was observed at hour 4 in *E. aerogenes*, *P. vulgaris*, *P. aeruginosa*, and *S. marcescens*. *K. pneumoniae*, *P. vulgaris*, and *P. aeruginosa* reached to the stationary phase by 6 hrs or 7 hrs. *S. marcescens* reached stationary phase at 8 hrs. While *E. aerogenes*, *E. coli* and *P. fluorescens* stayed exponential up to 11 hrs. The final reading for *E. aerogenes*, *E. coli* and *P. fluorescens* indicated that growth occurred past 11 hrs. Increased growth was observed at the final reading at 24 hrs. The generation time for *E. aerogenes* was 85.91 mins, *E. coli* was 80.07 mins, *K. pneumoniae* was 9.15 mins, *P. vulgaris* was 64.26 mins, *P. aeruginosa* was 55.16 mins, *P. fluorescens* was 124.3 mins, and *S. marcescens* was 73.67 mins.

![A. Standard Growth Curve of Enterobacter aerogenes](image1)

![B. Standard Growth Curve of Escherichia coli](image2)
C. Standard Growth Curve of *Klebsiella pneumoniae*

D. Standard Growth Curve of *Proteus vulgaris*

E. Standard Growth Curve of *Pseudomonas aeruginosa*

F. Standard Growth Curve of *Pseudomonas fluorescens*
Figure 17. Standard Growth Curves of seven gram-negative bacillus cultures were performed in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs and a final reading taken at 24 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.
c. Acid-fast bacterium

A Standard Growth Curve of Acid-Fast bacterium, *M. smegmatis*, was generated as shown in Fig. 18. It had a long lag phase for 4 hrs and reached the stationary phase by 6 hrs. *M. smegmatis* had a generation time of 74.30 mins.

Figure 18. A Standard Growth Curve of Acid-Fast bacterium, *M. smegmatis*, was performed in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs and a final reading taken at 24 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.
d. Candida albicans

A Standard Growth Curve of *C. albicans* was generated as shown in Figure 19. It showed that the lag phase lasted for 3 hrs. Finally, reaching the stationary phase by 7 hrs. The calculated generation time for *C. albicans* was 87.1 mins.

![Standard Growth Curve of Candida albicans](image)

Figure 19. A Standard Growth Curve of Fungi, *C. albicans*, was performed in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs and a final reading taken at 24 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.

The generation times for all of these microorganisms are longer than the reported times. This may be because growth was monitored in a 96-well plate and agitation at 125 rpm less than the regular growth at 250 rpm. In addition, a total of 100μl of culture was used in each study. The nutrient may be a limiting factor for their growth thus many of the organisms reached to the stationary phase earlier than expected.

*Bacillus* spp., *Enterobacter aerogenes*, *Escherichia coli*, and *Pseudomonas fluorescens* were still in the exponential phase at hour 11. The growth was monitored for 24 hrs for these organisms. The results suggested that they were continued growth throughout the monitoring time. The constructed growth curves of *Bacillus* spp. were
similar to growth curve observed by (Lalata, 2017); this may due to the formation of endospores.

3. The Growth of Microorganisms in Different Percentages of Sucrose

It has been reported that sugar can affect the biofilm formation (Leme et al., 2006), thus the effect of sucrose on the growth of microorganisms was studied. Each microorganism was grown in sucrose concentrations of 0%, 0.1%, 0.5%, 1%, and 5% and growth curves were generated accordingly (Fig. 20-24) at optical growth temperature. The growth in different conditions were compared with the control. Through the monitoring process, the generation time and final cell density were summarized in Table 3 for each bacterium.

a. Gram-positive Bacteria

i. Gram-positive Bacillus

The growth pattern of gram-positive bacillus in different sugar concentrations was very similar to the control as shown in Fig. 20. Sucrose does not affect the length of the log phase, but the end cell density changed. The generation time was determined in different sucrose concentrations in each bacterium, summarized in Table 3. B. cereus at 0%, 0.1%, 0.5%, 1% and 5% have a generation time of 43.56mins, 44.52 mins, 42.08 mins, 43.53 mins, and 76.61mins, respectively. B. megaterium at 0%, 0.1%, 0.5%, 1% and 5% have a generation time of 65.39mins, 67.49 mins, 60.97 mins, 48.66 mins, and 53.33 mins, respectively. B. subtilis 0%, 0.1%, 0.5%, 1% and 5% at 77.50 mins, 93.87 mins, 77.86 mins, 74.09 mins, and 91.83 mins, respectively. These results suggested that with increasing amounts of sucrose concentrations the growth of Bacillus spp. was enhance compared with the control, except B. cereus in 5% sucrose. The shortest generation in each bacterium is different in different sucrose concentrations: for B. cereus (0.5%) was 42.08 mins, B. megaterium (1%) was 48.66 mins, and B. subtilis (1%) was 74.09 mins. The cell density of each Bacillus spp. was monitored until 11 hrs and evaluated at each sucrose concentration. The results for B. cereus indicated that the cell density was higher in all sucrose concentrations than the control. B. megaterium had the shortest generation time in
1% sucrose, but the final cell density was higher in 0.1 and 0.5% sucrose after 11 hrs. The cell density of *B. subtilis* was higher than control for 0.1%, 0.5% and 1%.
Figure 20. Growth Curves of three gram-positive bacillus cultures with different sucrose concentrations were generated in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.

