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**“The Synergistic Effects of Modified Green Tea Polyphenols [GTP], Epigallocatechin-3-gallate [EGCG], Lipid-based Tea Polyphenol [LTP], Curcuminoids, and Red Algae Polysaccharides on Antibiotics against Potentially Pathogenic Bacteria.”**

Bobak Haghjoo

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"The Synergistic Effects of Modified Green Tea Polyphenols [GTP], Epigallocatechin-3-gallate [EGCG], Lipid-based Tea Polyphenol [LTP], Curcuminoids, and Red Algae Polysaccharides on Antibiotics against Potentially Pathogenic Bacteria."

by

Bobak Haghjoo

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**"The Synergistic Effects of Modified Green Tea Polyphenols [GTP], Epigallocatechin-3-gallate [EGCG], Lipid-based Tea Polyphenol [LTP], Curcuminoids, and Red Algae Polysaccharides on Antibiotics against Potentially Pathogenic Bacteria."**

**By Bobak Haghjoo**

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## Abstract

Green tea leaves contain many polyphenolic compounds such as (-)-epicatechin, (-)-epicatechin-3-gallate, (-)-epigallocatechin, and (-)-epigallocatechin-3-gallate(EGCG). Green tea polyphenols (GTPs) have been implicated to have distinct properties that combat the harmful effects of cell proliferation. These compounds contain certain anti-viral and anti-microbial mechanisms that inhibit growth and perhaps reverse the process in which replication occurs. In this study, varied concentrations of GTP, Lipophilic Tea Polyphenol (LTP), Curcumin, and Red Algae Polysaccharides were used separately and in synergism with the most commonly used antiseptics and antibiotics to study the effect on different species of gram positive and gram negative bacteria. The antiseptic study consisted of using the disk diffusion method. The antibiotic portion of the study utilized the Kirby-Bauer Method with an antibiotic disk dispenser consisting of twelve unique and commonly prescribed antibiotics. The zones of inhibition were measured in MDL and categorized as being resistant, intermediate, or susceptible to the chemical agent used.

The results suggested that synergistic effects of EGCG and LTP varied dependent upon microorganism, strain, classification, and antibiotic used against certain strains. The most studied organisms found to have had some form of impact when EGCG and LTP were combined with the antibiotic against the growth of the organism. It was found to work efficiently against *Escherichia coli* and *Staphylococcus epidermidis*. These organisms serve as an essential model for potentially pathogen strains that become pathogenic either due to plasmid exchange or developing other mechanisms to evade antibiotic resistance. It was also found that LTP had a greater synergistic effect with the antibiotics against the growth of the microorganism. The ampicillin-resistant strains of *Escherichia coli* were also focused upon to

determine if strains that develop antibiotic resistance can still become susceptible to treatment of the antibiotic in conjunction with the polyphenol to inhibit the mechanism of antibiotic resistance. The red algae polysaccharides also shown promise to inhibit the growth of many of these potentially pathogenic microorganism models. The spore study indicated that there was a certain extent of inhibition—up to 64%. The CFU Time-Kill portion of the study also indicated that after a polyphenolic treatment, the ability to inhibit the growth of microorganism up to 2 hours became 99.99999% (almost 100%).



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## Introduction

Chinese Green Tea is an orally ingested beverage popular in Asian and Western communities. The tea is derived from an herbal plant with a binomial latin name of *Camellia sinensis*. The greatest cultivation of this plant is areas surrounding mainland China, South and Southeast Asia. Recorded as the second most popular beverage consumed worldwide after water, Green tea has been observed to have several medical benefits, some of which include reduction in cholesterol level, protection against cardio-vascular diseases, cancer, etc<sup>18</sup>. The earliest record of the consumption of this beverage for medicinal purposes dates back to about 5000 years ago in China. Eliminating alcohol and toxins from the body, tea was utilized to improve urine and blood flow, relieve joint pains and build resistance against diseases<sup>12</sup>. These stimulating and detoxifying properties of tea have been attributed to phenolic compounds, namely epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epicatechin (EC), and caffeine (CN)<sup>33</sup>.

