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Comparative Evaluation of the Antibacterial, Anti-biofilm and

Anti-spore effects of Theaflavins and Palmitoyl-EGCG

By

Gabriella M. Appice

Submitted in partial fulfillment of the requirement for the
degree of Master of Science in Biology from the
Department of Biological Sciences of Seton Hall University

May 2017

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Abstract

Tea, one of the most common beverages, originates from the leaves of the *Camellia sinensis* plant. Two major groups of tea are fermented black tea and unfermented green tea. Theaflavins (TFs) are the major polyphenols present in black tea, while mono-palmitoyl-epigallocatechin-gallate (pEGCG) is a modified green tea polyphenol. In this study, the antibacterial effects of TF and pEGCG were evaluated against six selected bacteria, *Staphylococcus epidermidis, Streptococcus mutans, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Proteus vulgaris,* using an antibacterial assay. A viability assay using SYTOX® staining and flow cytometry was also used to determine the effect of these compounds on *S. epidermidis* and *S. mutans.* The anti-biofilm effects of the compounds were also investigated using Congo red assay, resazurin assay, polymerase chain reaction and flow cytometry. Finally, TF and pEGCG were also evaluated on the effects of inhibiting sporulation and germination in *Bacillus* spp. The results indicate that 0.2% TF and 0.2% pEGCG contain strong antibacterial effects against all bacteria tested with an IC_{50} range of approximately 0.05-0.1%. In addition, the viability assay showed that both compounds effectively inhibit the growth of the bacteria as early as 3 hours and can maintain their effect for 24 hours. Results from anti-biofilm assays showed that TF and pEGCG are also highly effective in inhibiting the formation of biofilm. Qualitatively, the compounds prevented the formation of biofilm, indicated by the absence of black colonies on the Congo red assay. Quantitatively 0.5% and 1% concentrations of both TF and pEGCG inhibited biofilm formation in the four biofilm forming bacteria tested. PCR and gel electrophoresis was also performed to study the effect of TF and pEGCG on biofilm forming genes in *S. epidermidis* and *S. mutans.*

Results indicated the presence of bands in the control sets, but absent in the treated samples for both sets of genes, *aap* and *brpA*. In addition, flow cytometry was used to further understand the effects of these compounds on *S. epidermidis* and *S. mutans.* Results showed that 0.5% TF is able to inhibit approximately 86% of biofilm formation in *S. epidermidis* and 88% in *S. mutans,* and 1% pEGCG is able to inhibit about 53% in *S. epidermidis* and 85% in *S. mutans.* Finally, results from sporulation and germination assays indicate that both compounds are capable of inhibiting these processes in spore-forming bacteria. 1% TF and 1% pEGCG inhibit sporulation as well as the germination process. 1% TF inhibits approximately 77% of germination whereas 1% pEGCG inhibits 99.9% of germination in *B. cereus.* These results suggest that tea polyphenols are not only viable antibacterial alternatives, but also potentially promising anti-biofilm and anti-spore agents.

Introduction

Recently there has been an increasing concern with the growing number of antibiotic resistant pathogens. Bacteria are able to desensitize against the antibiotics that once were able to kill them. Antibiotic resistance is a major public health problem with more than 2 million infections and 23,000 deaths per year caused by antibiotic-resistant microorganisms in the United States alone (Michaelidis et. al., 2016). Due to the emerging resistance to drugs by microorganisms there has been an increasing interest in developing new antibacterial therapies. Researchers have turned to nature and are studying the effects of natural antimicrobial compounds, specifically those that are plant-derived (Friedman, 2007).

For centuries, medicinal plants have been used to control human diseases, and in the last years, there has been an increased interest in plant stimulant beverages, such as tea, which has shown to possess anti-cariogenic activity (Ferrazzano et. al., 2009).

Tea, one of the most common beverages worldwide, originates from the leaves of the *Camellia sinensis* plant (Chan et. al., 2011). Two major categories of tea are unfermented green tea and and fully fermented black tea. The polyphenols present in the two types of tea are responsible for possessing antibacterial properties. Polyphenols exhibit a wide range of biological effects, such as anti-inflammatory, antioxidant, antiviral, antibacterial, and anti-cancer (Xu et. al., 2017). In black tea the major polyphenol present is theaflavin (TF) and in green tea the major polyphenol is (-)-epigallocatechin-gallate (EGCG), which in this study, was modified as mono-palmitoyl-epigallocatechin-gallate (pEGCG). Recent literature has reported that both green tea and black tea have potential antimicrobial effects.

Bacteria are categorized into two major groups, Gram-positive and Gram-negative due to the properties of their cell walls. Gram-positive bacteria possess a thick peptidoglycan layer in their cell wall, whereas Gram-negative bacteria possess a very thin peptidoglycan layer and an outer membrane containing lipopolysaccharides. Pathogenic gram positive bacteria include species from genera *Streptococcus* and *Staphylococcus,* which are both cocci, as well as spore forming bacilli, *Bacillus* and *Clostridium.* Although Gram-positive bacteria are typically more sensitive to antibiotics due to the lack of an outer membrane, some have become antibiotic resistant, the most known being MRSA (methicillin-resistant *Staphylococcus aureus*). These resistant gram-positive infections continue to be concerning; however, the rise in antibiotic resistant gram-negative bacteria has now become the most pressing issue in bacterial resistance (Vasoo et. al., 2015). Due to the presence of an outer membrane, Gram-negative bacteria are less susceptible to antibiotics making them extremely pathogenic. Gram-negative bacteria cause various types of infections in humans, ranging from a foodborne illness all the way to the plague. A few examples of Gram-positive bacteria are *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus mutans* (*S. mutans*), and *Bacillus cereus* (*B. cereus*).

S. epidermidis is typically arranged in grape-like clusters. It is normally a part of the human skin flora and is usually not pathogenic. *S. epidermidis* is capable of forming biofilm, which is an important virulence factor (Vandecandelaere et. al., 2017). *S. epidermidis* also serves as a model for the *Staphylococcus* genus, specifically for mutant strains such as MRSA.

S. mutans is also a Gram-positive cocci species and is arranged in a chain. It is typically found in the oral cavity and is known for causing dental caries due to its ability to also form a biofilm (dental plaque) (Subramaniam et. al., 2012).

B. cereus is a Gram-positive rod-shaped species capable of forming endospores. These spores are resistant to heat, cold, radiation, desiccation, and disinfectants (Turnbull, 1996), Due to the production of spores, *B. cereus* is most known for causing foodborne illnesses. The spores survive cooking or pasteurization and are able to germinate and multiply (Turnbull, 1996).

A few examples of Gram-negative bacteria are *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Proteus vulgaris* (*P. vulgaris*).

E. coli is a Gram-negative rod-shaped species found in the normal gut flora of animals and humans (Agin et. al., 1997). It is an opportunistic pathogen meaning that if introduced elsewhere it may cause serious infection. It is most known for causing foodborne illnesses due to fecal contamination or improper meat processing.

P. aeruginosa, a Gram-negative, rod-shaped bacterium, is an extremely opportunistic human pathogen found in soil and water. Due to its ability to thrive in moist environments, it is also found on medical equipment making it one of the major causes of nosocomial infections (Hameed et. al., 2016).

P. vulgaris is also a gram-negative, rod-shaped species. It is typically found in the intestines of humans and animals as well as in soil and water. It is also an opportunistic pathogen known to cause wound infections.

S. epidermidis, S. mutans, E. coli, and *P. aeruginosa* are some of the bacterial species studied that are capable of forming biofilms. Biofilms are complex microbial

communities developed on solid surfaces (Sanchez et. al., 2014). They are composed of colonies of bacterial cells either by the same species or a culmination of multiple species. The bacteria produce exopolysaccharides, which are the major component of the biofilm backbone and help to maintain the integrity of the biofilm as well as protecting against harmful agents (Saini et. al., 2015). Biofilms are unique in that the waste of one bacteria may be a nutrient source for another. The bacteria are able to communicate with another in a process known as quorum sensing. Quorum sensing involves the regulation of specific gene expression through the accumulation of stimuli that mediate intercellular communication (Saini et. al., 2015). Quorum sensing allows for the survival of the biofilm, such as urging the growth of beneficial bacteria and discouraging the growth of competitors.

Biofilm formation begins after successful attachment of planktonic bacterial cells to a solid surface. The cells produce a matrix made up of exopolysaccharides and continue to develop via quorum sensing. As the biofilm continues to mature, new cells may arrive and add to the biofilm while others might detach and go elsewhere. The biofilm is constantly evolving and growing making it nearly impossible to remove once it has successfully matured and established on a surface. The only way to effectively remove a biofilm is to physically scrape if off (Hayrapetyan et. al., 2015). This gives rise to problems since the bacteria are able to establish biofilms on medical equipment such as catheters and endotracheal tubes, leading to further infections in patients. In the United States alone, nosocomial infections (healthcare-associated infections) account for about 1.7 million infections and 99,000 associated deaths each year (Centers for Disease Control and Prevention Report, 2007).

Isolation and characterization of genes involved in biofilm formation may help to further understand the formation of biofilms. Previous studies have indicated the roles of sucrose and glucosyltransferases in *S. mutans* biofilm formation, while others have implicated several genes that are associated with genetic competence, such as *brpA,* which plays a regulatory role in biofilm formation (Motegi et. al., 2006). Whereas some genes may be directly involved in biofilm formation, such as *brpA* in *S. mutans,* others may be indirectly related. Many factors regarded as multifunctional proteins have important roles during several phases of biofilm formation and surface colonization, such as the accumulation associated protein (Aap) and the extracellular matrix binding protein (Embp) (Büttner et. al., 2015). The continued study of genes involved in biofilm formation may lead to ways to learn how to potentially prevent them from occurring.

Bacteria have different ways in order to survive. Some may produce biofilms as previously mentioned, whereas others, such as *Bacillus* and *Clostridium,* undergo a process called sporulation. Sporulation, or endospore formation, is the process where a cell produces a non-reproductive structure called a spore to withstand unfavorable conditions. The genetic information of the mother cell is preserved in the spore. When the spore is reintroduced to favorable conditions, it reactivates and germinates into a new vegetative cell (Ali et. al., 2017). Some common illnesses due to spores are: anthrax caused by *Bacillus anthracis, C. diff* colitis caused by *Clostridium difficile,* and food poisoning caused by *Bacillus cereus.* Recent estimates indicate that there are approximately 84,000 cases of illnesses caused by *B. cereus* annually in the United States alone (Tajkarimi, 2007).

The aim of this study was to investigate the antimicrobial effects of black tea polyphenol, TF, and green tea polyphenol, pEGCG, against six selected bacteria using antibacterial assay. The compounds' inhibitory effects on biofilm formation, sporulation and germination were also evaluated. The anti-biofilm effects were investigated using Congo red assay, resazurin assay, polymerase chain reaction, and flow cytometry. The anti-spore effects were evaluated using sporulation and germination inhibition assays.

Materials and Methods

Bacterial culture maintenance

For this study, Gram-positive species: *Staphylococcus epidermidis* (*S. epidermidis*) (Item # 155556A), *Streptococcus mutans* (*S. mutans*) (ATCC® Number: 25175), *Bacillus cereus* (*B. cereus*) (Item # 154870A) and *Bacillus megaterium* (*B. megaterium*) (Item # 154900A), and Gram-negative species, *Escherichia coli* (*E. coli*) (Item # 155065A), *Pseudomonas aeruginosa* (*P. aeruginosa*) (Item # 155250A), and *Proteus vulgaris* (*P. vulgaris*) (Item # 155240A), were obtained from Carolina Biological (Carolina, Burlington, NC) and ATCC® (Manassas, VA). All bacterial cultures were grown at 37 °C with consistent shaking at 250 rpm. All cultures were maintained in Tryptic Soy Broth (TSB) (Difco™, Sparks, MD).

Compound solution preparation

Theaflavin (TF) was obtained from *DH Nutraceutical, LLC.* The 2% stock solution was made in EtOH/diH_2O (1:1). Mono-palmitoyl-epigallocatechin-gallate (pEGCG) was obtained from *Camillex,* LCC (Augusta, GA). The 10% stock solution was prepared using DMSO. The compounds were kept at 4 °C.

Antibacterial assay

An antibacterial assay was used to observe the antibacterial effects of the natural compounds. The overnight bacterial cultures and 48-well clear microtiter plate were used in this assay. Each well contained 40 µL of bacteria and various concentration of TF (final concentration of 0.2%, 0.5%, and 1%) or various concentration of pEGCG (final

concentration of 0.05%, 0.1% and 0.2%) with a total volume of 1000 μL in each well. The growth of the bacteria with or without natural compounds was monitored by taking the optical density at 600 nm (OD_{600nm}) hourly for up to 8 hours with SpectraMax M5 (Molecular Devices, Sunnyvale, CA). Over the monitoring period, the plate was maintained at 37 °C with shaking.

Viability assay

A viability assay was performed using control samples of *S. epidermidis* and *S. mutans* as well as cells treated with 0.5% TF, 1% pEGCG, and combination of both compounds (0.25% TF and 0.5% pEGCG). Cells were stained with 5 μM SYTOX® in order to detect dead cells. Samples were run and analyzed using MACSQuant® Analyzer 10 every 3 hours for 24 hours.

Congo red assay

Congo red assay was performed to screen the formation of biofilm. Tryptic Soy Agar (TSA) (Difco™, Sparks, MD) with 2% sucrose (Amresco, Solon, OH) and 10X Congo red (Amresco, Solon, OH) were added to each well of a 24-well plate. A total of 50 μL of bacteria with either 50 μL TSB or 50 μL compound was added to the appropriate well. The plate was incubated for 24 hours at 37 °C.

Resazurin assay

Resazurin assay was performed to quantify the formation of biofilm. After incubation, planktonic cells were removed and the wells were washed twice with Phosphate buffered

saline (PBS) to remove any non-adherent cells. Following the wash, 120 μL Resazurin Solution was added to each well. Fluorescence was measured at λ_{ex}=560 nm and λ_{em}=590 nm at 30 minute intervals for 4 hours.