ii. Gram-positive Cocci

The growth pattern for all sugar concentrations for *M. luteus*, *S. aureus*, *S. epidermidis*, and *S. mutans* are very similar to the control as shown in Fig.21. The generation time was determined in different sucrose concentrations and each bacterium. *E. faecalis* at 0%, 0.1%, 0.5%, 1% and 5% had generation time of 84.41 mins, 77.61 mins, 52.28 mins, 57.73 mins, and 192.4 mins, respectively. *M. luteus* at 0%, 0.1%, 0.5%, 1% and 5% had generation time of 73.15 mins, 77.87 mins, 83.94 mins, 84.57 mins, and 48.89 mins, respectively. *S. aureus* at 0%, 0.1%, 0.5%, 1% and 5% had generation time of 94.71 mins, 100.3 mins, 93.55 mins, 105.9 mins, and 94.39 mins, respectively. *S. epidermidis* at 0%, 0.1%, 0.5%, 1% and 5% had generation time of 56.87 mins, 68.69 mins, 98.87 mins, 65.04 mins, and 82.68 mins, respectively. *S. mutans* at 0%, 0.1%, 0.5%, 1% and 5% had generation time of 51.56 mins, 52.71 mins, 53.84 mins, 59.21 mins, and 42.43 mins, respectively. The results suggest that with increasing amounts of sucrose concentrations, gram positive cocci growth was enhanced, except for *S. epidermidis*. The shortest generation in each bacterium is different; for *E. faecalis* (0.5%) was 52.58 mins, *M. luteus* (5%) was 48.89 mins, *S. aureus* (0.5%) was 93.55 mins, *S. epidermidis* (0%) was 56.87 mins, and *S. mutans* (5%) was 42.43 mins. At all sucrose concentrations *S. mutans* was observed to have a final cell density higher than the control. *S. epidermidis* had a higher cell density in sucrose concentrations of 0.1%, 0.5% and 1%. Through the turbidity study, the final cell density of *E. faecalis* was highest in sucrose concentrations 0.1% and 0.5%. *S. aureus* was observed most cells with 0.5% and 1% sucrose.
b. Gram-negative Bacteria

The growth patterns of gram-negative bacteria in different sugar concentrations are shown in Fig. 22. The generation time was determined in different sucrose concentrations in each bacterium. The shortest generation in each bacterium was: 71.55 mins for *E. aerogenes* (5%), 48.89 mins for *E. coli* (0.5%), 53.46 mins for *K. pneumoniae* (1%), 64.26 mins for *P. vulgaris* (0%), 50.65 mins for *P. aeruginosa*, 98.46 mins for *P. fluorescens* (0.1%) and 68.19 mins for *S. marcescens* (1%). All gram-negative bacillus showed that in all sucrose concentrations growth was enhanced, except *P. vulgaris*. The generation time was determined in different sucrose concentrations and each bacterium as summarized in Table 3. *E. aerogenes* at 0%, 0.1%, 0.5%, 1% and 5% had generation time of 85.91 mins, 75.66 mins, 71.82 mins, 64.53 mins, and 71.55 mins, respectively. The generations times calculated for *E. coli* at 0%, 0.1%, 0.5%, 1% and 5% were, 80.07 mins, 98.07 mins, 65.57 mins, 103.1 mins, and 89.62 mins, respectively. *K. pneumoniae* at 0%,
0.1%, 0.5%, 1% and 5% had generation times of 59.15 mins, 69.39 mins, 96.08 mins, 53.46 mins, and 65.68 mins, respectively. The generation times of *P. vulgaris* at 0%, 0.1%, 0.5%, 1% and 5% were calculated to be 64.26 mins, 82.74 mins, 74.74 mins, 74.43 mins, and 84.09 mins, respectively. *P. aeruginosa* at 0%, 0.1%, 0.5%, 1% and 5% generation times were calculated to be 55.16 mins, 63.82 mins, 54.14 mins, 50.65 mins, and 60.50 mins, respectively. *P. fluorescens* grown in 0%, 0.1%, 0.5%, 1% and 5% had generation times of 124.7 mins, 98.46 mins, 109.0 mins, 138.2 mins, and 160.2 mins, respectively. *S. marcescens* at 0%, 0.1%, 0.5%, 1% and 5% had generation times of 73.67 mins, 74.49 mins, 68.74 mins, 68.19 mins, and 90.53 mins, respectively. The shortest generation time in each bacterium was different; *E. aerogenes* (5%) was 71.55 mins; *E. coli* (0.5%) was 48.89 mins; *K. pneumoniae* (1%) was 53.46 mins; *P. vulgaris* (0%) was 64.26 mins; *P. aeruginosa* (1%) was 50.65 mins; *P. fluorescens* (0.1%) was 98.46 mins; and *S. marcescens* (1%) was 68.19 mins. Final cell densities of both *P. vulgaris* and *P. aeruginosa* were higher at sucrose concentration of 1%, which correlates with shortest duration of growth of *P. aeruginosa*. *E. coli* and *S. marcescens* both had higher cell density at 0.1%, 0.5% and 1% sucrose concentrations. *K. pneumoniae* 11 hrs cell density reading did not increase with any sucrose concentration. While *E. aerogenes* had an increase in cell density at 0.1% and 0.5%, *P. fluorescens* had an increase at 0.5%, and 1%.
G.