Tea was first introduced in European countries from China by Portuguese and Dutch explorers as a medicinal herb<sup>12</sup>. Tea is cultivated in three particular types - green, black and oolong. Each of these teas differs in their processing. Tea extraction techniques have undergone a great deal of modification from the conventional extraction methods to the novel Microwave-assisted extraction adopted to yield higher, faster, less labor intensive, eco-friendly extracts. Some of the conventional methods include heat reflux extraction, Soxhlet extraction and ultrasonic extraction. It has been noted that time plays a very critical role in Microwave-assisted extraction method. The extraction of polyphenols and caffeine was highest at the 4 minute mark after which the levels decreased. 4 minutes is thereby considered the standard extraction time in Microwave-assisted extraction (MAE). Also, these



yields of MAE for 4 minutes after pre-leaching for 90 minutes were consistently higher than extraction at room temperature for 20 hours, ultrasonic extraction for 90 minutes and heat reflux extraction for 45 minutes respectively <sup>29</sup>. It can also be observed that MAE offers better results without altering the antioxidant activity of the phenolic compounds <sup>34</sup>. This form of extraction is very easily performed. In a high pressure reactor (HPR) 100, tea residues are suspended in 20 ml of water. This reactor is then centrally placed in a microwave apparatus. For about 2 minutes, the reactor is heated to 110-230 (temperature range) degrees Celsius and a magnetic stirring bar is used for homogenous heating. This temperature is automatically controlled by Full name and then (PID) and is measured using a thermocouple- thermometer. The reactor is then immediately cooled after heating in an ice bath. The extraction is obtained from the tea residues via filtration using a no.3 filter paper <sup>36</sup>.

Due to its ease of extraction and a direct link that has been tested between the anticarcinogenic, antiatherogenic, antioxidant and antimicrobial activity of tea and its specific polyphenolic compositions, green tea has experienced popularity in the scientific realm. Its non-toxicity has led it to be one of the prime contenders for cancer treatment as officially acknowledged in Japan. Topical application of EGCG, a phenolic compound to mouse skins initiated with 7,12-dimethylbenz(*a*)anthracene inhibited tumor promotion of both teleocidin, one of the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters, and okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, in two-stage carcinogenesis experiment. TPA and okadaic acid do not function in a similar pattern. However, the application of EGCG caused a dramatic decrease in the specific binding of these molecules to their binding sites in mouse skin ranging from 100% to 30-40%. This can possibly indicate that EGCG caused resistance to the binding of the tumor promoter, now

referred to as “the sealing effect of EGCG”. Pretreatment with EGCG also was found to restrain the tyrosine phosphorylation of full name and then (EGF) receptor in human lung cancer cell line A431 cells treated with EGF. As a result, it can be concluded that EGCG might also be immune to the interaction of hormones and various growth factors with their receptors on mouse skin. These observations suggest that there are structural and functional similarities between EGCG and chaperones that regulate different protein activities and this could be a new line of research for cancer prevention <sup>25</sup>.

These chemopreventive effects of green tea have also been studied in conjugation with curcumin, individually and in combination settings. Curcumin is the principal curcuminoid of turmeric and a natural phenol. Primarily used as a food additive, this yellow pigment is extracted from the rhizome of herb *Curcuma longa* <sup>38</sup>. Its therapeutic benefits have been explored since 1900 BC as a part of Indian Ayurvedic treatment. However, its first documented use can be traced back to 1937 when it was used to treat Biliary disease. Thereafter, curcumin’s medicinal properties have been tested in inflammatory diseases, neoplastic diseases, cardiovascular and neurodegenerative diseases, diabetes, cystic fibrosis and other ailments. Many studies have also demonstrated the anti-tumor effects of curcumin. The phenol has been suggested of suppressing proliferation and metastasis, along with inducing apoptosis in various malignant tumors by altering the signal pathways, colorectal cancer being one of them.