DNA isolation

The DNA extraction method presented in this paper is modified from Cheng and Jiang, 2006 (Cheng et. al., 2006). 1 ml cell suspension was centrifuged at 8,000 x *g* for 2 min at room temperature. After removal of supernatant, cells were washed twice with 400 μL STE Buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) and centrifuged again at 8,000 x *g* for 2 min at room temperature. After removal of supernatant, cells were resuspended in 200 μL 1X TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 100 μL Phenol:Chloroform:Isoamyl Alcohol (25:24:1; pH 8.0) was added, followed by a vortex-mixing step for 90 s. The samples were subsequently centrifuged at 13,000 x *g* for 5 min at 4 °C to separate the aqueous phase from organic phase. 140 μL upper aqueous phase was transferred to a clean 1.5 mL tube. 60 μL 1X TE was added to the tube followed by 100 μL Chloroform. Tubes were mixed by rapid inversion multiple times and centrifuged again at 13,000 x *g* from 5 min at 4 °C to separate upper aqueous phase from lower organic phase. 140 μL of upper aqueous phase was transferred to a new 1.5 mL microfuge tube. 55 μL 1X TE and 5 μL RNase (2 mg/mL RNase A/5000 u/mL RNase T1) were added and incubated at 37 °C for 10 min to digest RNA. 100 μL chloroform was added to the tube, mixed well and centrifuged for 5 min at 13,000 x *g* at 4 °C. 140 μL of upper aqueous phase was transferred to new 1.5 mL tube. 60 μL 1X TE and 100 μL chloroform were added followed by centrifugation again at 13,000 x *g* for 5 min at 4

°C. This was repeated a total of 3 times. The final aqueous phase containing purified DNA was stored at -20 °C. The purity and yield of the DNA were assessed using the A_{260}/A_{280} reading.

Polymerase chain reaction (PCR) and gel electrophoresis

Positive control was prepared by adding 2.5 µL DMSO, 6 µL nuclease free dH_2O, 12.5 µL 2x Master Mix (Promega and 2x GoTaq), 1 µL 400nM forward primer, 2 µL DNA Template, and 1 µL 400 nM reverse primer. Negative control was prepared by adding 2.5 µL DMSO, 8 µL nuclease free dH_2O, 12.5 µL 2x Master Mix (Promega and 2x goTaq), 1 µL 400nM forward primer, and 1 µL 400nM reverse primer. Samples were spun down with an annealing time of 45 seconds. Samples were loaded and ran through 35 cycles of PCR. Gel was prepared with 1% Agarose in 1X TAE.

Biofilm viability study with SYTOX®

Flow cytometry was performed to determine the effect of TF and pEGCG on biofilm inhibition of *S. epidermidis* and *S. mutans.* Bacteria were treated with 0.5% TF, 1% pEGCG, and combination of both compounds (0.25% TF and 0.5% pEGCG). Samples were incubated at 37 °C for 24 hours to allow biofilm formation to occur. After incubation, cells were stained with 5 µM SYTOX® and analyzed using MACSQuant® Analyzer 10.

Sporulation inhibition assay

Spore-forming bacteria, *B. cereus,* was starved with diH_2O, 1% TF or 1% pEGCG and for 24 hours. Schaeffer-Fulton stain procedure was performed to observe spore formation inhibition.

Germination inhibition assay

B. cereus was starved in diH_2O for 48 hours to induce spore formation. The cultures were then split into two tubes: TSB only and TSB with TF (1% final concentration) or TSF with pEGCG (1% final concentration). Tubes were then incubated at 37 °C at 250 rpm for 6 hours. Viable count was then used to determine viable cells.

Statistical analysis

All experiments were performed in triplicate and statistical analyses were carried out using Student's t-test. Results with p values less than 0.05 are considered statistically significant.

Results

Antibacterial assay

Six selected bacteria, three gram positive and three gram negative, were treated with or without TF and pEGCG in order to demonstrate the effect of these compounds on bacterial growth. Figures 1-6 illustrate the growth curves of these bacteria with different concentrations of TF and pEGCG. Figure 1A shows the growth curve of *S. epidermidis* with various concentrations of TF. The treatment of TF on *S. epidermidis* compared to control (no treatment) showed significant inhibition of growth demonstrating the antibacterial effect of the compound (p = 0.005). Figure 1B shows the growth curve of *S. epidermidis* treated with various concentrations of pEGCG. This figure provides information on the minimum inhibitory concentration (MIC) and the Inhibitory Concentration 50% (IC_{50}) of the compound. The results indicate that the MIC for *S. epidermidis,* is 0.2% pEGCG showing a significant inhibition of growth (p = 0.00045) with an IC_{50} of 0.1% pEGCG.

Figures 2A and 2B show the effect of TF and pEGCG on *S. mutans.* TF effectively inhibits the growth of *S. mutans* at all concentrations. A 0.2% concentration of pEGCG significantly inhibits the growth of *S. mutans* completely (p = 0.013), and the IC_{50} is 0.1% pEGCG. Figure 3 indicates that both TF and pEGCG are effective in inhibiting *B. cereus.* TF had a MIC of 0.2% and pEGCG with a MIC of 0.2% and IC_{50} of 0.1%.

Figures 4-6 demonstrate the effect of TF and pEGCG on three gram-negative species, *E. coli, P. vulgaris,* and *P. aeruginosa.* Figure 4A shows that all concentrations of TF inhibit the growth of *E. coli,* with a MIC of 0.2%. Figure 4B denotes that 0.2%

pEGCG is the MIC and 0.1% pEGCG is the IC_{50}. TF and pEGCG are also effective in inhibiting the growth of *P. aeruginosa* and *P. vulgaris* with concentrations of 0.2% (Figures 5 and 6).

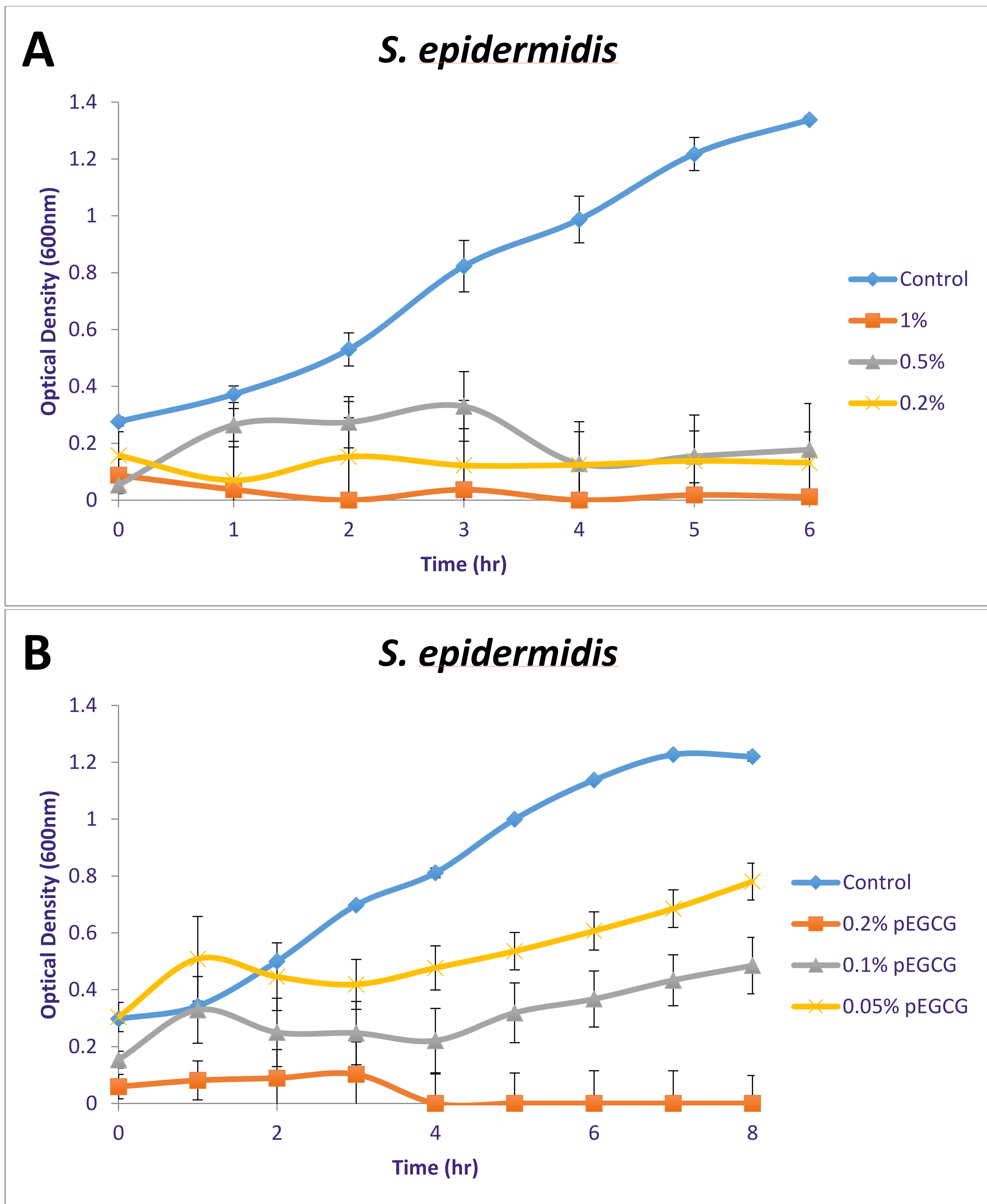

Figure 1. Growth curve of *S. epidermidis* A) *S. epidermidis* treated with various concentrations of TF. B) *S. epidermidis* treated with various concentrations of pEGCG.

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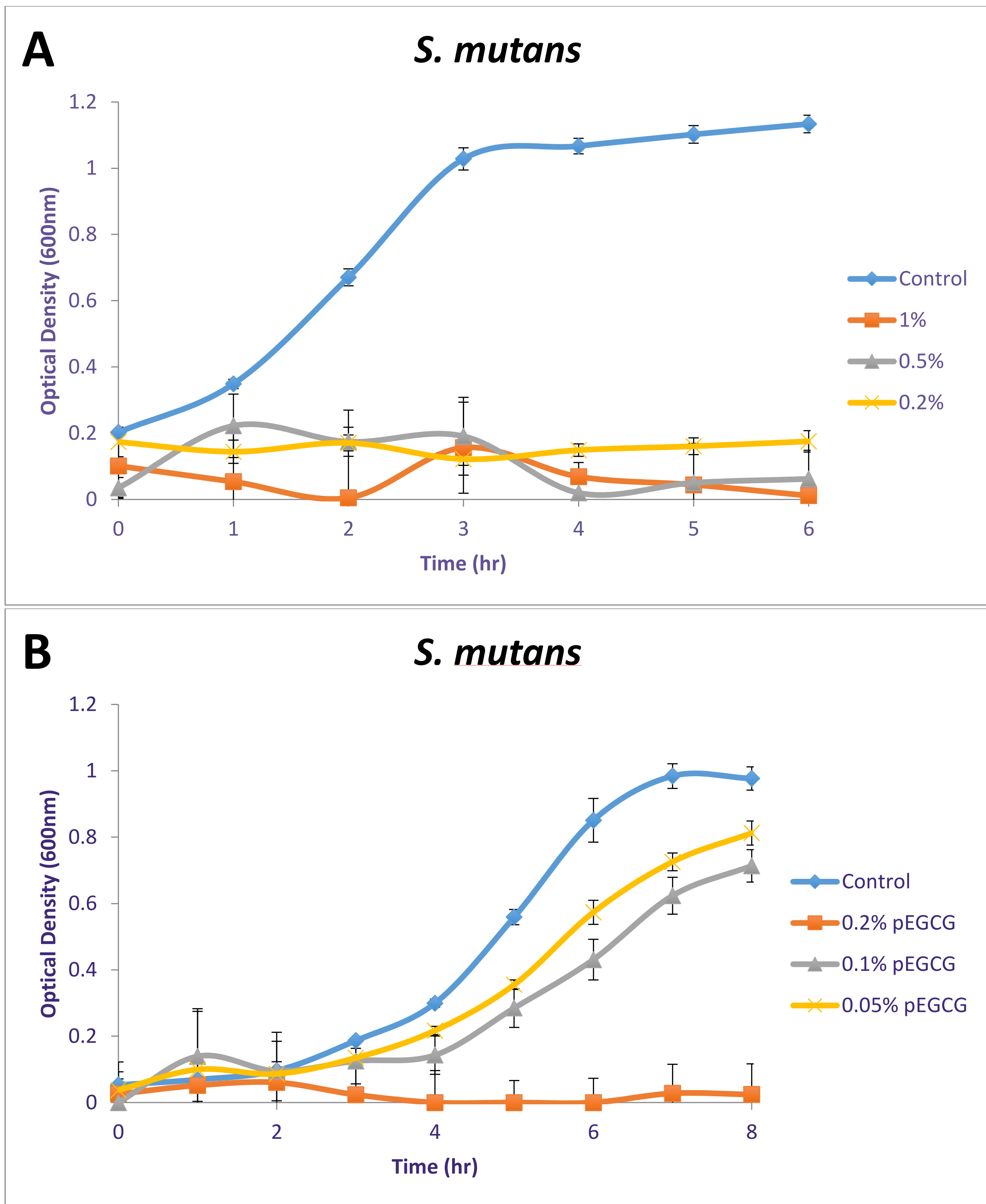


Figure 2. Growth curve of *S. mutans* A) *S. mutans* treated with various concentrations of TF. B) *S. mutans* treated with various concentrations of pEGCG.