Figure 22. Growth Curves of seven gram-negative bacillus cultures with different sucrose concentrations were generated in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs and a final reading taken at 24 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.

c. Acid-fast bacterium

The growth pattern of gram negative bacteria in different sugar concentrations are shown in Figure 23. The growth pattern of *M. smegmatis* decreased with increasing amounts of sucrose concentrations as shown in Table 3. The shortest generation time for *M. smegmatis* was (0%) 74.30 mins. *M. smegmatis* at 0%, 0.1%, 0.5%, 1% and 5% had a generation time of 74.30 mins, 97.92 mins, 92.55 mins, 82.47 mins, and 89.28 mins, respectively. At 0.1% and 0.5%, the final density was higher compared to the control after 11 hrs.
Figure 23. A Growth Curve of Acid-fast positive bacterium, *M. smegmatis*, with different sucrose concentrations were generated in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs and a final reading taken at 24 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600 nm.

**d. Candida albicans**

The growth of *C. albicans* was monitored in sucrose concentrations shown in Figure 24. The growth pattern of *C. albicans* increased with amounts of sucrose concentrations. The shortest generation time for *C. albicans* was (0.5%) 86.14 mins observed in bold and highlighted green in Table 3. *C. albicans* at 0%, 0.1%, 0.5%, 1% and 5% had generation times of 87.1 mins, 100.2 mins, 86.14 mins, 87.78 mins, and 91.07 mins, respectively. The final cell density of *C. albicans* did not increase compared to control.
Figure 24. A Growth Curve of Fungi, *C. albicans*, with different sucrose concentrations were generated in triplicates with the mean and SD represented. The growth curve was obtained through the turbidity study from 0 to 11 hrs. The x-axis represents the time in hrs and the y-axis represents the absorbance at 600nm.
<table>
<thead>
<tr>
<th>Bacterial Name</th>
<th>Generation time in Sucrose Concentrations</th>
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<tr>
<td></td>
<td>Gram-Positive Bacillus</td>
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<tr>
<td></td>
<td>0%</td>
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<tr>
<td><strong>B. cereus</strong></td>
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<td>Cell Density</td>
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<td><strong>B. megaterium</strong></td>
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<tr>
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<td>77.50</td>
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<td>Cell Density</td>
<td>+</td>
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<tr>
<td></td>
<td>Gram-Positive Cocci</td>
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<td><strong>E. faecalis</strong></td>
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<td>Cell Density</td>
<td>+</td>
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<td><strong>M. luteus</strong></td>
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<td>-</td>
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<tr>
<td><strong>S. aureus</strong></td>
<td>94.71</td>
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<tr>
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<tr>
<td><strong>S. mutans</strong></td>
<td>51.56</td>
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<tr>
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<tr>
<td></td>
<td>Gram-Negative Bacillus</td>
</tr>
<tr>
<td><strong>E. aerogenes</strong></td>
<td>85.91</td>
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<tr>
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<td><strong>K. pneumoniae</strong></td>
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<tr>
<td>Fungi</td>
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<td>C. albicans</td>
<td>87.1</td>
</tr>
<tr>
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<td>-</td>
</tr>
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</table>

Table 3. The generation times of 16 microorganisms are shown for control and bacterium grown in 0.1%, 0.5%, 1%, and 5% of sucrose, calculated in minutes.

In summary, the generation times varied depending on organisms as well as sucrose concentrations. The generation times shortened in most microorganisms in the presence of sucrose, except for S. epidermidis, P. vulgaris, and M. smegmatis. The growth of these three bacterial spp. was not enhanced in any sucrose concentration.

4. The Effect of EGCG-S on All Microorganisms

To determine the effect of EGCG-S on all microorganisms, CFU Assay was used. The cultures were treated with 250μg/mL EGCG-S for 2 hrs and 24 hrs, using serial dilution and were plated on TSA Y-plates in triplicate. The colonies were counted 24 hrs later after incubation and the percentage of inhibition was calculated for each microorganism. Sucrose (0.1%) was also used to study the effect of sugar on EGCG-S efficacy as an antimicrobial agent.
a. Gram-positive Bacteria

i. Gram-positive Bacillus

The effects of EGCG-S (250μg/mL) on gram positive endospore formers are shown in Fig. 25. *Bacillus* spp. at 2 hours treatment, all had inhibition up to ~96 to 97%. At 24 hrs, the percentage of inhibition reached 99%. In the presence of 0.1% sucrose, for 2 hr. treatment, the percentage of inhibition changed compared to the controls: 87% for *B. cereus*, 98% for *B. megaterium*, and 91% for *B. subtilis*. After 24-hour treatment, the inhibition reached near 100%. All *Bacillus* spp. were very sensitive to EGCG-S treatment with 250μg/mL for both 2 hours and 24 hours treatment. The results indicated that EGCG-S can effectively inhibit the growth of *Bacillus* spp. In the presence of 0.1% sucrose at 2 hrs, the % of inhibition reduced but at 24 hours, the inhibition was nearly complete. This suggested that sucrose concentration does impact the inhibition of *Bacillus* spp.; prolong exposure to EGCG-S is needed to reach complete inhibition.
C.