Colorectal cancer is third leading cause of morbidity and mortality in cancer related deaths. Amongst the various causes, dietary habits and lifestyles have been closely related to the development of this form of cancer. Studies have shown that it is possible to prevent the advancement of colon cancer with the use of natural polyphenols and flavanoids Wister rats



suffering from 1, 2 dimethylhydrazine (DMH) induced colon carcinogenesis were tested to study the effects of green tea and curcumin, individually and in combinatorial trials. These Wistar rats had to first go through a 32 week diet regimen. After the 32 weeks, the incidence, number and size of colorectal cancer were measured. In order to examine aberrant crypt foci (ACF), methylene blue staining was employed. Aberrant crypt foci are abnormal tube like glands found in the colon and rectum; precursors of the colorectal polyps. Their presence in the colon is one of the guiding indicators that may suggest a change that might eventually lead to cancer. Consequently, after staining, PCNA immunostaining and TUNEL assay were used to corroborate proliferation indices and apoptotic indices. It was recorded that the consumption of curcumin and catechins (individual and combination) helped decrease the number of ACF per rat. However, the combination administration had the most significant repressive effect. The incidence and proliferation index was substantially lower in the treatment groups in comparison to the positive control group. The apoptotic index, on the other hand was extremely high in treatment groups when matched up to the positive control group. This synergistic effect of green tea and curcumin on the prevention of colon cancer can be another avenue that must be further explored <sup>38</sup>.

The medical benefits of green tea have also intercepted the cardiovascular world. In Japanese populations, green tea has been directly associated to the lower incidences of coronary artery diseases. In one of the studies, twenty-two male volunteers that spanned the age of twenty-two and thirty-two years old has been enrolled to observe the effects of the intake of green tea polyphenols. The LDL resistance and in-vivo oxidation was also observed as the primary focus of the study. These men were then divided into control and tea groups after a week of observation. EGCG levels detected in the tea group before the experiment

accounted to none. The plasma EGCG levels at the end of the experiment were 56 nmol/ L after ingesting 300 mg of green tea polyphenol extract twice a day for an entire week in the tea group. Apart from the  $\beta$ -carotene levels, plasma concentrations of  $\alpha$ -tocopherol, ascorbic, lipids, and lipid peroxides did not change before and after the experiment in either group.  $\beta$ -carotene were considerably higher in the tea group. LDL was then incubated with 5  $\mu$ M  $\text{Cu}^{2+}$  and the oxidation was measured through absorbance. Subsequently, the measured lag time was extended by 13.7 minutes in the tea group. No such increase in the lag time was observed in the control group. The study, therefore, highly suggests that the daily consumption of a seven to eight cups of green tea approximation may increase the ratio of resistance of LDL against the process of in vivo oxidation leading to an overall beneficial health. This implies that the risk of cardiovascular diseases can be reduced <sup>26</sup>.

Chinese green tea extract has also been found to strongly inhibit the growth of major food-borne pathogens, *Escherichia coli* O157:H7, *Salmonella Typhimurium* DT104, *Listeria monocytogenes*, *Staphylococcus aureus*, and a diarrhea food poisoning bacteria *Bacillus cereus* in varied levels of effectiveness <sup>33</sup>. EGCG and ECG were the most active of the four phenolic compounds that were indentified using a bioassay-guided fractionation technique followed by reversed-phase high-speed counter-current chromatography (HSCCC) that separated and purified all the tea components. It was observed that these two active compounds acted by altering the bacterial morphology, a possible result of disturbed cell division.

This is of particular interest since EGCG has been suggested to be highly effective against *S. aureus* and its methicillin resistant *S. aureus* (MRSA). *S. aureus* is of major concern to the dairy industry worldwide since it has been associated with bovine mastitis in



high rates causing tremendous economic losses. This pathogen has become an increasing problematic infection with the response in evolutionary resistance to a broad spectrum of antibiotics. As a positive marker of a recent study, it was concluded that after about 5 to 6 hours of incubation under assay conditions, 500µg/ mL of green tea extract was able to completely inhibit the growth of both susceptible and resistant strains of this bacteria <sup>33</sup>.