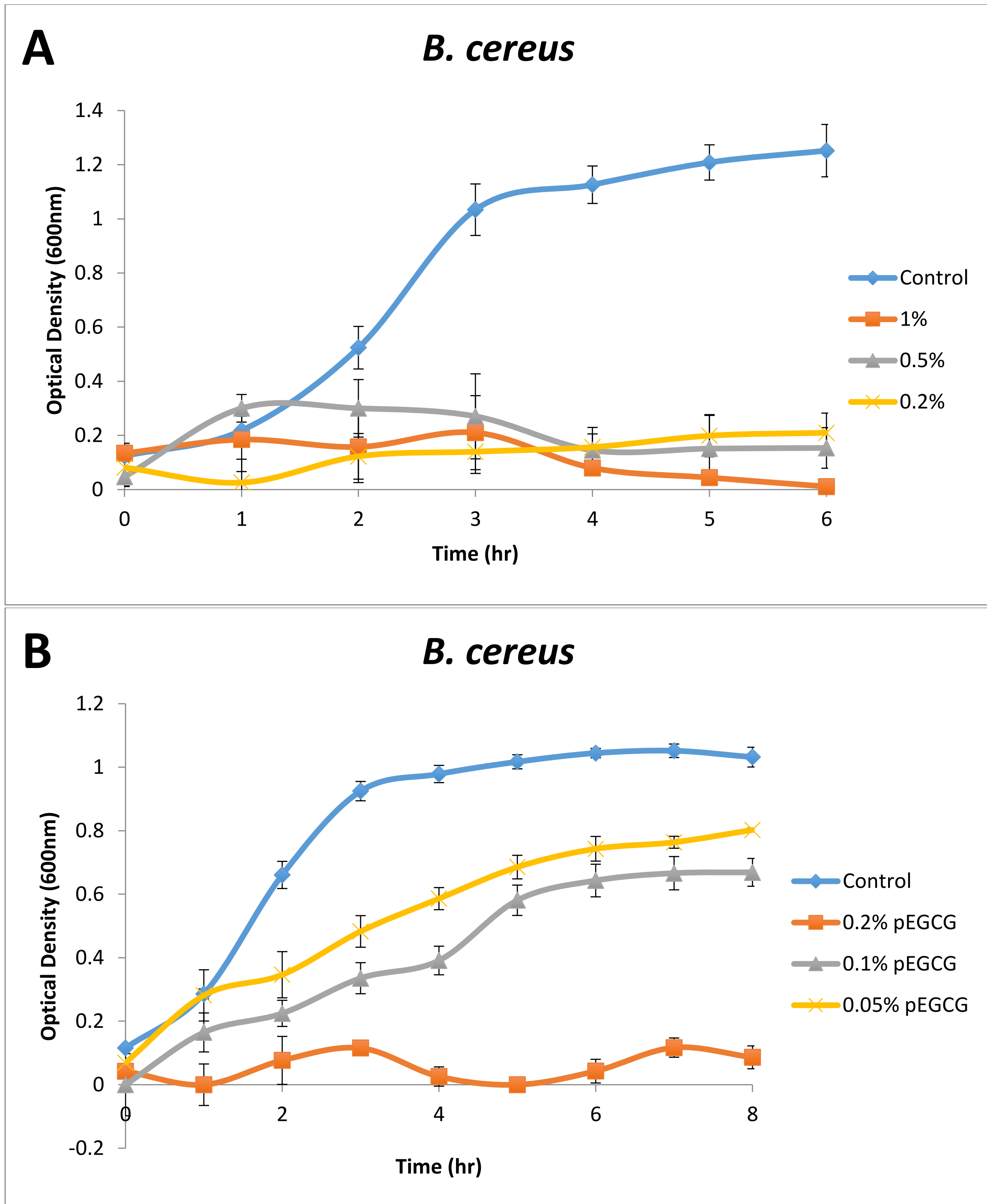


Figure 3. Growth curve of *B. cereus* A) *B. cereus* treated with various concentrations of TF. B) *B. cereus* treated with various concentrations of pEGCG.

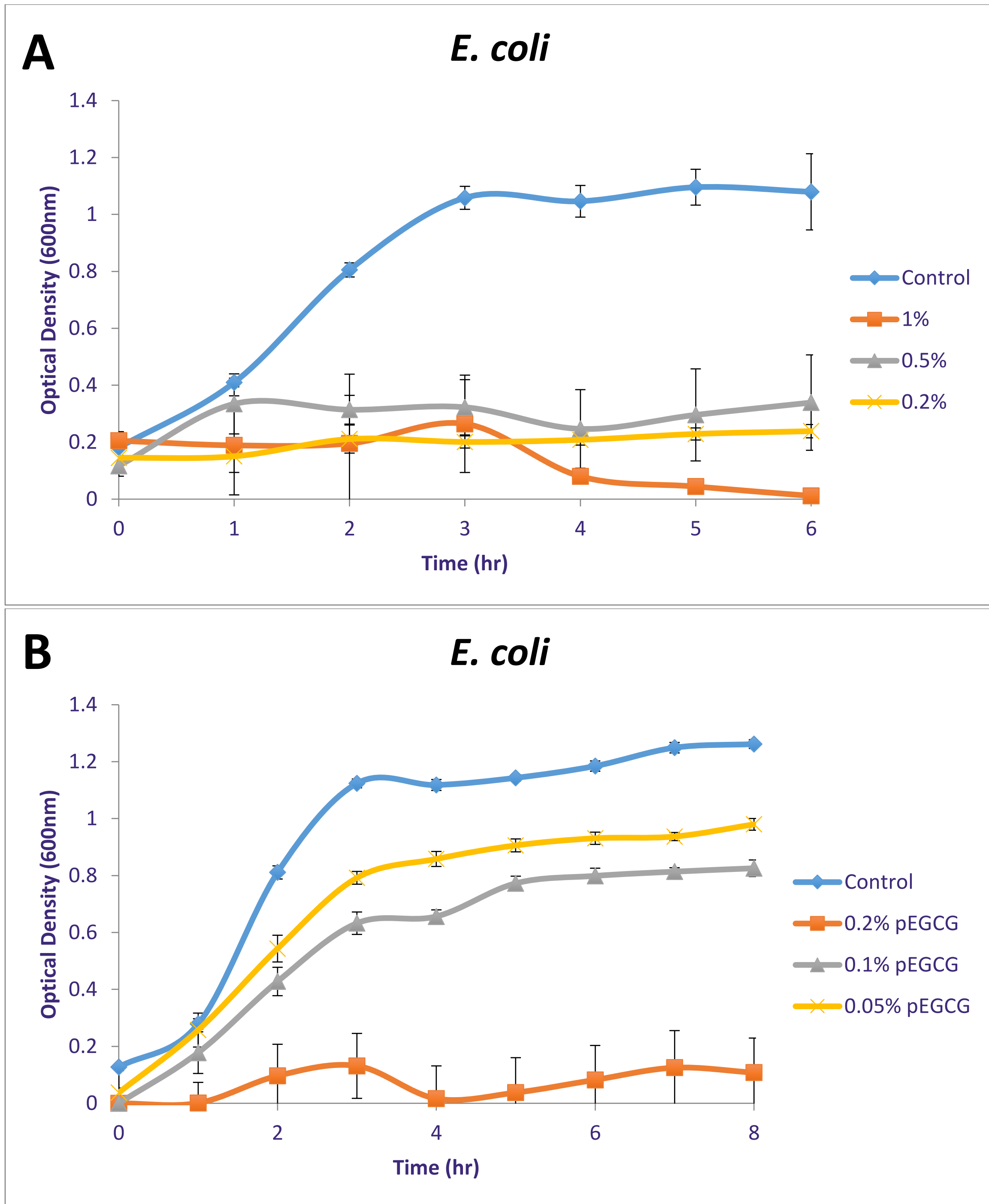


Figure 4. Growth curve of *E. coli* A) *E. coli* treated with various concentrations of TF. B) *E. coli* treated with various concentrations of pEGCG.

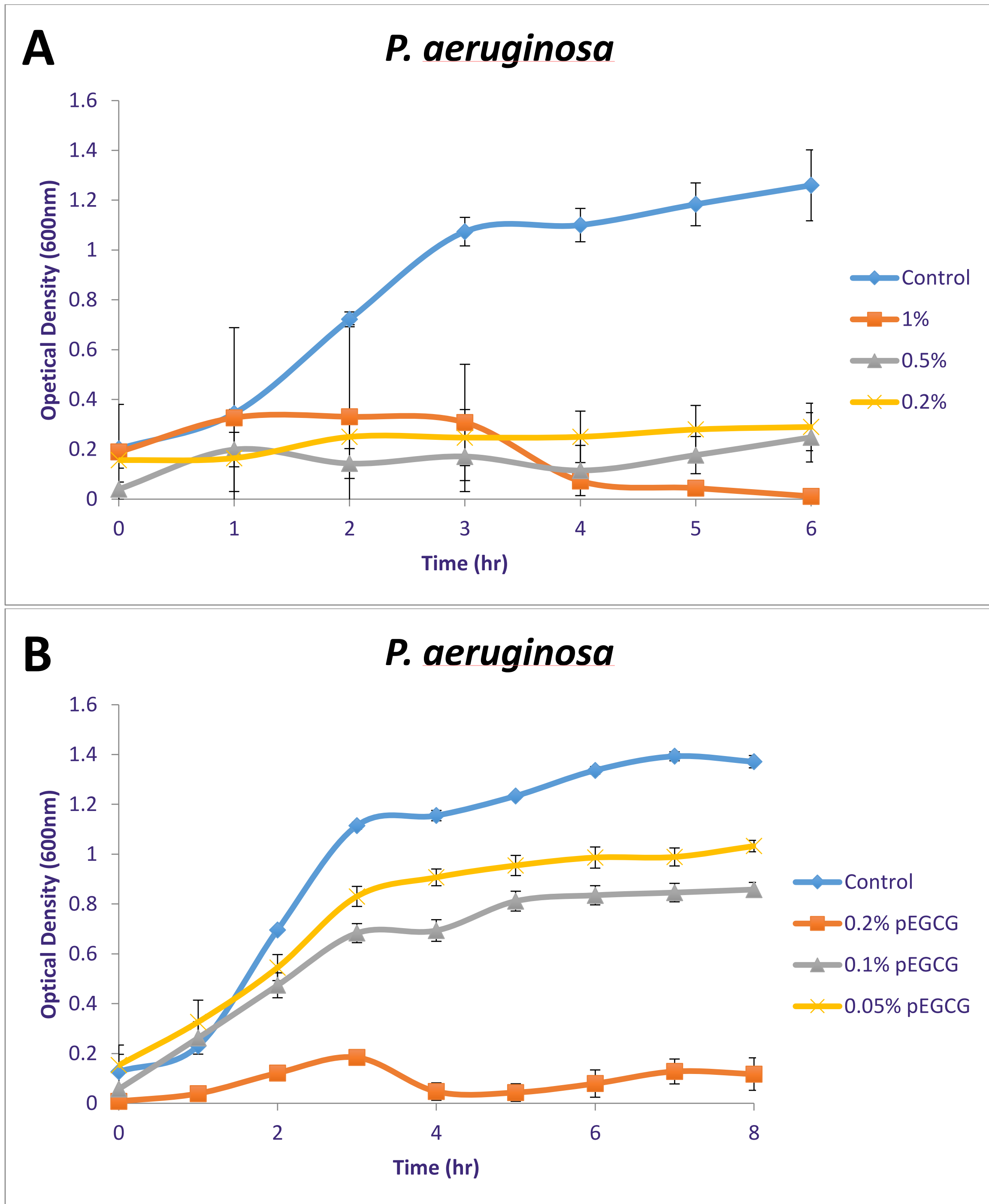


Figure 5. Growth curve of *P. aeruginosa* A) *P. aeruginosa* treated with various concentrations of TF. B) *P. aeruginosa* treated with various concentrations of pEGCG.

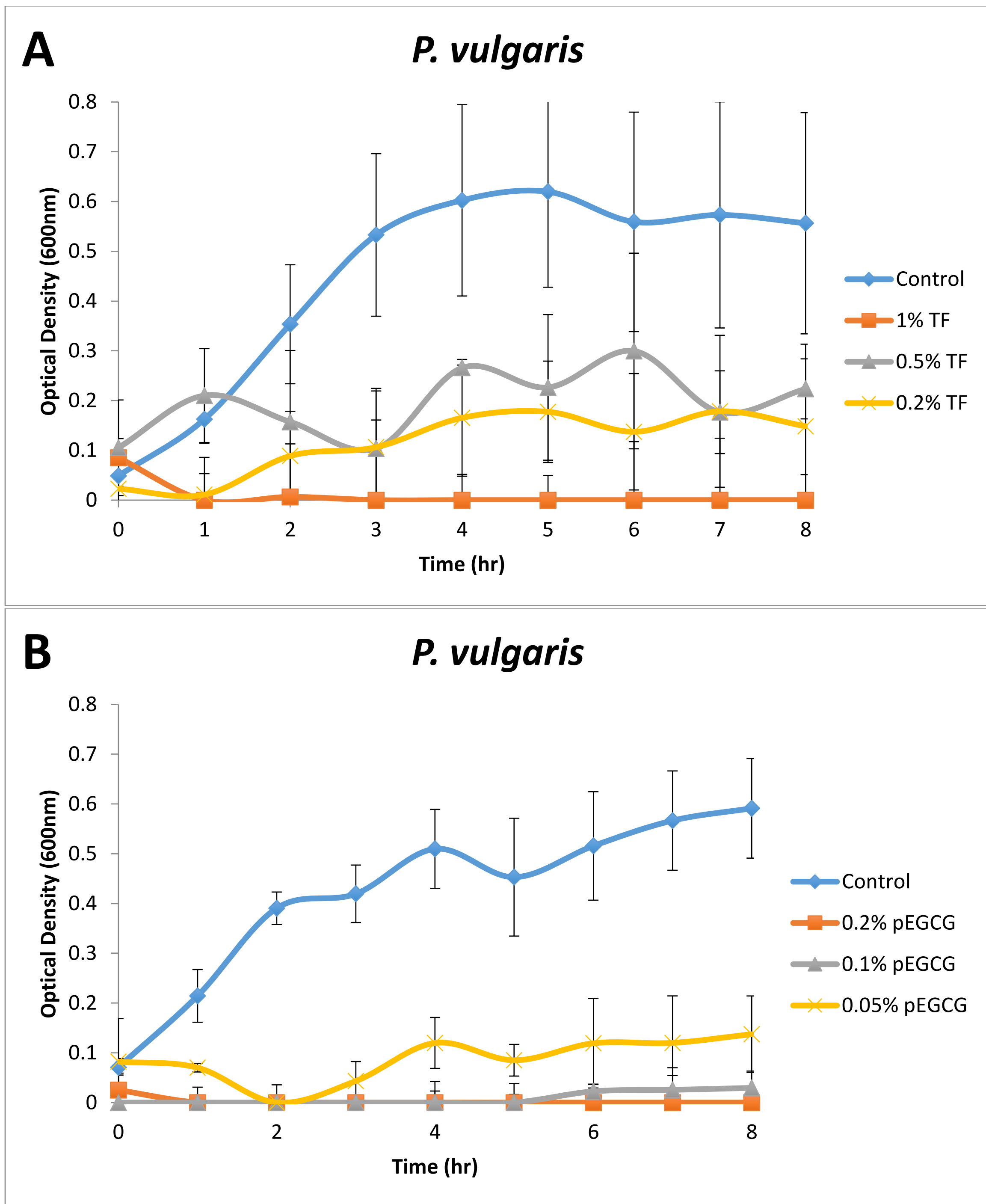


Figure 6. Growth curve of *P. vulgaris* A) *P. vulgaris* treated with various concentrations of TF. B) *P. vulgaris* treated with various concentrations of pEGCG.