Figure 25. The Percent of Inhibition of three Bacillus spp. at sucrose concentrations of 0% and 0.1% for 2 and 24 hr treatment with 250μg/ml of EGCG-S.

ii. Gram-positive Cocci

All Gram-positive coccus spp. were susceptible to the EGCG-S treatment with 250μg/mL within 2 hrs and 24 hrs as shown in Figure 26. For 2 hrs of treatment, the % of inhibition ranged from 92 to 99%, and for 24 hours of treatment, the % of inhibition all reached 99% except S. aureus, which is 93.9%. In the presence of 0.1% sucrose, 2 hrs treatment, the % of inhibition was reduced in all the bacteria except E. faecalis. M. luteus had the lowest percent of inhibition at 69.2%; S. aureus at 77.1%. Growth in 0.1% sucrose had a decreased inhibition for most cocci spp. at 2 hrs. This indicated that sucrose is able to enhance the growth and hinder the activity of EGCG-S on these bacteria and may require more than 2 hrs treatment time. After treatment for 24 hrs in 0.1% sucrose, all coccus spp. had inhibition between 95.43 to 99.96%.
A. The Effect of EGCG-S on the Growth of *Enterococcus faecalis* at 0 and 0.1% Sucrose

B. The Effect of EGCG-S on the Growth of *Micrococcus luteus* at 0 and 0.1% Sucrose

C. The Effect of EGCG-S on the Growth of *Staphylococcus aureus* at 0 and 0.1% Sucrose

D. The Effect of EGCG-S on the Growth of *Staphylococcus epidermidis* at 0 and 0.1% Sucrose
E. 

Figure 26. The Percent of Inhibition of five gram-positive cocci bacteria at sucrose concentrations of 0% and 0.1% for 2 and 24 hrs treatment with 250μg/ml of EGCG-S.

b. Gram-negative Bacteria

The effects of treatment of EGCG-S on Gram-negative bacteria are shown in Figure 27. Two hr treatment with 250μg/ml of EGCG-S for all gram-negative bacteria, except K. pneumoniae, was able to inhibit growth between 92.1 to 99.96%. K. pneumoniae was inhibited by 71.93%. After 24 hr. treatment, all organisms were inhibited from 99.22 to 99.96%. In the presence of 0.1% sucrose, Gram-negative bacteria inhibition with 250μg/ml of EGCG-S had decreased to 81.98 to 99.42%. Twenty-four-hour treatment was able to inhibit bacterial growth from 97.10 to 99.99%.
A. The Effect of EGCG-S on the Growth of *Enterobacter aerogenes* at 0 and 0.1% Sucrose

B. The Effect of EGCG-S on the Growth of *Escherichia coli* at 0 and 0.1% Sucrose

C. The Effect of EGCG-S on the Growth of *Klebsiella pneumoniae* at 0 and 0.1% Sucrose

D. The Effect of EGCG-S on the Growth of *Proteus vulgaris* at 0 and 0.1% Sucrose
Figure 27. The Percent of Inhibition of seven gram-negative bacteria are shown for sucrose concentrations 0% and 0.1% for 2 and 24 hrs treatment with 250μg/ml of EGCG-S.
c. Acid-fast Bacterium

The results of treatment of *M. smegmatis* with 250μg/mL of EGCG-S is shown in Fig. 28. It indicated 2 hr treatment of *M. smegmatis* in 0% sucrose and 0.1% sucrose was able to inhibit 78.84 and 66.44% of cell growth, respectively. Twenty-four-hour treatment for both concentrations of sucrose had inhibition of 99.2%.

![The Effect of EGCG-S on the Growth of Mycobacterium smegmatis at 0 and 0.1% Sucrose](image)

Figure 28. The Percent of Inhibition of *M. smegmatis* at sucrose concentrations of 0% and 0.1% for 2 and 24 hrs treatment with 250μg/ml of EGCG-S.

d. Candida albicans

*C. albicans* was vulnerable to the treatment with EGCG-S (Fig. 29) at 2 hrs for both control and 0.1% sucrose, with 89.18% and 90.65% inhibition, respectively. Control treatment was inhibited to 97.55% after 24 hrs. Inhibition of *C. albicans* after 24 hrs with the addition of 0.1% sucrose had 90.41% of inhibition. This indicated that sucrose reduced the activity of EGCG-S in treated *C. albicans*. 
Inhibition of most microorganisms were observed within 2 hrs treatment with 250μg/ml of EGCG-S at no sucrose condition. The minimum inhibitory concentration (MIC) of EGCG-S for all these organisms is 250μg/ml except for *K. pneumoniae* and *M. smegmatis*. These two organisms require longer time (24 hours) to reach the MIC. A decrease in inhibition at 0.1% sucrose was observed in most organisms. This indicates that sucrose has an impact on the effectiveness of EGCG-S.

Figure 29. The Percent of Inhibition of *C. albicans* at sucrose concentrations of 0% and 0.1% for 2 and 24 hrs treatment with 250μg/ml of EGCG-S.
<table>
<thead>
<tr>
<th>Bacterial name</th>
<th>Percent of Inhibition in Sucrose Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gram-Positive Bacillus</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
</tr>
<tr>
<td>Time</td>
<td>2 hours</td>
</tr>
<tr>
<td>B. cereus</td>
<td>96.58</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>96.67</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>95.57</td>
</tr>
<tr>
<td></td>
<td>Gram-Positive Cocci</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>92.05</td>
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<tr>
<td>M. luteus</td>
<td>94.09</td>
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<tr>
<td>S. aureus</td>
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<tr>
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<td>S. mutans</td>
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</tr>
<tr>
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<td>Gram-Negative Bacillus</td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>92.91</td>
</tr>
<tr>
<td>E. coli</td>
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<td>K. pneumoniae</td>
<td>71.93</td>
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<tr>
<td>P. vulgaris</td>
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<td>P. aeruginosa</td>
<td>99.96</td>
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<td>P. fluorescens</td>
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<td>M. smegmatis</td>
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</tr>
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<td></td>
<td>Fungi</td>
</tr>
<tr>
<td>C. albicans</td>
<td>89.18</td>
</tr>
</tbody>
</table>