The utilization of green tea polyphenols (-)-epicatechin, (-)-epicatechin-3-gallate, (-)-epigallocatechin, and (-)-epigallocatechin-3-gallate(EGCG) as a measure against illness and disease, prevention or cure, in human beings is becoming an increasingly novel approach in Western Science & Medicine <sup>13</sup>. These compounds found exclusively in green tea have been implicated to have distinct properties that combat the harmful effects of many potentially pathogenic bacteria <sup>1</sup>. The composition of these derivatives contains unique properties that are anti-viral and anti-microbial in nature and are a sought out measure in the possible usage of future medical intervention for therapeutic purposes.

One of the several diseases that impacts human beings globally are from the strain of bacteria that causes tuberculosis (genus *Mycobacterium*). These organisms have become an increasing problematic infection with the response in evolutionary resistance to a broad-spectrum of antibiotics <sup>3</sup>. Thus, there is a crucial need to develop novel approaches that reconstitutes the antimicrobial activity of antibiotics and the determination of the synergistic effects found from green tea compound derivatives.



The polyphenols found in green tea leaves are classified into a group of antioxidants that possess characteristics that are beneficial for the natural processes of biologically living organisms. These antioxidants have properties that are also antimicrobial in nature and may be used against many potentially pathogenic strains of several disease-causing bacteria. The establishment and understanding of these synergistic properties against microorganisms are essential for the development of novel therapeutic agents <sup>8</sup>.

Curcumin is another major phenolic compound (organism *Curcuma longa*) that is becoming increasingly popular in Microbiology, Food and Chemical Toxicology, and Western medicine. There has been reported synergistic effect of this compound with insulin as a potential remedy for a natural anti-diabetic therapeutic formulation <sup>19</sup>. The clinical observations of this compound have been concluded in a variety of *in-vivo* and *in-vitro* experimentations <sup>37</sup>.

The synergistic effect of the phenolic compounds of green tea and curcumin have been studied in carcinogenesis—specifically antagonistically to the compounds that give rise to colon cancer. Methylene blue staining, a common staining technique used in Microbiology, was able to analyze colorectal cancer incidence <sup>38</sup>. These compounds could have a great impact synergistically against potential pathogenic bacteria that invade human beings through inhalation, ingestion, or injection. They could also provide a protective barrier or measure against pathogenic bacteria that are able to bypass the skin or manipulate mechanisms that benefit pathogenesis.

Our hypothesis is that the compounds and derivatives that are found in green tea leaves and other polyphenolic compounds may have clinical applications against several

strains of bacteria that are potentially pathogenic. In addition, the combination of phenolic compounds from different natural products display synergistic properties that increase the effects of antibiotics and antiseptics against infection. Spore evaluations will also be performed and studied to determine if Green Tea Polyphenols have any effect on inhibiting growth of *Bacillus* microorganisms.

1. The following objectives are proposed to test the hypothesis: Screening and profiling the effect of GTP and other phenolic compounds on microorganisms.

In this study, GTP, LTP (lipophilic green tea polyphenols), curcumin, and polysaccharides will be used to evaluate their antimicrobial activity on differentiated groups of bacterial organisms. The groups will consist of gram positive, gram negative, and acid-fast positive species of microorganisms. The gram positive species consist of *Staphylococcus epidermidis*, *Bacillus megatarium*, and *Enterococcus faecalis* (pathogenic strain). The gram negative species consist of *Enterobacter aerogenes*, *Proteus vulgaris*, *Serratia marcescens*, and *Escherichia coli*. The acid-fast organism that will be tested is *Mycobacterium smegmatis*. The antimicrobial activity and the effectiveness of the phenolic compounds with antibiotics and antiseptics are measured by the Zone of Inhibition using the established disk diffusion method.