Table 1. MIC and IC_{50} concentrations of TF and pEGCG for 6 bacterial species.

	Theaflavin (TF)		Palmitoyl-EGCG (pEGCG)	
	MIC	**IC_{50}**	**MIC**	**IC_{50}**
S. epidermidis	0.2%	< 0.2%	0.2%	0.05-0.1%
S. mutans	0.2%	< 0.2%	0.2%	~ 0.1%
B. cereus	0.2%	< 0.2%	0.2%	~ 0.1%
E. coli	0.2%	< 0.2%	0.2%	~ 0.1%
P. aeruginosa	0.2%	< 0.2%	0.2%	~ 0.1%
P. vulgaris	0.2%	< 0.2%	0.2%	< 0.2%

Viability assay

To further understand the effect of these compounds on the bacteria, a time-coursed study was performed where *S. epidermidis* and *S. mutans* were stained with SYTOX® and were monitored every 3 hours with MACSQuant® Analyzer 10 flow cytometer. In Figure 7, TF is able to significantly inhibit *S. epidermidis* as quickly as 3 hours ($p = 8.3 \times 10^{-6}$), whereas pEGCG is more effective at 6 hours. Both compounds are able to hold their effect for up to 24 hours after initial treatment. In Figure 8, TF is significantly inhibiting *S. mutans* at 3 hours and is able to hold that effect for 24 hours ($p = 4.2 \times 10^{-6}$), where pEGCG is most effective at 24 hours.

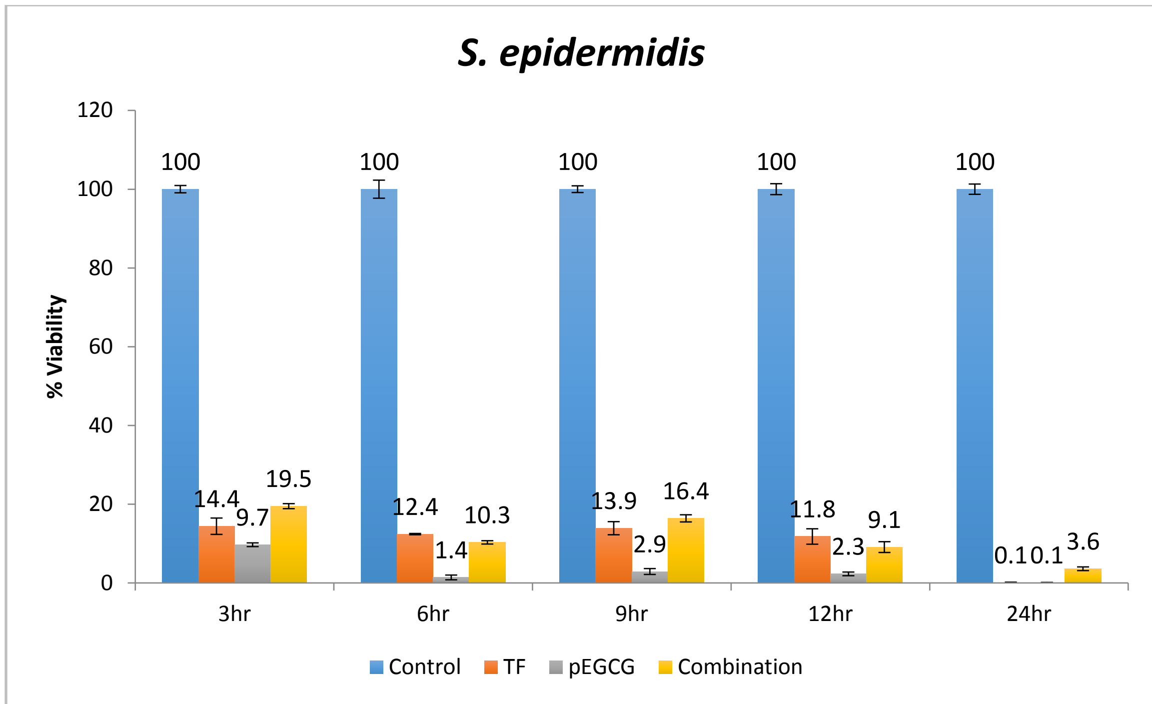


Figure 7. Percent viability of *S. epidermidis*. The percent viability of the compounds on *S. epidermidis* at 3 hour intervals.

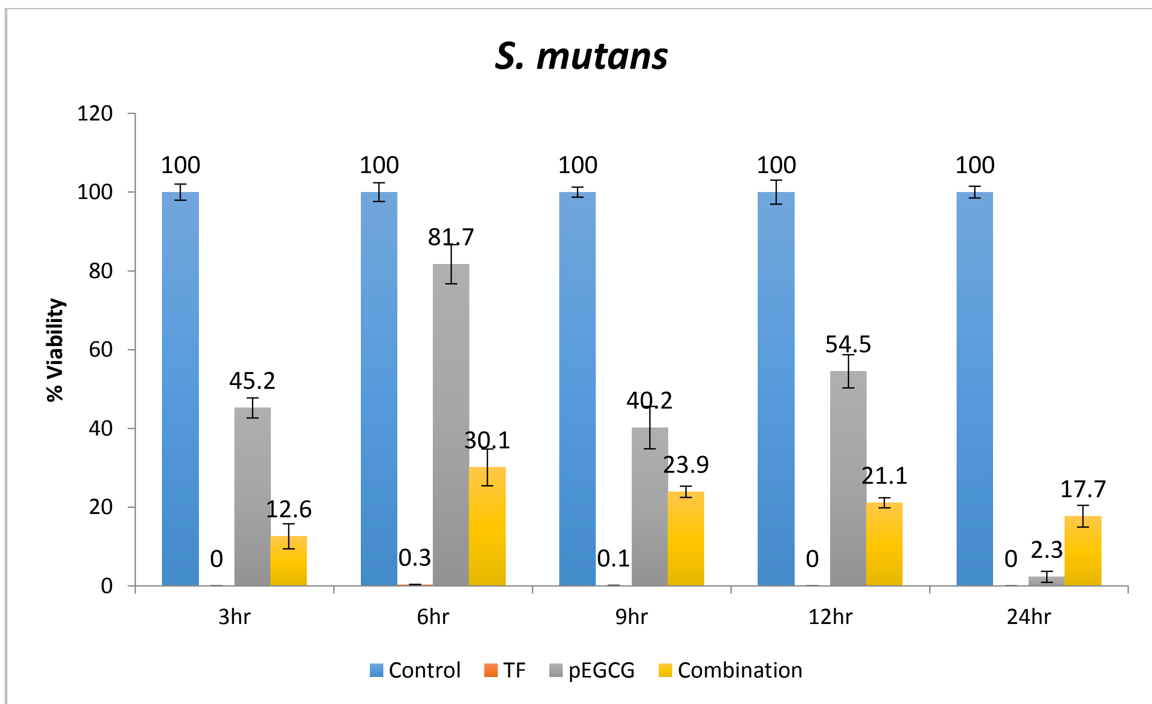


Figure 8. Percent viability of *S. mutans*. The percent viability of the compounds on *S. mutans* at 3 hour intervals.

Congo red assay

A qualitative assay was performed using Congo red to detect the production of a biofilm. When a biofilm is formed, the Congo red media will form black colonies. Two known biofilm forming bacteria were selected for this assay, *S. epidermidis and S. mutans.* The bacteria were treated with either 1% TF or 2% pEGCG to test the effect of these compounds on biofilm formation. Both TF and pEGCG inhibited *S. epidermidis* from forming a biofilm and TF was also able to inhibit *S. mutans* from forming a biofilm (results not shown).

Resazurin assay

Results from the Congo red assay suggested that TF and pEGCG are effective in inhibiting biofilm formation. A quantitative assay using Resazurin was carried out to quantify biofilm reduction. Resazurin is a blue compound that is reduced to a pink fluorescent resorfin in the presence of a biofilm (O'Brian et al., 2000). Four biofilm-forming bacteria were selected for this assay: *S. epidermidis, S. mutans, E. coli,* and *P. aeruginosa.* TF at concentrations of 0.5% and 1% show to be the most effective in significantly inhibiting the production of biofilm in *S. epidermidis* (p = 0.0011 and p = 0.0012) (Figure 9A) with a percent inhibition of approximately 99.9% for both concentrations (Figure 9B). 0.2% TF is less effective with a percent inhibition of approximately 50%. In comparison, all concentrations of pEGCG (0.2%, 0.5%, and 1%) were equally effective in inhibiting *S. epidermidis* biofilm formation with a percent inhibition of approximately 99.9% (Figure 10). In Figure 11, all concentrations of TF are able to inhibit the production of biofilm in *S. mutans* species with 99.9% inhibition. 0.5% and 1% pEGCG are significantly effective in inhibiting *S. mutans* biofilm formation (p =

0.00025 and $p = 0.00026$); 0.2% pEGCG is the least effective in inhibiting biofilm formation (Figure 12). In Figure 13 and 15, all concentrations of TF (0.2, 0.5 and 1%) were effective in inhibiting biofilm formation in *E. coli* (Figure 13B) and *P. aeruginosa* (Figure 15B), whereas only 0.5 and 1% pEGCG were effective in inhibiting biofilm formation (Figures 14 and 16). 0.2% pEGCG did not inhibit biofilm production in *E. coli* (Figure 14B) and *P. aeruginosa* (Figure 16B).

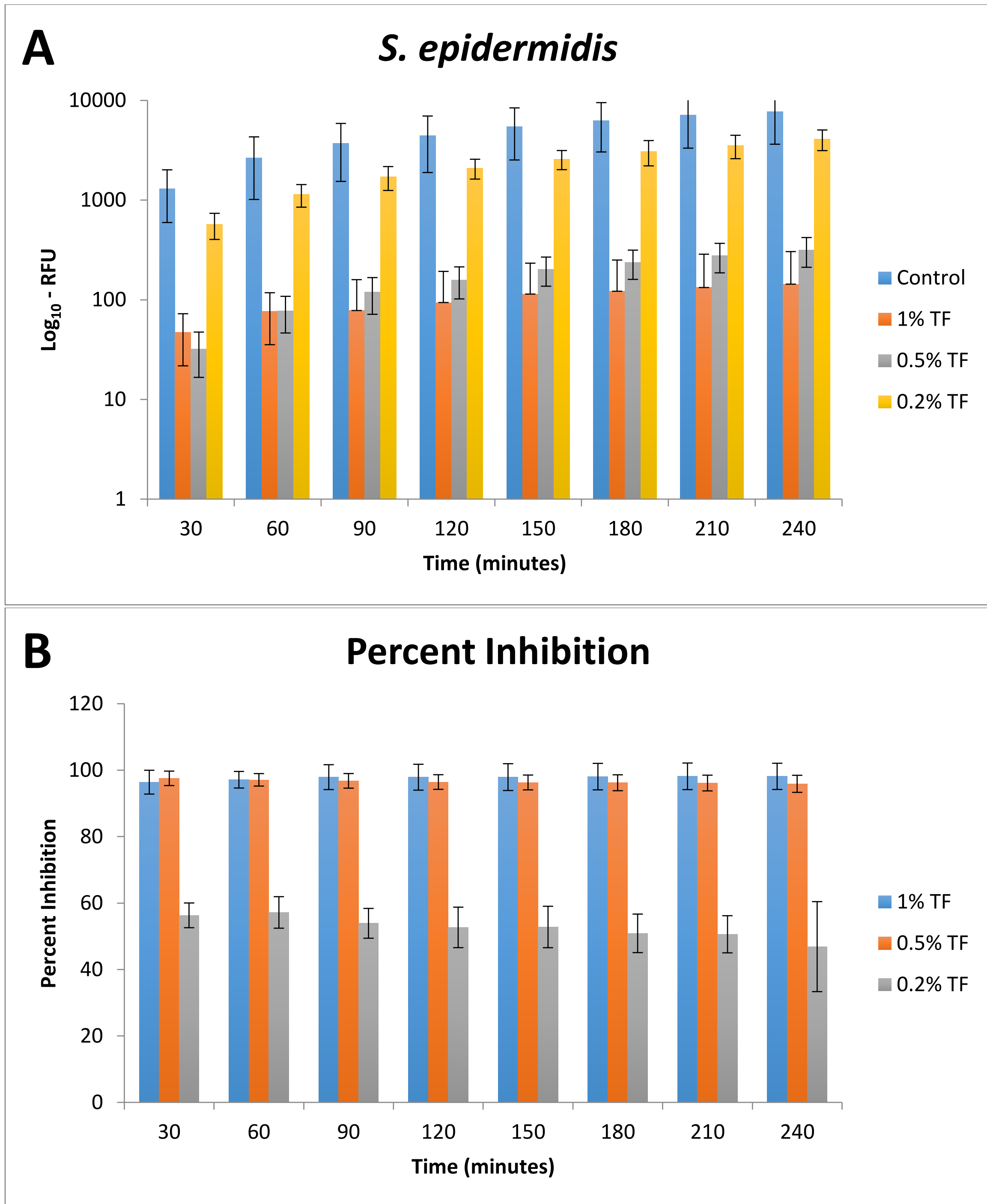


Figure 9. Resazurin assay of *S. epidermidis* with TF A) *S. epidermidis* treated with various concentrations TF. B) Percent Inhibition of TF on *S. epidermidis* biofilm formation.