Table 4. Summary of Percent Inhibition of 17 microorganisms grown in 0 and 0.1% sucrose treated with 250μg/ml EGCG-S for 2 and 24 hrs.
e. Live/Dead Assay

LIVE/DEAD® Baclight™ Assay was performed to differentiate the viability of live cells (green) from dead cells (red). Overnight cultures were stained with LIVE/DEAD dye and viewed under 1000X using Zeiss fluorescence microscope (Fig. 30-32). Bacteria treated with 250μg/ml of EGCG-S for 24 hrs were stained red, indicating that all treated samples were dead.

a. Gram-positive Bacteria

i. Gram-positive Bacillus

<table>
<thead>
<tr>
<th>Bacteria Name</th>
<th>Control</th>
<th>Treated with 250μg/mL of EGCG-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td>![Control Image]</td>
<td>![Treated Image]</td>
</tr>
<tr>
<td>B. megaterium</td>
<td>![Control Image]</td>
<td>![Treated Image]</td>
</tr>
</tbody>
</table>
Figure 30. Fluorescent microscopy of five gram-positive bacillus bacteria at 1000x.

ii. Gram-positive Cocci

<table>
<thead>
<tr>
<th>Bacteria Name</th>
<th>Control</th>
<th>Treated with 250μg/mL of EGCG-S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td><img src="image1" alt="Control Image" /></td>
<td><img src="image2" alt="Treated Image" /></td>
</tr>
<tr>
<td><em>M. luteus</em></td>
<td><img src="image3" alt="Control Image" /></td>
<td><img src="image4" alt="Treated Image" /></td>
</tr>
</tbody>
</table>
Figure 31. Fluorescent microscopy of five gram-positive cocci bacteria at 1000x.
b. Gram-negative Bacteria

<table>
<thead>
<tr>
<th>Bacteria Name</th>
<th>Control</th>
<th>Treated with 250μg/mL of EGCG-S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. aerogenes</em></td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Figure 32. Fluorescent microscopy of two gram-negative bacteria at 1000x.

The Live/Dead Assay demonstrates that gram-positive and gram-negative bacteria were viable before treatment. Treatment with 250μg/ml of EGCG-S was able to kill all the bacteria used in this study within 24 hrs.

6. Effect of Green Tea Polyphenol EGCG-S on Bacterial Biofilm

Brain heart infusion agar with different sucrose concentrations at 0%, 0.1%, 0.5%, 1%, and 5% with Congo Red was used for this experiment. Congo red containing media was used to detect the presence of biofilm. Each organism was treated for 24 hrs in an Eppendorf tube with EGCG-S concentrations of 100μg/mL, 250μg/mL, and 500μg/mL, respectively. Untreated cultures were used as control for each condition. After treatment, 10μl of each sample was plated onto Congo red plates with various sugar concentrations.
and incubated 37°C for 24 hrs and 48 hrs for observation. Qualitative analysis was used to determine the presence of biofilm formation. The level of Biofilm formation was assigned a range (Fig. 33B). The possible outcomes: No growth, growth with no biofilm production, growth with biofilm production as shown in Fig. 34.

Figure 33. Congo Red Assay Set up (A) and Biofilm Formation Guide (B)

<table>
<thead>
<tr>
<th>Growth No biofilm</th>
<th>No Growth/No Biofilm</th>
<th>Severely inhibited +/-</th>
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<tr>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

Figure 34. Congo Red Assay Results. Visible outcome results for Congo Red Assay.
A. Congo Red Assay on Biofilm Formation

a. Gram-positive Bacteria

i. Gram-positive Bacillus

All *Bacillus* spp. at all sucrose concentrations were able to produce biofilm after 24 hrs. At 24 hrs, *B. cereus* had formed biofilm in samples treated with 100μg/mL and 250μg/mL EGCG-S. With 500μg/mL EGCG-S was able to inhibit growth and biofilm formation at 24 hrs. Biofilm formation was observed at 48 hrs for *B. cereus* at all conditions. Biofilm formation for *B. megaterium* and *B. subtilis* was observed at all sucrose concentrations and all concentrations of EGCG-S at 24 and 48 hrs.
### Bacillus megaterium

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### Bacillus subtilis

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<tr>
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</table>
Figure 35. Biofilm formation of three gram-positive bacillus cultures on Congo Red Assay with Sucrose

ii. Gram-positive Cocci

Biofilm formation was observed at all sucrose concentrations, except at 0% with 500μg/mL EGCG-S within 24 hrs. Biofilm formed at all sucrose concentrations and all EGCG-S concentrations by 48hrs. Both *M. luteus* and *S. mutans*, were able to inhibit biofilm formation at all sucrose concentrations treated with 500μg/mL EGCG-S at 24 and 48hrs. Observation of *S. aureus* 24 hrs had no biofilm formation at 500μg/mL EGCG-S at all sucrose concentrations. After 48 hrs, biofilm formation was observed at 1% and 5% sucrose with 500μg/mL EGCG-S. *S. epidermidis* treated with 500μg/mL EGCG-S was able to inhibit biofilm formation at 0%, 0.1%, and 5% sucrose concentrations at 24 and 48 hrs. Slight biofilm formation was observed at 24 and 48 hrs with 500μg/mL EGCG-S at 1% sucrose.
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**Micrococcus luteus**