2. Study the possible Synergistic effects of GTP and other phenolic compounds on antibiotics and antiseptics

Antiseptics such as alcohol, betadine, hydrogen peroxide, and Listerine will be used alone or in combination with phenolic compound GTP, LTP, curcumin and



polysaccharide to evaluate the antimicrobial activity by zone of inhibition as describe above. The synergistic effect will be determined by this study. Twelve selected antibiotics (*Ampicillin, Bacitracin, Cephalothin, Chloramphenicol, Doxycycline, Erythromycin, Gentamicin, Penicillin, Polymyxin, Rifampin, Streptomycin, and Tetracycline*) are used as controls and in combination with these compounds by measuring the zone of inhibition using Kirby-Bauer Method. The synergistic effect is determined in this region of study.

3. Study the effect of GTP on the antibiotic resistant bacteria and spores

a. Study the effect of GTP on ampicillin (amp) resistant bacteria *E. coli*

Different concentrations of GTP will be used to treat both amp sensitive and amp resistant *E. coli* to evaluate the effect of GPT on the antibiotic resistant strain. Time kill study will be carried out to quantitative study the potential anti-antibiotic resistant properties of GTP.

b. Study the effect of GTP on bacterial spores

*Bacillus megaterium*, an endospore former, will be used to study the effect of GTP on spores' germination. The cells will be starved to induce the spore formation, then heated treated to kill the vegetative cells. Quantities study on the formation of vegetative cell at different time intervals will be studied.

## Materials and Methods

### I. Culture of 8 Strains of Microorganisms and preparation of Polyphenols

There were 8 microorganisms in stock, constantly maintained and tested against for these trials. The organisms are listed as: *Esherichia coli*, *Staphylococcus epidermidis*, *Proteus vulgaris*, *Bacillus megatarium*, *Enterococcus faecalis* (most pathogenic strain), *Serratia marcasens*, *Enterobacter aerogenes*, and *Mycobacterium smegmatis*. They were used to screen and profile according to their characteristics depicted in our laboratory. The maintenance of each culture was kept throughout a constant, medium, and growth conditions such as temperature to ensure that each of the organisms had been tested in similar conditions while only changing one variable at a time. All the cultures were maintained in nutrient broth or nutrient agar plate. They were grown at 37°C incubation except *Serratia marcasens* which is kept at room temp. The fresh stocks were always prepared and stored at 4°C.

#### I.A. Preparation of Green Tea Polyphenol

The preparation of GTP was utilized by creating stock solution from fine grinded/extracted powder. The solution was created by taking the solute, in this case the extract, and inserting it into the sterile, autoclaved H<sub>2</sub>O solvent. The baseline study utilized 1% solution and was created by measuring 0.04grams of GTP, which was measured analytically by the digital scale in the microbiology laboratory, and dissolved into 4mL of solution. The final result created a 1% solution to use antiseptically and/or for antibiotic purposes.

#### I.B. Preparation of Lipophilic Tea Polyphenol

The preparation of LTP (the EGCG-ester derivative) was utilized by creating stock solution from fine grinded/extracted powder. The solution was created by taking the solute, in this case the extract, and inserting it into an organic solvent (i.e. DMSO, EtOH, etc.). The baseline study utilized 1% solution and was created by measuring 0.04grams of LTP, which was measured analytically by the digital scale in the microbiology laboratory, and dissolved into 4mL of solution. The final result created a 1% solution to use antiseptically and/or for antibiotic purposes. The organic solvent of choice was 95% ethanol as an organic solvent due to the harmful and toxic nature of DMSO. This allows an ideal scenario to study the effectiveness or using the LTP as a topical solution for antibacterial properties.

#### I. C. Preparation of Curcuminoids

The preparation of curcumin was utilized by creating stock solution from fine grinded/extracted powder. The solution was created by taking the solute, in this case the extract, and inserting it into the sterile, autoclaved H<sub>2</sub>O solvent. The baseline study utilized 1% solution and was created by measuring 0.08grams of curcumin, which was measured analytically by the digital scale in the microbiology laboratory, and dissolved into 8mL of solution. The final result created a 1% solution to use antiseptically and/or for antibiotic purposes. The reason to use more grams and more milliliters is due to the nature of the curcumin's ability to avoid dissolving. This allowed adequate amount of sample to continuously be re-suspended and to avoid any loss from aperture exhalation such as evaporation.