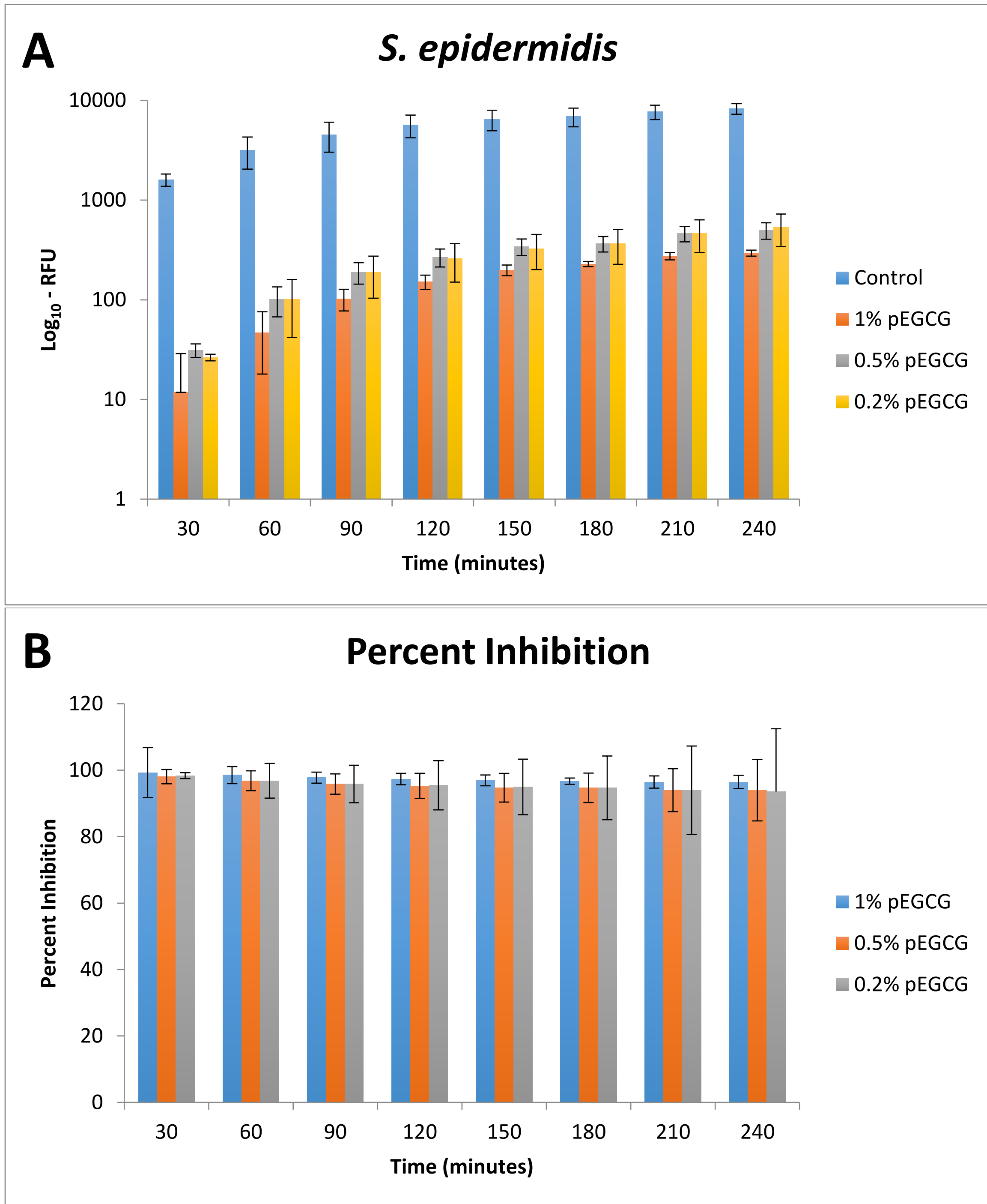


Figure 10. Resazurin assay of *S. epidermidis* with pEGCG A) *S. epidermidis* treated with various concentrations of pEGCG. B) Percent Inhibition of pEGCG on *S. epidermidis* biofilm formation.

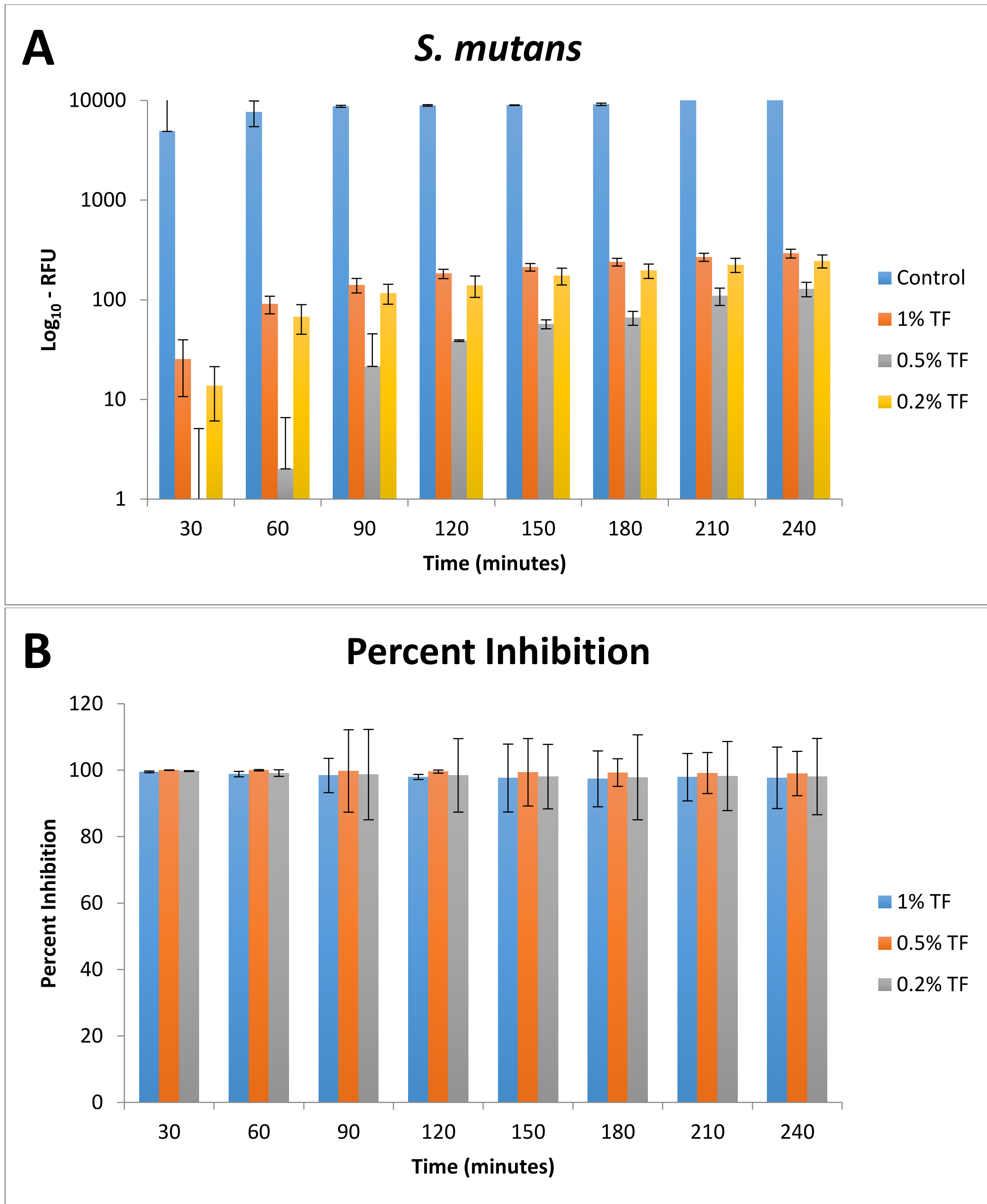


Figure 11. Resazurin assay of *S. mutans* with TF A) *S. mutans* treated with various concentrations of TF. B) Percent Inhibition of TF on *S. mutans* biofilm formation.

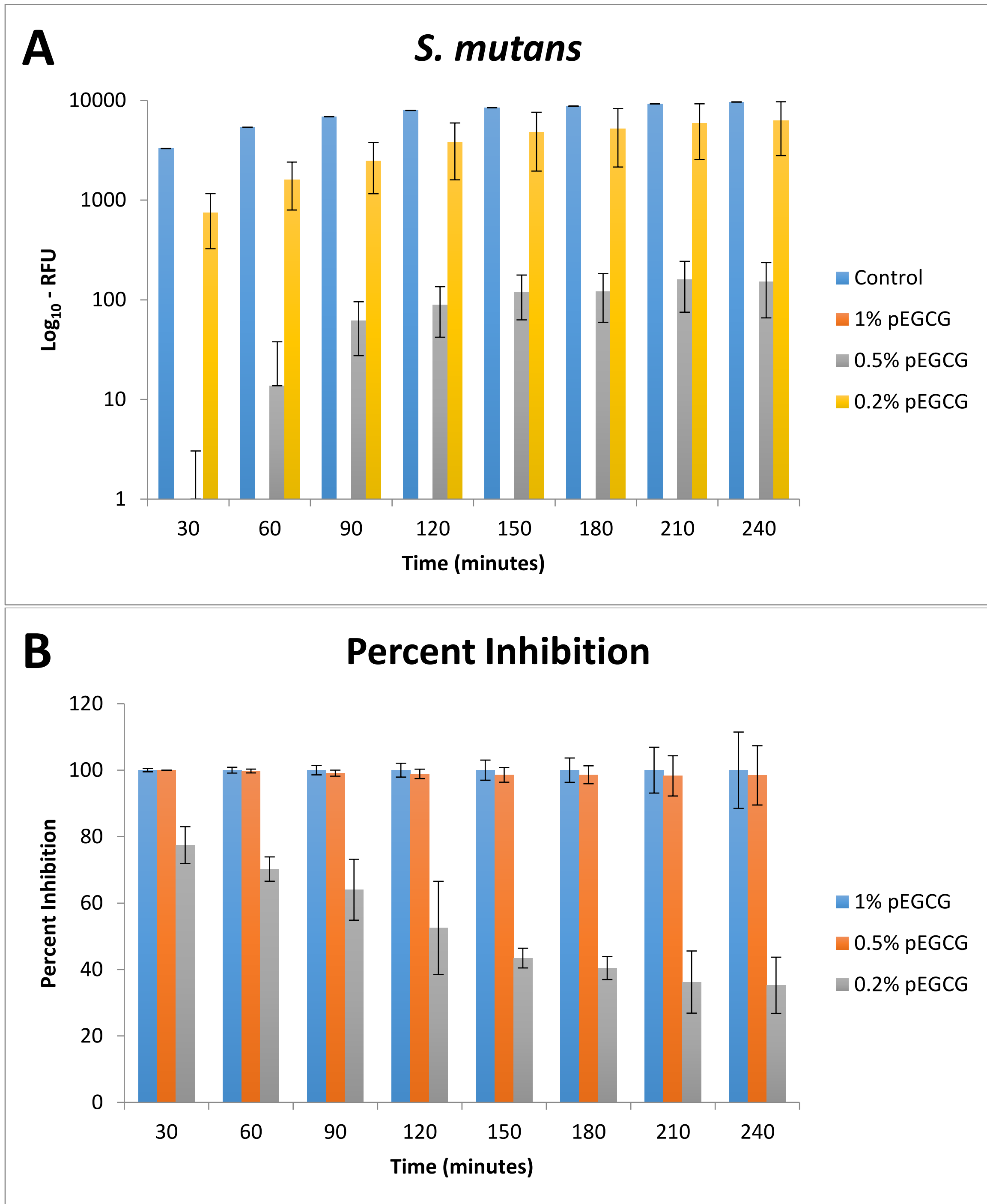


Figure 12. Resazurin assay of *S. mutans* with pEGCG A) *Streptococcus mutans* treated with various concentrations of pEGCG. B) Percent Inhibition of pEGCG on *S. mutans* biofilm formation.

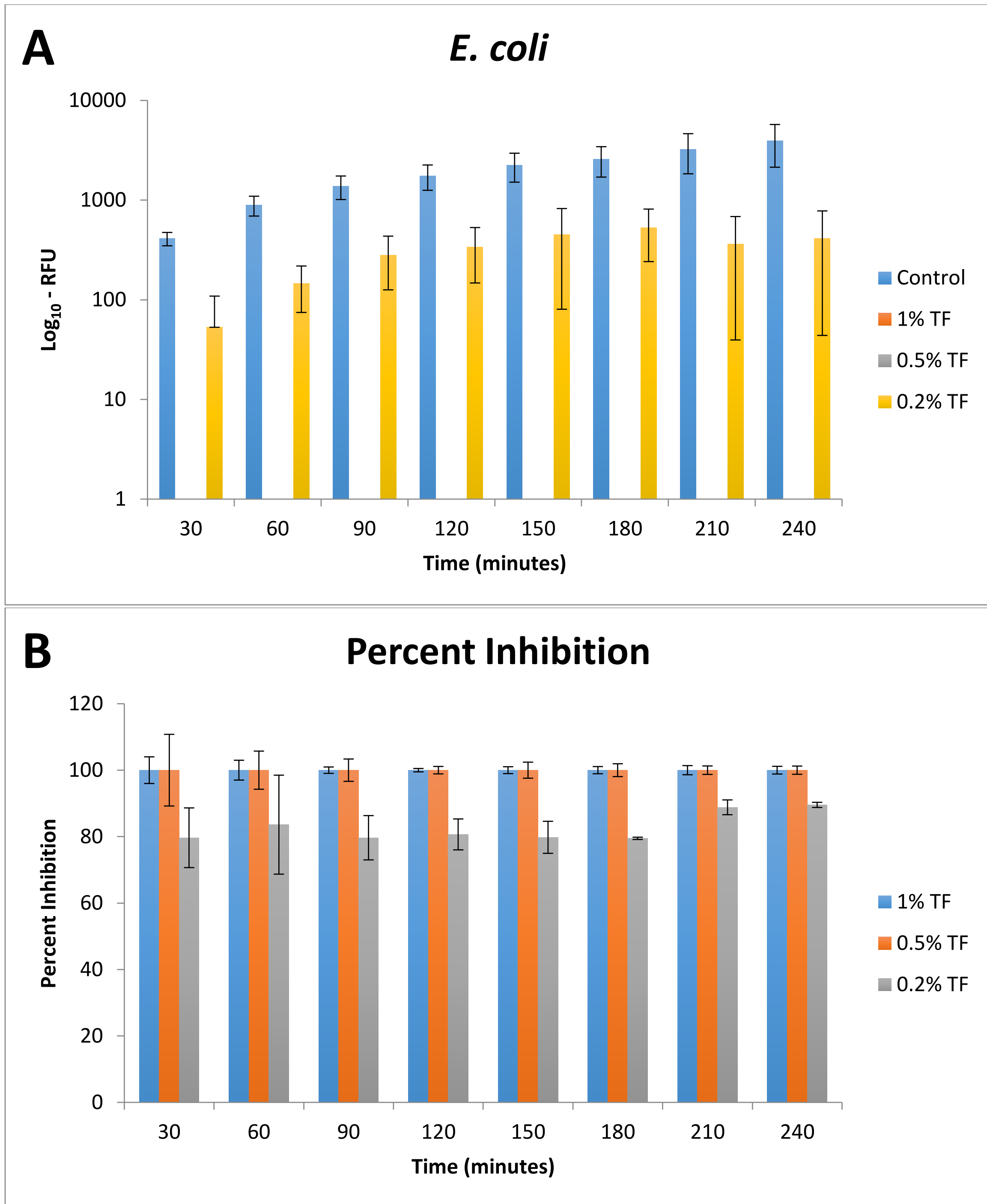


Figure 13. Resazurin assay of *E. coli* with TF A) *E. coli* treated with various concentrations of TF. B) Percent Inhibition of TF on *E. coli* biofilm formation.