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<tr>
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<td><strong>Growth -</strong></td>
<td><strong>Growth -</strong></td>
<td>++</td>
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<td><strong>No Growth</strong></td>
<td><strong>No Growth</strong></td>
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### Staphylococcus aureus

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### Staphylococcus epidermidis

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Figure 36. Biofilm formation of five gram-positive coccus cultures and *M. smegmatis* on Congo Red Assay with Sucrose
b. Gram-negative Bacteria

After 24 hrs, *E. aerogenes* treated with at 100μg/mL and 250μg/mL EGCG-S had formed biofilm at all sucrose concentrations. Concentration of 500μg/mL EGCG-S was able to inhibit biofilm formation after 24 hrs at all sucrose concentrations. *E. coli* biofilm formation was inhibited with 500μg/mL EGCG-S at both 24 and 48 hours at all sucrose concentrations. *K. pneumoniae* formed biofilm at all sucrose concentrations and all EGCG-S concentrations. *P. vulgaris* treated with 500μg/mL EGCG-S at sucrose concentrations of 0%, 0.1%, and 1% did not form biofilm at 24 and 48hrs. At 5% sucrose with 500μg/mL EGCG-S *P. vulgaris* was able to form biofilm at 24 and 48 hrs. Results for *P. aeruginosa* and *P. fluorescens* were the same, where biofilm formation was inhibited with 500μg/mL EGCG-S at 24 and 48 hrs at all sucrose concentrations. *S. marcescens* biofilm formation was inhibited at all sucrose concentrations with 500μg/mL EGCG-S at 24 and 48 hrs, except at 5% sucrose with 500μg/mL EGCG-S after 48 hrs biofilm formation was observed.

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| 48 hrs   | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |

*Enterobacter aerogenes*
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**Klebsiella pneumoniae**

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Proteus vulgaris

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Pseudomonas aeruginosa
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**Pseudomonas fluorescens**

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Figure 37. Biofilm formation of seven gram-negative bacillus cultures on Congo Red Assay with Sucrose

**c. Acid-fast Bacterium**

Biofilm formation at all sucrose concentrations with EGCG-S at 100µg/mL and 250µg/mL was observed within 24 hrs. EGCG-S at 500µg/mL was able to inhibit biofilm formation at all sucrose concentrations at 24 and 48 hrs.
Most of the bacteria evaluated formed biofilm as early as 24 hrs and by 48 hrs all bacterial spp. had formed biofilm in all the sucrose concentrations. For some gram-positive bacteria, *B. megaterium*, *B. subtilis* and *S. mutans*, biofilm was not obvious at 24 hrs at sucrose concentrations of 0% and 0.1%. For 1% and 5% sucrose, the biofilm formation was significant. For gram negative bacteria similar results were observed in *E. aerogenes* and *K. pneumoniae*. For *P. vulgaris*, 0% of sucrose the biofilm was not obvious but at 0.1% sucrose the biofilm was clearly observed. For *P. fluorescens* no biofilm formation was observed in all sucrose concentrations. Different concentrations of EGCG-S were used to study the effect on biofilm formation. Concentrations of 100 and 250μg/mL were not able to inhibit biofilm formation in all the microorganisms tested at all sucrose concentrations.

**Table 1:** Biofilm formation of *Mycobacterium smegmatis* on Congo Red Assay with Sucrose

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<th>Time (hrs)</th>
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<th>1% Sucrose</th>
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Figure 38. Biofilm formation of *M. smegmatis* on Congo Red Assay with Sucrose
The results indicated that EGCG-S at 500µg/ml was able to inhibit growth and biofilm formation in most bacteria. For *B. cereus*, *B. subtilis*, and *P. vulgaris* 24 hrs there was no notable biofilm formation observed but after 48 hrs the biofilm was observed. For *B. megaterium*, biofilm was observed at both 24 and 48 hrs. For *E. faecalis* and *K. pneumoniae*, 500µg/ml of EGCG-S was not able to inhibit biofilm formation. These organisms may require a higher concentration of EGCG-S to inhibit or decrease biofilm formation. It is clearly indicated that sucrose concentration affects biofilm formation by enhancing and reducing the duration of the formation of biofilm. The results suggested that 500µg/mL can inhibit most biofilm formation and possibly the minimum inhibitory concentration (MIC). Further experiments should be carried out to determine the MIC for some bacteria for which the results were not conclusive.

**B. Congo Red Assay on Pre-Formed Biofilm**

Biofilm formation was observed for controls of *S. aureus* grown on Congo Red plates containing 5% sucrose (Fig. 39A-C) for 24 hrs, (Fig 39A). To demonstrate the quantity and viability of *S. aureus* biofilm, images were obtained using inverted and fluorescence microscopy (Fig. 39B and C, respectively). Because the biofilm was extremely dense, an inverted microscope was used to view the plate. Live/Dead Assay indicated that there were viable bacteria and biofilm in the untreated cultures as shown in in Fig. 39C. Once biofilm formation was established, the plates were utilized to determine the effectiveness of EGCG-S in treating pre-formed biofilm. EGCG-S (50µL of 500µg/mL) was added to the center of the pre-formed biofilm and treated for 24 hrs. Inverted and fluorescence microscopy were utilized to compare results to the control. Results indicated that the treatment of *S. aureus* using 50µL of 500µg/mL of EGCG-S was able to revert the black agar to red (Fig. 39B’) and indicated by yellow arrow in Fig. 39A’. Examination using the Live/Dead Assay demonstrates that 50µL of 500µg/mL of EGCG-S was able to kill *S. aureus* that formed biofilm. (Fig. 39.C’). The results suggested that EGCG-S at 500µg/mL was able to kill the biofilm formed cells. This result is promising and further study should be carried out to evaluate other biofilm forming bacteria.
<table>
<thead>
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<th><strong>S. aureus</strong> untreated in 5% sucrose-100x</th>
<th><strong>S. aureus</strong> treated with 500μg/mL of EGCG-S in 5% sucrose - 250x</th>
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*Note: Images A, B, and C show different treatments and conditions of *S. aureus*.*
Figure 39. Control and Treated Biofilm formation of *S. aureus* on Congo Red with 5% sucrose observed using Interested and Fluorescence microscopy. A-C represent controls of *S. aureus* grown on Congo Red plate containing 5% sucrose, inverted microscopy, and fluorescence microscopy, respectively. A’-C’ represents the treatment of *S. aureus* with 500ug/ml EGCG-S, using the same methods, of controls, to evaluate treatment.