#### 1.D. Preparation of Red Algae Polysaccharides



The preparation of Red Algae Polysaccharides was utilized by creating stock solution from a thick, liquid substance of high viscosity. The solution was created by taking the solute, in this case the already thickened liquid, and inserting it into the sterile, autoclaved H<sub>2</sub>O solvent. The baseline study utilized 1% solution and was created by measuring 0.04grams of GTP, which was measured analytically by the digital scale in the microbiology laboratory, and dissolved into 4mL of solution. The final result created a 1% solution to use antiseptically and/or for antibiotic purposes. This allowed an adequate ability to pipette the solution using a micropipetter as the thick substance would create issues in performing that step.

## II. **Preparation of medium for microorganisms**

**Nutrient Broth (NB) and Nutrient Agar plates (NA)** were used and prepared according to manufacturer standard. The recommended amount of sample was 38 grams into 1 Liter of deionized water and heat-stirred on hot plate with magnetic stirs until the completely dissolved. The sample was autoclaved at 121°C for 30 min. The preparation is followed by step II.C and poured onto petri dish (85mm in diameter) and stored at 4°C for further inoculations and testing.

### A. **Mueller-Hinton Agar plates (MH Plates)**

Mueller-Hinton Broth agar was used and measured according to manufacturer standard. The recommended amount of sample was 28 grams into 1 Liter of deionized water and heat-stirred on hot plate with magnetic stirring until completely dissolved. The media were autoclaved at 121°C for 30 min. The ingredients of the Mueller-Hinton Broth were infused beef extract, (300.0g), casamino acid (17.5g), starch (1.5g), and agar (17.0g). The preparation is

followed by step II.C. and poured onto petri dish (135mm diameter) and stored at 4°C for further inoculations and testing.

### **B. Autoclave Parameters and usage**

The Autoclave machine provided adequate sterilization for the water compounds and media preparations listed in steps 'B' and 'C'. This ensures that there contaminants, pollutants, and other bacteria have been eradicated from the solution if any were present during the preparation procedure. The parameters used were liquid cycles set at 121°C for an interval of 30 minutes and 15 PSI (pounds per square inch). First, the items that are required to be autoclaved must be inserted into the machine. The machine should be manually set to 121°C and a pressure of 15 PSI. The timing can be set to 15 minutes or up to 30 minutes. Dependent upon the actual item for sterilization, the user must be select a liquid or wrapped cycle. Afterwards, the user may start the cycle and wait for the pressure and steam of the machine to sterilize the equipment or broth.

### **III. Minimum Inhibitory Concentrations (MIC)**

The MIC procedure allows the quantitative and macroscopic observation of the amount of antibiotics and extracts necessary to inhibit microbial growth. The method utilized microdilutions of 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32. The starting concentration of the samples for ampicillin was measured to match the original concentration established by BD® Diagnostic Systems Sensi-Disc™ (Catalog 260640). The serial dilution method was established against the deep-well nutrient agar broths. The 24-well plates were incubated for 24 hours under 37°C. The inhibitory concentration was monitored by 1) The turbidity of the



sample and 2) the absorbance of the microorganisms at an OD<sub>686nm</sub> using a Thermo-Scientific GENSYS 20™.

#### **IV. *Escherichia coli* Ampicillin-Resistant Plasmids w. Green Fluorescent Protein (GFP)**

The ampicillin-resistant strain of *Escherichia coli* was isolated from a pure colony that was originally grown on Luria Broth (LB) in the presence of ampicillin and L-arabinose. The colony was isolated from the medium by the usage of aseptic technique and continuous streaking onto new LB plates that had been supplemented with 100µl of ampicillin and 100µl of L-arabinose. The concentration of ampicillin was standardized at 100ng/ml. The concentration of L-arabinose was created using serial dilutions and set in a 1:10 ratio to create a solution of 5% percent. Prior to the inoculation of the ampicillin-resistant plasmid onto the new media, the plates had been supplemented with the above compounds for an hour to ensure that the solutions can have an adequate time to penetrate the agar and dry. The solution that was added to the agar was also placed onto spinning-plates and spun to evenly distribute the compounds on the agar by the usage of glass-rods. The even distribution allowed for the accuracy and adequacy of the compounds to be present on the LB plates so that the plasmid containing cells can multiply in the presence of ampicillin.