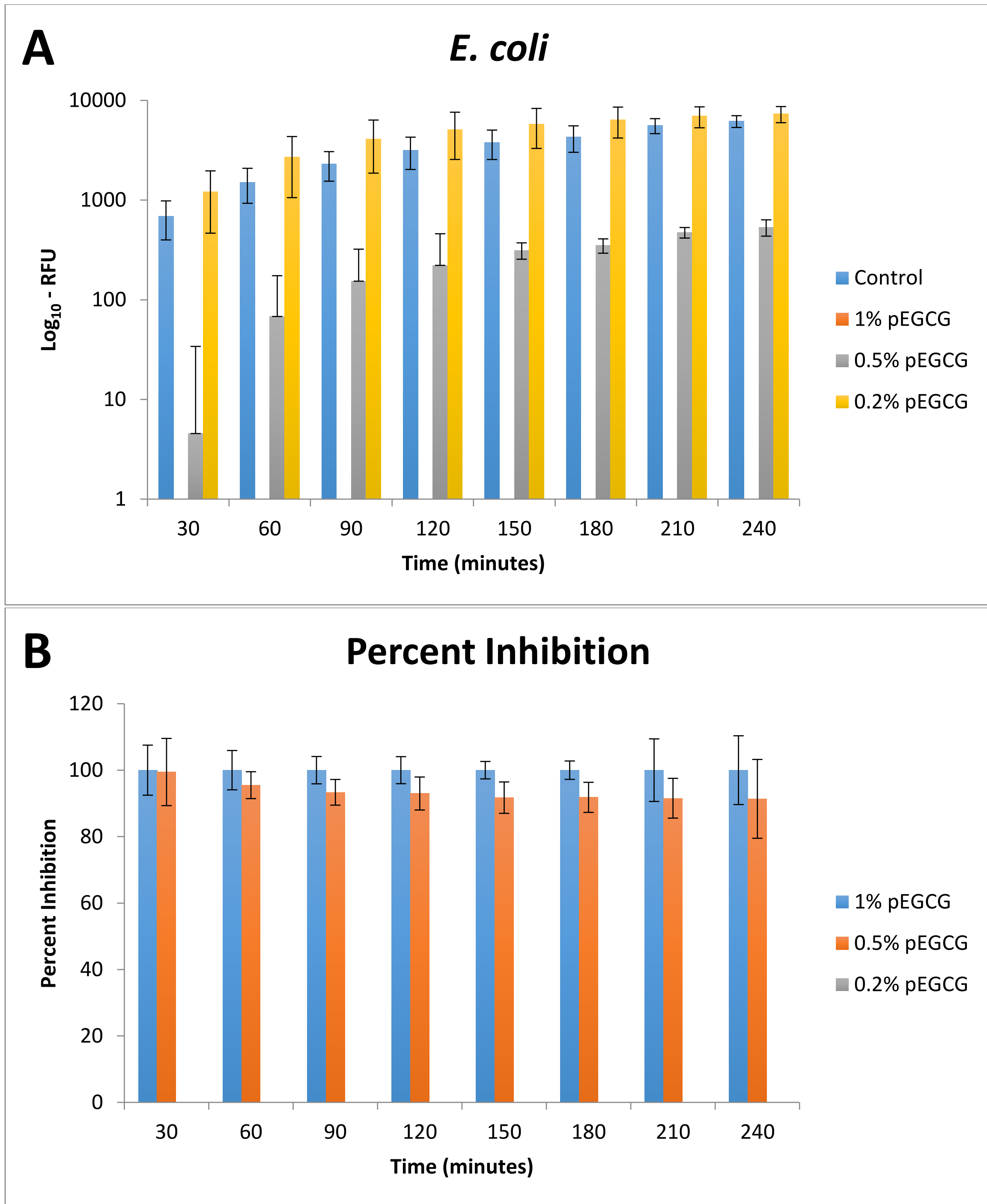


Figure 14. Resazurin assay of *E. coli* with pEGCG A) *E. coli* treated with various concentrations of pEGCG. B) Percent Inhibition of pEGCG on *E. coli* biofilm formation.

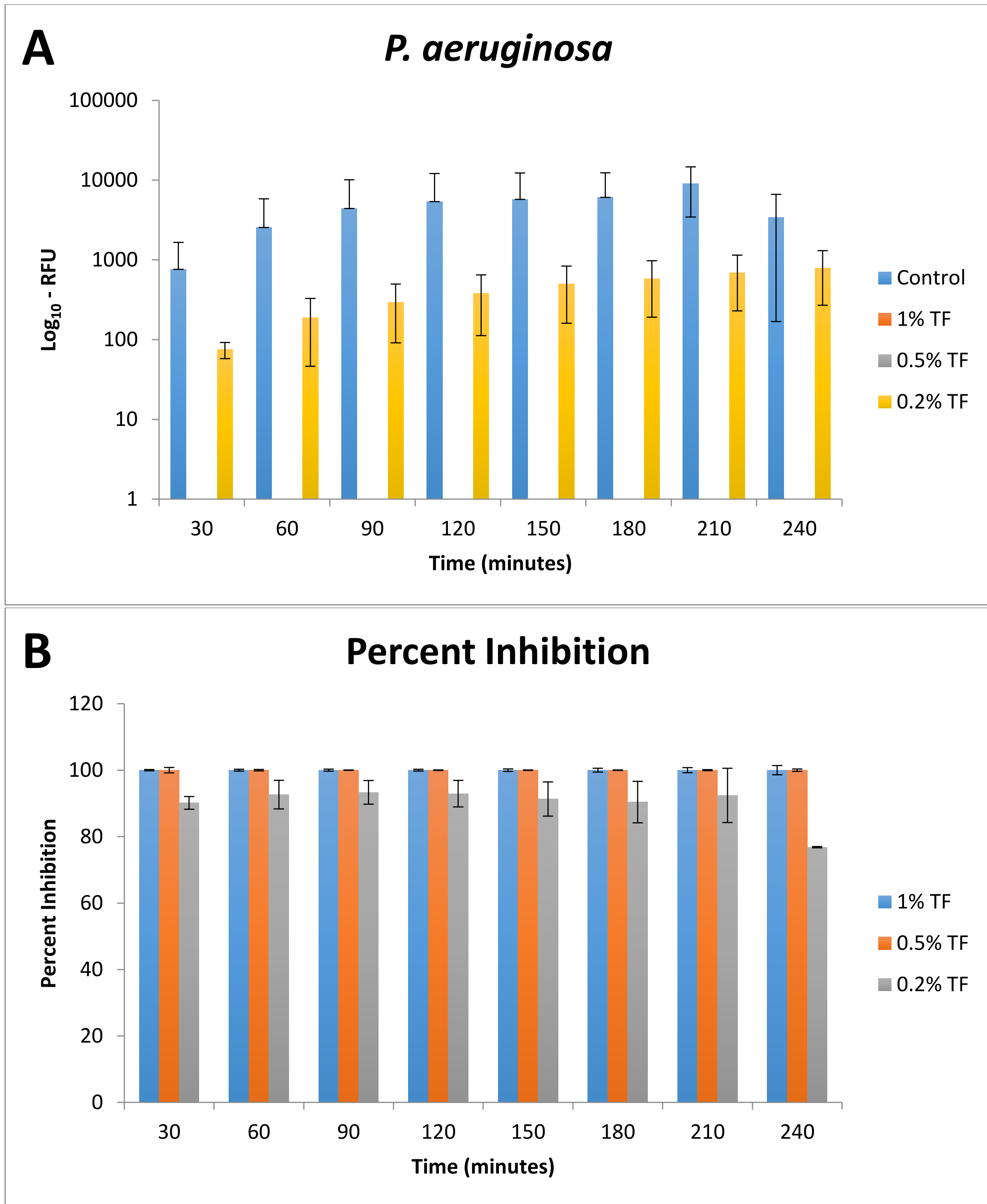


Figure 15. Resazurin assay of *P. aeruginosa* with TF A) *P. aeruginosa* treated with various concentrations of TF. B) Percent Inhibition of TF on *P. aeruginosa* biofilm formation.

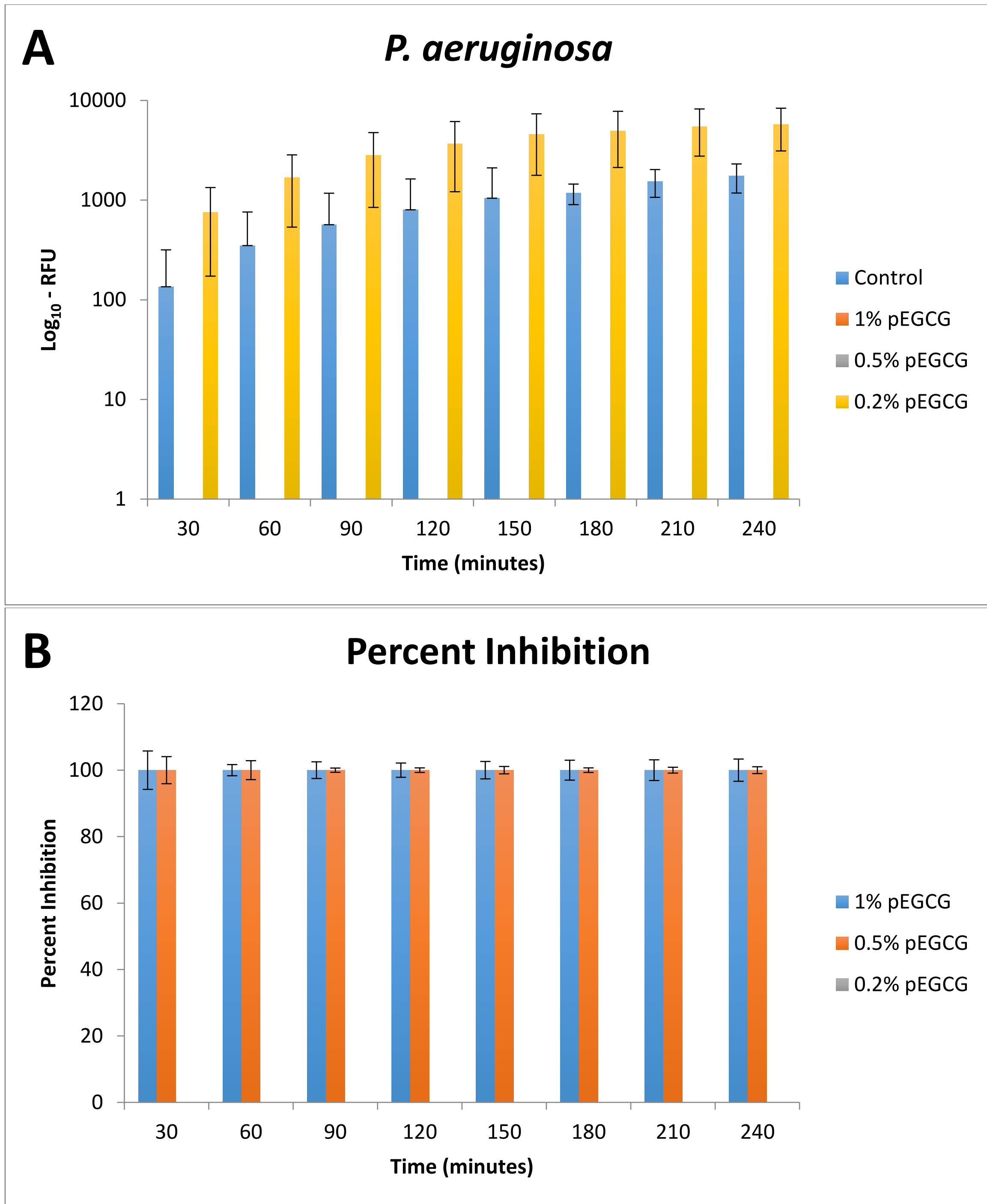


Figure 16. Resazurin assay of *P. aeruginosa* with pEGCG A) *P. aeruginosa* treated with various concentrations of pEGCG. B) Percent Inhibition of pEGCG on *P. aeruginosa* biofilm formation.

Polymerase chain reaction (PCR) and gel electrophoresis

Based on both the Congo red and Resazurin results, it is apparent that TF and pEGCG are capable of inhibiting biofilm formation; however, the mechanism in which these compounds work is not well understood. Primers were designed for genes involved in biofilm formation for two selected bacteria, *S. epidermidis* and *S. mutans,* to determine whether these compounds work at the molecular level. The *aap* gene for *S. epidermidis* and the *brpA* gene for *S. mutans* were observed. Primers for these genes were designed using NCBI. The genes responsible for biofilm formation in *S. epidermidis* were studied, yielding two sets of primers: *aap*_f, *aap*_r, Similarly, the gene responsible for biofilm formation in *S. mutans* yielded primers: *brpA*_2f, *brpA*_2r, *brpA*_3f, *brpA*_3r (Figure 17). DNA was isolated from *S. epidermidis* and *S. mutans* without treatment of compounds as well as treatment with 0.5% TF, 1% pEGCG, and a combination of TF and pEGCG. Gel electrophoresis was performed to observe the effect of these compounds. The *aap* gene for *S. epidermidis* was present in the control with a band at 524bp; however when *S. epidermidis* was treated with TF, pEGCG, and the TF/pEGCG combination, the band was no longer present (Figure 18). The *brpA* gene for *S. mutans* was present in the control for both sets of primers, but was absent in all the samples treated with TF, pEGCG and TF/pEGCG combination (Figure 19).