8. Pre and Post Application of EGCG-S Using Rapid Agar Plate Assay (RAPA)

The effectiveness of 250μg/mL of EGCG-S was evaluated on pre- and post-application using RAPA.

**a. Gram-positive Bacteria**

**i. Gram-positive Bacillus**

All gram-positive bacillus spp. were used to demonstrate the inhibitory effects of 250μg/mL of EGCG-S with pre- and post-application as shown in Figure 40. Pre-application had a higher inhibition percentage compared to post-application of three *Bacillus* spp. Pre-Application of EGCG-S was able to inhibit all *Bacillus* spp. from 64.92 to 74.57%. The inhibition of growth of three *Bacillus* spp. with post-application of EGCG-S was between 36.43 to 68.53%.

![Graph](image-url)
iii. Gram-positive Cocci

Figure 41 represents the pre- and post-application of gram-positive cocci spp. with EGCG-S. Post-application of EGCG-S had an increased inhibition for *E. faecalis* and *S. epidermidis* at 42.28% and 39.80%, respectively. Inhibition for *M. luteus*, *S. aureus*, and *S. mutans* was increased with pre-application 57.92%, 61.71%, and 91.73%, respectively.
A. Pre & Post Application of EGCG-S on *Enterococcus faecalis*

B. Pre & Post Application of EGCG-S on *Micrococcus luteus*

C. Pre & Post Application of EGCG-S on *Staphylococcus aureus*

D. Pre & Post Application of EGCG-S on *Staphylococcus epidermidis*
b. Gram-negative Bacteria

Pre- and post-application of EGCG-S on gram-negative bacteria was illustrated in Fig. 42. This indicated that post-application of EGCG-S was effective for most gram-negative bacteria, except for *P. fluorescens*. Pre-application inhibited growth of *P. fluorescens* 59.11% compared to pre-application with 43.70%. Post-application was able to inhibit growth of *E. aerogenes*, *E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, and *S. marcescens* by 38.06, 58.04, 20.19, 25.91, 39.69, and 53.84%, respectively.
A. Pre & Post Application of EGCG-S on *Enterobacter aerogenes*

B. Pre & Post Application of EGCG-S on *Escherichia coli*

C. Pre & Post Application of EGCG-S on *Klebsiella pneumoniae*

D. Pre & Post Application of EGCG-S on *Proteus vulgaris*
Figure 42. Percent of Inhibition of seven gram-negative bacillus cultures using Pre- and Post-Application of EGCG-S

c. Acid-fast Bacterium

The results of pre- and post-application of EGCG-S on *M. smegmatis* is shown in Fig. 43. Post-application of EGCG-S with *M. smegmatis* was inhibited by 37.65% compared to 27.60% for pre-application.
Figure 43. Percent of Inhibition of *M. smegmatis* using Pre- and Post-Application of EGCG-S.

d. *Candida albicans*

*C. albicans* sensitive to EGCG- with both pre- and post- application (Fig. 44). The inhibition was over 99.6% inhibition.

Figure 44. Percent of Inhibition of *C. albicans* using Pre- and Post-Application of EGCG-S.
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<tr>
<td>Gram-negative Bacillus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. aerogenes</td>
<td>23.75</td>
<td>38.06</td>
</tr>
<tr>
<td>E. coli</td>
<td>30.05</td>
<td>58.04</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>15.10</td>
<td>20.19</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>6.32</td>
<td>25.91</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>30.61</td>
<td>39.69</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>59.11</td>
<td>45.70</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>28.20</td>
<td>53.84</td>
</tr>
<tr>
<td>Acid-fast bacterium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>27.60</td>
<td>37.65</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>99.68</td>
<td>99.72</td>
</tr>
</tbody>
</table>

Table 5. Summary of percent of inhibition pre and post-application using EGCG-S.

In summary, *C. albicans* growth for pre- and post-application indicated that inhibition with 250μg/mL of EGCG-S was sufficient to hinder growth. Most of the bacteria were observed to have lower inhibition for pre- and post-application with 250μg/mL of
EGCG-S. EGCG-S at 500μg/mL may be required to inhibit growth for pre- and post-applications.

9. Evaluation of the effectiveness of formulated tea as antimicrobial product

These studies used formulations of ProtecTea V 0 and ProtecTea V 0.1 to evaluate their inhibitory effect on *C. albicans* and spores of *Bacillus* spp. ProtecTea V 0 (Pro 0) is the formulated product without tea, and ProtecTea V 0.1 (Pro 0.1) is the formulated product with both EGCG-S and palmitoyl-EGCG (p-EGCG).