#### **V. *Escherichia coli* Ampicillin-Resistant Plasmids w/o Green Fluorescent Protein (GFP)**

The *Escherichia coli* specific strain that did not utilize and contain the green fluorescent protein (GFP) gene was derived from the University of California and labeled as pUC19. The treatment and cultivation of this microorganism had a similar ampicillin-



resistant gene that was expressed solely in the presence of ampicillin. The *Escherichia coli* were grown on Luria Broth (LB) in the presence of ampicillin and L-arabinose. The plasmid containing cells and continual cultivation was performed by the usage of aseptic technique and continuous streaking onto new LB plates that had been supplemented with 100µl of ampicillin and 100µl of L-arabinose. The concentration of ampicillin was standardized at 100ng/ml. The concentration of L-arabinose was created using serial dilutions and set in a 1:10 ratio to create a solution of 5% percent. Prior to the inoculation of the ampicillin-resistant plasmid onto the new media, the plates had been supplemented with the above compounds for an hour to ensure that the solutions can have an adequate time to penetrate the agar and dry. The solution that was added to the agar was also placed onto spinning-plates and spun to evenly distribute the compounds on the agar using glass-rods. The even distribution allowed for the accuracy and adequacy of the compounds to be present on the LB plates so that the plasmid containing cells can multiply in the presence of ampicillin.

## VI. Spore-Study

### Preparation of bacterial spores:

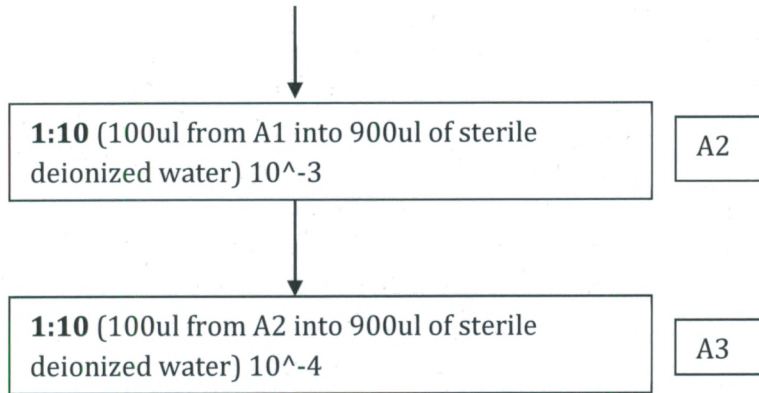
*B. meg* cells will be put into sterile deionized water for 2 hours to starve the cells. After 2 hours, determine absorbance reading at 686 wavelength using spectrometer 20 in order to determine dilution measurement. The OD reading should be around 0.5. Pipette out 3ml of the starve *B. meg* cells into 9ml of sterile deionized water, total of (12ml). The 12ml tube will be separated into 2 tubes of 6ml, which will be used for serial dilution. ( $10^{-2}$  to  $10^{-4}$ ) The flow chart is for the student to follow.

### Serial Dilution:

a) One tube with the 6ml will be used for the serial dilution:

The flowchart indicates a step-by-step direction on how to perform the serial dilution of the *Bacillus megaterium* spore study. This preparation ensures that an adequate dilution was performed in order to have the optimal amount of colonies to view upon the nutrient agar.