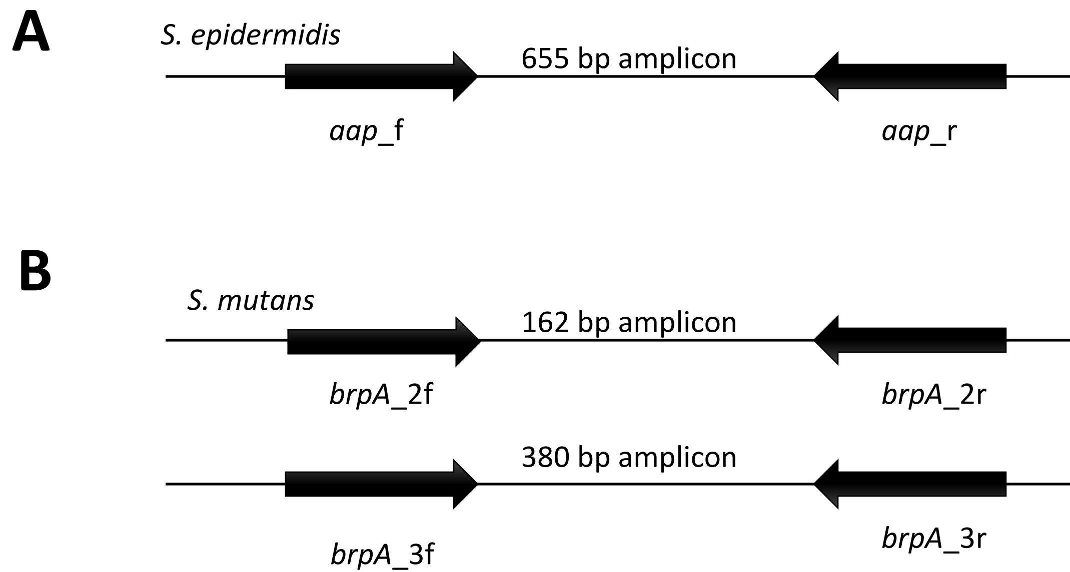


Figure 17. Primer design. Two sets of primers (forward and reverse) were designed for the selected genes responsible for biofilm formation in *S. epidermidis* and *S. mutans.* A) *S. epidermidis* primers: *aap*_f + *aap*_r. B) *S. mutans* primers: *brpA*_2f + *brpA*_2r and *brpA*_3f + *brpA*_3r

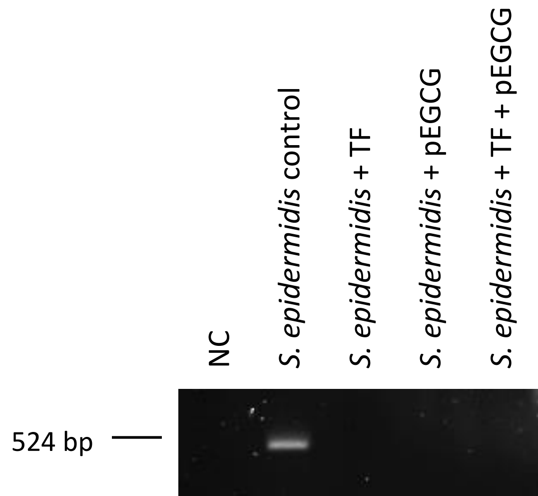


Figure 18. Gel electrophoresis on *S. epidermidis* biofilm gene. PCR was performed to amplify genes of interest. The *aap* gene responsible for biofilm formation in *S. epidermidis* was observed under control and treated conditions

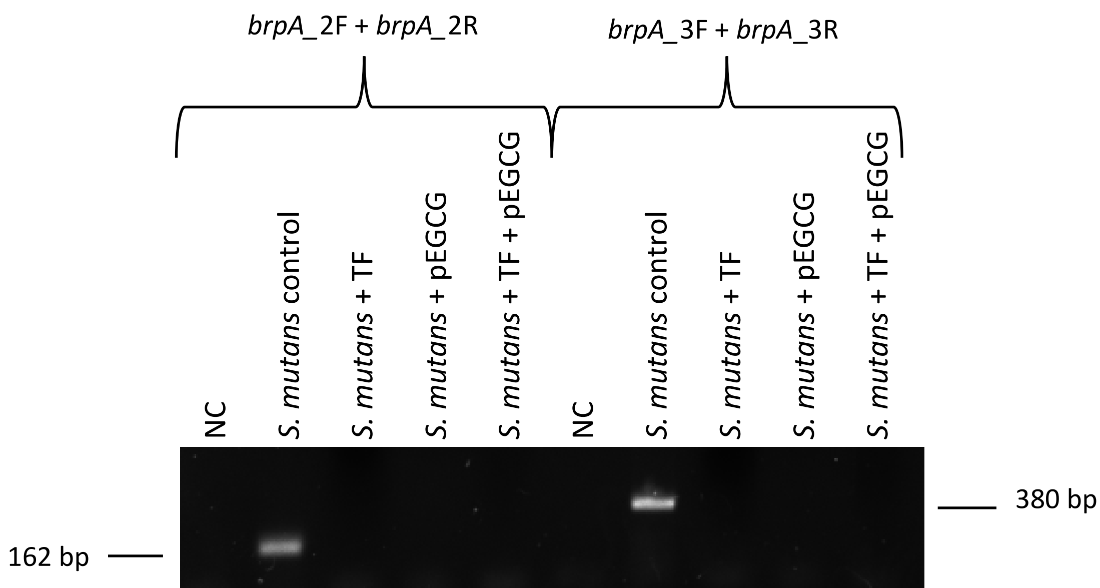


Figure 19. Gel electrophoresis on *S. mutans* biofilm gene. PCR was performed to amplify genes of interest. The *brpA* gene responsible for biofilm formation in *S. mutans* was observed under control and treated conditions.

Biofilm viability study with SYTOX®

To further understand the effect of TF and pEGCG on the production of biofilm in *S. epidermidis* and *S. mutans,* a viability assay using flow cytometry was performed. In this study, the bacteria were stained with SYTOX®. *S. epidermidis* and *S. mutans* were treated with 0.5% TF, 1% pEGCG, and a combination of the two compounds. The compounds are all shown to be effective in inhibiting the production of biofilm in *S. epidermidis.* TF is shown to inhibit approximately 86% of the biofilm formation in *S. epidermidis,* pEGCG inhibits approximately 57% and the combination of the two compounds inhibits 73% (Figure 20). Results show that the compounds are also effective in inhibiting biofilm formation in *S. mutans.* TF inhibits approximately 88% and pEGCG inhibits about 85% of biofilm formation in *S. mutans.* The TF/pEGCG combination, however, only inhibits about 62% (Figure 21).

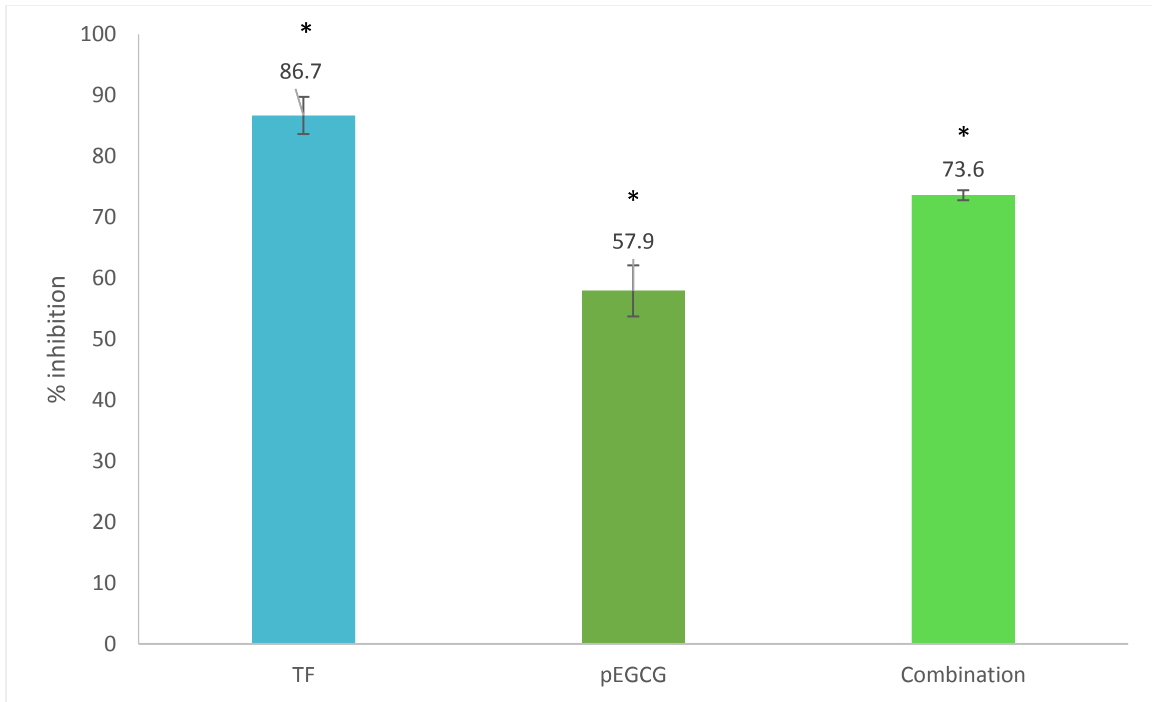


Figure 20. Percent inhibition of TF and pEGCG on *S. epidermidis*. The percent inhibition of the compounds on *S. epidermidis* was calcaluated based on the flow cytometry results. Asterisk (*) indicates statistically significant ($p < 0.05$).

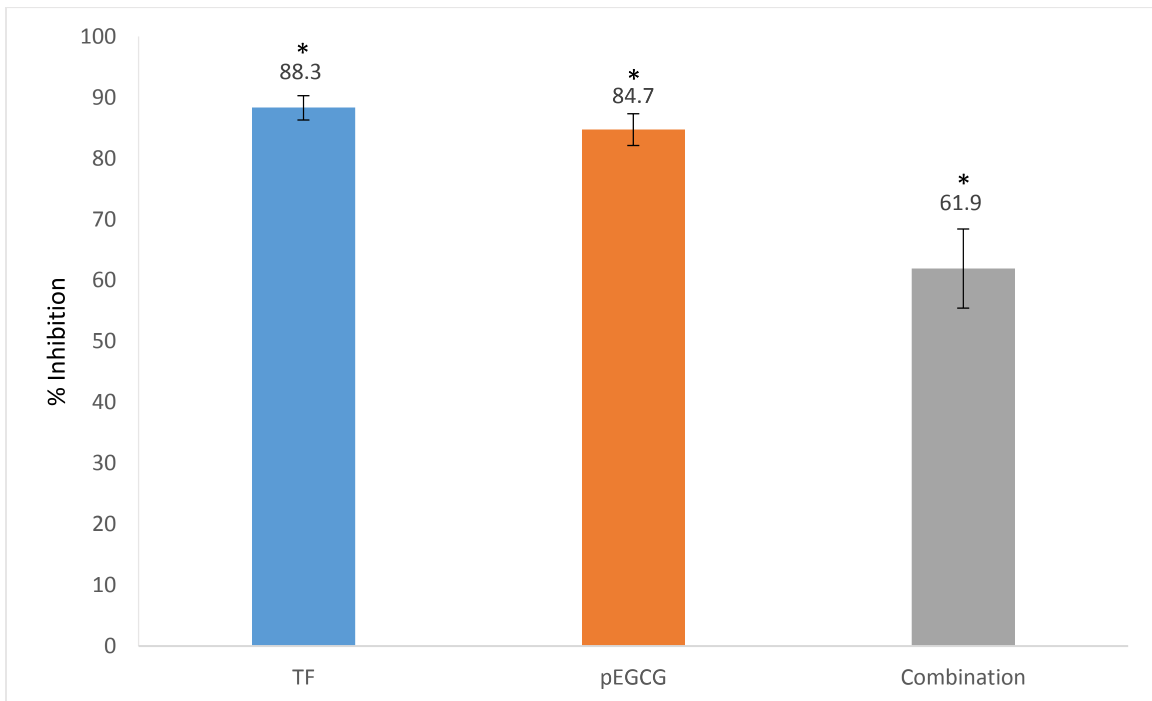


Figure 21. Percent inhibition of TF and pEGCG on *S. mutans*. The percent inhibition of the compounds on *S. mutans* was calcaluated based on the flow cytometry results. Asterisk (*) indicates statistically significant ($p < 0.05$).

Sporulation inhibition assay

B. megaterium and *B. cereus* were treated with TF and pEGCG in order to determine the effect of the compounds on sporulation. *B. megaterium* and *B. cereus* cultures were centrifuged and resuspended in either: diH_2O, TF or pEGCG for 24 hrs. A spore stain was performed in order to observe the effect of the compounds on sporulation for these two spore-forming bacteria. In Figure 22, spores are present in the control (A and D), but are absent in the treated cells; therefore indicating that the compounds, TF and pEGCG, are able to inhibit the process of sporulation in both *Bacillus* spp.