**a. Candida albicans Suspension Study**

The Suspension Study demonstrated the effectiveness of Pro 0 and Pro 0.1 on *C. albicans* within 60 seconds (Fig. 45). Treatments with Pro 0 and Pro 0.1 were able to inhibit growth by 78.11 and 97.46%, respectively. The addition of EGCG-S enhances the effectiveness.

![Figure 45. Percent of Inhibition of Suspension Study with *C. albicans*.](image-url)
b. Spore Study

To evaluate if the formulated tea product works on the spores, three different endospore formers, *B. cereus*, *B. megaterium* and *B. subtilis*, were used. They were treated with Pro 0 and Pro 0.1 for 30 seconds and 1 minute, respectively, and CFU was determined as shown in Figure 50. Within 30 seconds of treatment, inhibition of 12.5 to 41.67% was observed in Pro 0 treated samples. The growth of *Bacillus* spp. using Pro 0 was inhibited 64.94 to 79.16 % after 60 seconds. Growth of *B. cereus*, *B. megaterium* and *B. subtilis* was inhibited 86.79, 91.11 and 90.58%, respectively, within 30 seconds treatment with Pro 0.1. While all *Bacillus* spp. were inhibited from 95.97 to 99.4% with Pro 0.1 for 60 seconds.
Figure 47. The percent of inhibition by CFU of Bacillus spp. treated with ProtecTea V 0 and ProtecTea V 0.1 for 30 and 60 seconds.

<table>
<thead>
<tr>
<th></th>
<th>ProtecTea V 0</th>
<th>SD</th>
<th>Log Redu</th>
<th>ProtecTea V 0.1</th>
<th>SD</th>
<th>Log Redu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 sec.</td>
<td>60 sec.</td>
<td></td>
<td>30 sec.</td>
<td>60 sec.</td>
<td></td>
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<tr>
<td>\textit{B. cereus}</td>
<td>41.67</td>
<td>79.16</td>
<td>2.12</td>
<td>0.81</td>
<td>86.79</td>
<td>97.89</td>
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<tr>
<td>\textit{B. megaterium}</td>
<td>12.5</td>
<td>72.09</td>
<td>2.83</td>
<td>1.09</td>
<td>91.11</td>
<td>99.4</td>
</tr>
<tr>
<td>\textit{B. subtilis}</td>
<td>12.29</td>
<td>64.94</td>
<td>2</td>
<td>1.29</td>
<td>90.58</td>
<td>95.97</td>
</tr>
</tbody>
</table>

Table 7. Summary of Percent Inhibition with ProtecTea V 0 and ProtecTeaV 0.1 on \textit{Bacillus} spp. for 30 and 60 seconds.

ProtecTeaV 0.1 is an effective compound for inhibition of \textit{C. albicans} and spores from \textit{Bacillus} spp. with 60 seconds treatment.
Conclusion

The study of green tea polyphenol EGCG-S was used to determine the effectiveness of an antimicrobial against bacterial growth, biofilm formation, endospores, and fungi. The use of carbohydrate source, sucrose, was able to enhance bacterial growth and biofilm formation of most bacteria. The minimum inhibitory concentration (MIC) of most bacterial growth was 250μg/ml within 2 hour treatment inhibition was observed between 92 to 99.7% for gram-positive and 92.1 to 99.96 % for gram-negative bacteria, except for *K. pneumoniae* and *M. smegmatis*. These two organisms require longer treatment times or the MIC may require higher EGCG-S concentration. A decrease in inhibition at 0.1% sucrose was observed in most organism. This indicates that sucrose has an effect on the effectiveness of EGCG-S. From this study the use of Live/Dead Assay also determined that treatment with 250μg/ml of EGCG-S was able to kill the organisms studied. Different concentrations of EGCG-S were used to study their effect on biofilm formation using Congo Red Assay. At 24 hrs, biofilm for most bacteria was observed, except for *B. megaterium*, *B. subtilis* and *S. mutans*. After 48 hrs all bacteria were observed to produce biofilm at all sucrose concentrations. Biofilm was not inhibited with EGCG-S at 100 and 250μg/mL. Results using 500μg/ml of EGCG-S was able to inhibit growth of most bacteria, except for *E. faecalis* and *K. pneumoniae*, did not inhibit biofilm formation. MIC for these organisms need to be further evaluated. Preformed biofilm of *S. aureus* indicated that treatment with 500μg/mL of EGCG-S was able to kill the biofilm formed cells. Pre- and post-application with EGCG-S at 250μg/mL had low inhibition percentages for all bacteria. Pre- and post-application of *C. albicans* had over 99.6% inhibition. Pre- and Post-application of 250μg/mL of EGCG-S with 0.1% sucrose had decrease inhibition for all bacteria. Pre- and post-application with at 0% and 0.1% sucrose EGCG-S at 500μg/mL may be able to inhibit bacterial growth. Compound ProtecTeaV 0.1 was effective on *C. albicans* and endospores of *Bacillus* spp. within 60 seconds. EGCG-S was observed to be a promising antimicrobial agent.
**Future Study**

1. Determine CFU of EGCG-S with sucrose concentrations of 0.5%, 1% and 5% on all microorganisms
2. RAPA will be carried out with higher concentrations of EGCG-S
3. Determine the effectiveness of ProtecTeaV on all bacteria
4. Molecular study to determine the mode of action of EGCG-S on different bacteria
References