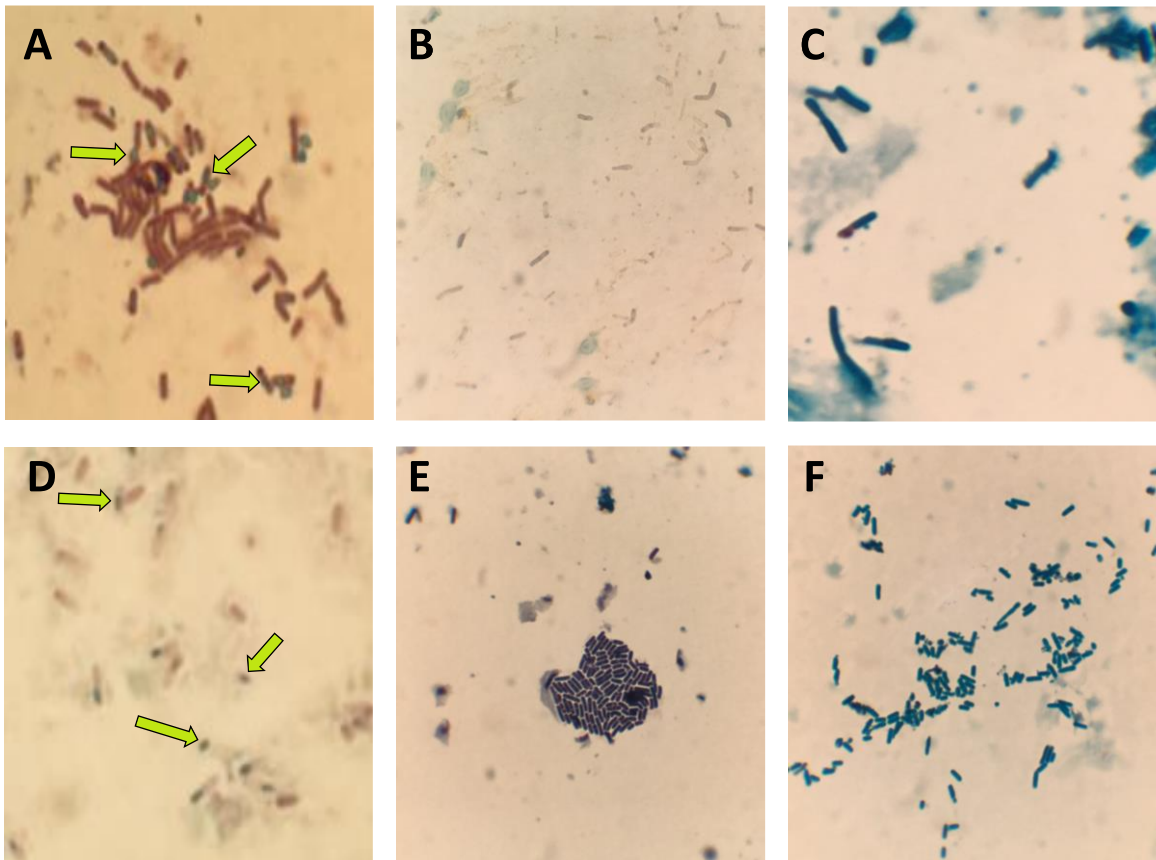
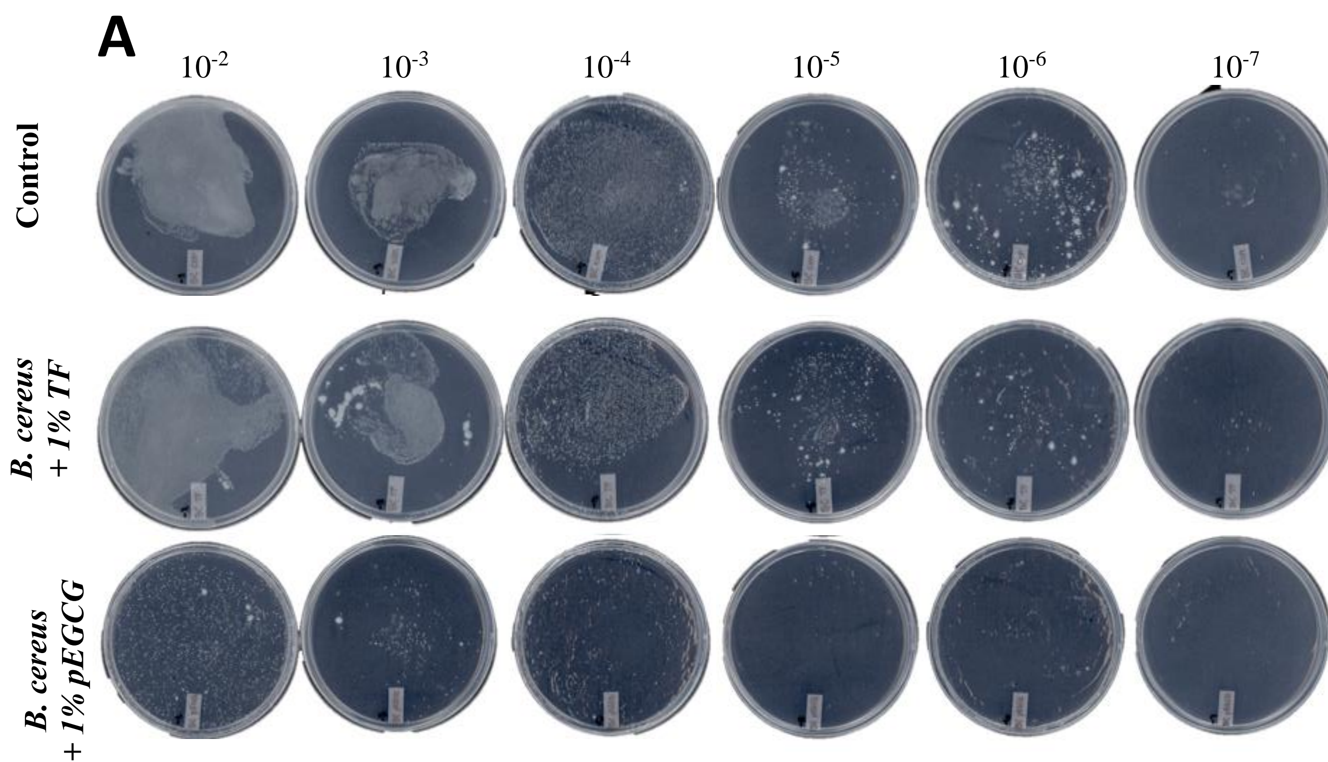


Figure 22. Microscopic observations: spore stain. A) *B. megaterium* control B) *B. megaterium* treated with 1% TF C) *B. megaterium* treated with 1% pEGCG D) *B. cereus* control E) *B. cereus* treated with 1% TF F) *B. cereus* treated with 1% pEGCG

Germination inhibition assay

TF and pEGCG have both shown to be effective in inhibiting biofilm formation and sporulation. The effects of these compounds on inhibiting germination in spore-forming bacteria were also studied. In this study, *B. cereus* was used to evaluate the germination process (Figure 23A). TF is able to inhibit approximately 76% of germination with a log fold reduction of 1.77, and pEGCG inhibits approximately 99.9% of the process in *B. cereus*, which is a log fold reduction of 3.44 (Figure 23B).



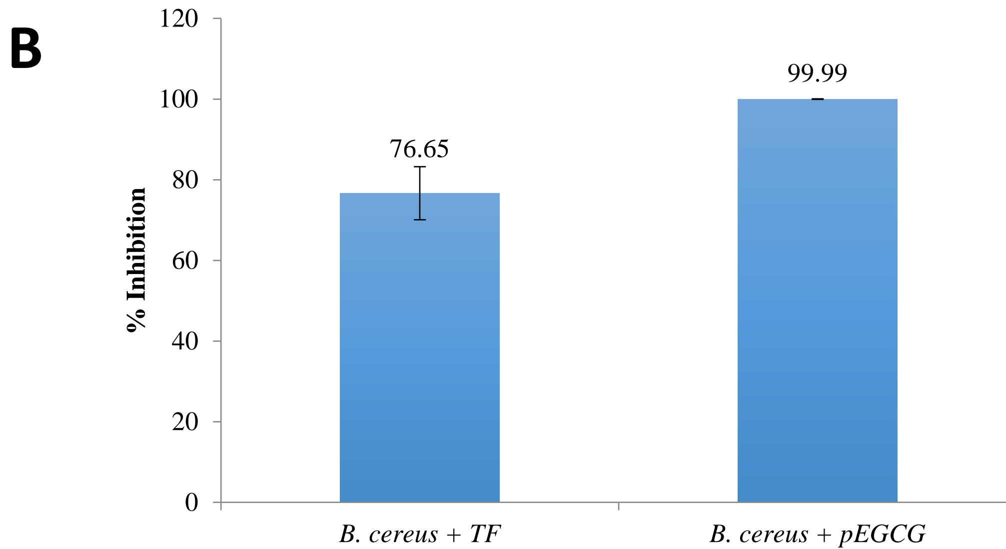


Figure 23. Germination inhibition assay. A) Colonies were seen up to the 10^{-7} dilution in the control plates, 10^{-6} dilution for the plates treated with 1% TF, and 10^{-5} dilution for the plates treated with 1% pEGCG. B) The percent inhibition of the compounds on *B. cereus* was calcaluated based on the viable count.

Discussion

As of late, the increase in antibiotic resistant pathogens has led to the interest in studying natural compounds, plant-derived in particular, as potential therapeutic agents. For millennia, tea has been used as a medicinal beverage, especially in its native country of China (Ferrazzano et. al., 2009). Tea is characterized by the presence of phenolic catechins such as epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea. During fermentation, these catechins are oxidized yielding secondary polyphenols such as theaflavins, the major polyphenols in black tea (Ferrazzano et. al., 2009). In this study, the antibacterial effects of black tea and green tea polyphenols, theaflavin (TF) and palmitoyl-EGCG (pEGCG), were evaluated against a variety of bacteria and their specific defense mechanisms including biofilm and spore formation.

The results from the antibacterial assay showed that at 0.2% concentration of both TF and pEGCG effectively inhibited the growth of bacteria (Figures 1-6). Therefore the minimum inhibitory concentration (MIC) for both compounds is 0.2% for all bacteria tested. It can also be observed that the IC_{50} for TF for most of the selected bacteria is less than 0.2% and for pEGCG the range is between 0.05 - 0.1% (Table 1). These results indicate that the compounds are capable of inhibiting the growth of bacteria. The results from the viability assay using flow cytometry showed that both compounds, TF and pEGCG, are effective after a 3 hour treatment and are able to maintain their effect for up to 24 hours (Figure 7 and 8). These results further conclude that these compounds possess antibacterial properties against both gram negative and gram positive bacteria.

Due to the rise in nosocomial infections caused by bacterial biofilms, the anti-biofilm effects of TF and pEGCG were also evaluated. Four bacteria that are known to

cause biofilms, *S. epidermidis, S, mutans, E. coli,* and *P. aeruginosa,* were studied using Congo-red and resazurin assays. Congo-red is an indicator dye; when a bacteria is inoculated and incubated on Congo-red media, the formation of black colonies indicates the presence of biofilm formation. In the same sense, resazurin is also an indicator. The resazurin assay is based on the reduction of resazurin by metabolically active cells, where the blue compound is reduced to a fluorescent pink resorufin (Peeters et. al., 2008). The fluorescence is measured using a spectrophotometer, which then indicates the presence of biofilm formation. The results from the resazurin assay indicate that both TF and pEGCG are effective in inhibiting the formation of biofilm in the four selected bacteria (Figures 9 – 16).

Although it is inferred that TF and pEGCG are capable of inhibiting the formation of biofilm, the mechanism in which these compounds work is not completely understood. In order to further understand how these compounds are inhibiting the production of biofilm, molecular analysis was conducted. Two bacteria were selected for this study, *Staphylococcus epidermidis* and *Streptococcus mutans. S. epidermidis* is a model organism for its pathogenic family member, *Staphylococcus aureus,* which is capable of becoming antibiotic resistant, the most known strain being methicillin-resistant *Staphylococcus aureus* (MRSA). *Streptococcus mutans* is one of the major causing agents of dental plaque and dental caries. Genes that are involved in biofilm formation for each of these bacteria were identified and tested. The *brpA* gene plays a regulatory role in biofilm formation in *S. mutans,* and the *aap* gene, which produces an accumulation associated protein (Aap) that is involved in biofilm formation in *S. epidermidis*. The results from the polymerase chain reaction (PCR) and gel

electrophoresis studies showed that TF, pEGCG and a combination of TF and pEGCG effect the presence of these genes compared to the control (Figure 18 – 19).

Although the use of Congo-red, resazurin and PCR are effective in showing the effect of TF and pEGCG on biofilm formation, these methods are typically time consuming. In an effort to be able to rapidly detect the effects of these compounds on biofilm formation, flow cytometry was used. Flow cytometry offers a multi-parametric description of individual cells, which can be applied to study microbial communities (Rubbens et. al., 2017). *S. epidermidis* and *S. mutans* biofilms were stained with SYTOX®, a Live/Dead stain. Results showed that *S. epidermidis* biofilm was inhibited 86.7% with TF, 52.9% with pEGCG and 73.6% with TF/pEGCG combination (Figure 20). This indicates that TF alone is able to inhibit majority of biofilm formation in *S. epidermidis.* pEGCG is not as effective in inhibiting biofilm formation alone, but in combination with TF is able to increase percent inhibition. *S. mutans* biofilm was inhibited 88.3% with TF, 84.7% with pEGCG, and 61.9% with TF/pEGCG combination (Figure 21). These results indicate that TF and pEGCG are more effective in inhibiting biofilm formation alone. The combination of the two compounds was less effective in inhibiting biofilm formation.

Recently, microbiologists, food safety researchers, and the general public, to name a few, are concerned with the growing number of foodborne illness outbreaks caused by pathogens, such as *Bacillus cereus* (Friedman, 2006). *Bacillus* spp. are capable of producing spores, which are able to withstand harsh conditions. In this study, *B. megaterium* and *B. cereus* were first treated with 1% TF and 1% pEGCG, to observe the potential effectiveness in inhibiting sporulation (Fig. 22). Figure 22A and Figure 22D

represent the control sample, where *B. megaterium* and *B. cereus* are untreated with diH_2O to induce the cells to sporulate. When the cells were treated with the compounds, TF and pEGCG, there is a decrease in spores present in the samples indicating that the compounds are effective in inhibiting sporulation of these bacterial cells.

Once it was observed that these compounds were effective in inhibiting sporulation, they were then tested for their effectiveness of inhibiting germination. *Bacillus cereus* spores were treated with TSB, 1% TF and 1% pEGCG to allow for germination to occur. Based on the results of the viable plate count (Fig. 23A), it was calculated that TF showed 76.6 % inhibition and pEGCG had 99.9 % inhibition (Fig. 23B). This indicates that pEGCG was more effective in inhibiting germination of *B. cereus* spores compared to TF.

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