**1:100** (10ul of *B. meg* + 990ul of sterile deionized water)  $10^{-2}$

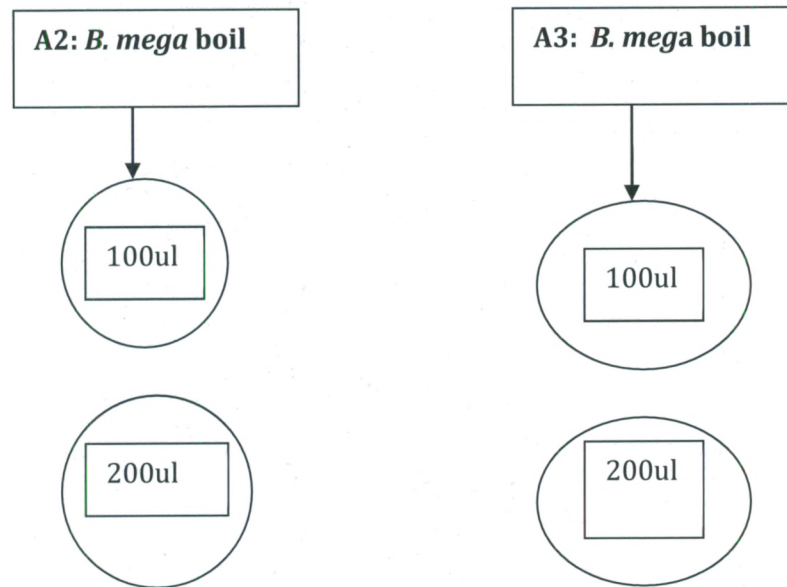


Use Micropipette out 500ul of A2 into (2) eppendorf tubes and 500ul of A3 into (2) eppendorf tubes, then heat at 90 Celsius for 20 minutes. After 20mins, add 0.05gram of GTP into one tube of A2 and A3. Wait for 1 hour before plating each tube into NA plates. The plating protocol is shown as follows as on page 27.



Protocol:

a) Nutrient agar plating: pre-warm the NA plates at 37c and plate as follows:



- b) A2: (B. meg boil + GTP)  
a. Pipette 100ul into NA plate and 200ul into another NA plate
- c) A2: (B. meg)  
a. Pipette 100ul into the 1<sup>st</sup> NA plate and 200ul into the 2<sup>nd</sup> NA plate
- d) A3: (B.meg)  
a. Pipette 100ul into the 1<sup>st</sup> NA plate and 200ul into 2<sup>nd</sup> NA plate. One plate has 100ul and the second plate has 200ul, total of 2 plates for B.meg.
- e) A3: (B. meg + GTP)  
a. Pipette 100ul into 1<sup>st</sup> NA plate and 200ul into 2<sup>nd</sup> NA plate

All the plates were incubated at 37c for 24 hours. After 24 hours, results will be collected by total plate count (TPC).

## VII. *Escherichia coli* Bacteriocidal Time-Kill Assays

3 ml of sterile DI H<sub>2</sub>O was added to a sterile test tube. *Escherichia coli*, obtained from a pure colony grown on a nutrient agar plate, was inoculated into this test tube using a sterile cotton swab. The solution was homogenized using a vortexer (spelling?) A Spec 20 set at a wavelength of 686 nm was used to measure the Optical Density of the solution. 1 ml of sterile DI H<sub>2</sub>O was added to a 1 ml cuvette and used as a blank. 1 ml was pipetted out of the test tube inoculated with *E. coli* and transferred to a 1ml cuvette. The Optical Density reading was recorded at 0.1. A 1:100 dilution was then performed by transferring 100 µl to a new sterile test tube and adding 9.9 ml of sterile DI H<sub>2</sub>O. The solution was vortexed. This step was performed following each dilution or transfer. A 1:10 dilution was performed by transferring 1 ml from this test tube into a new sterile test tube labeled: "E 10<sup>-3</sup>" then adding 9 ml of sterile DI H<sub>2</sub>O. 1ml was pipetted out of the "E 10<sup>-3</sup>" test tube and transferred to a sterile test tube labeled "E 10<sup>-4</sup>". 9 ml of sterile DI H<sub>2</sub>O was added to this test tube, performing a 1:10 dultion. 4.5 ml was pipetted out of "E 10<sup>-3</sup>" and transferred to a sterile test tube labeled "E 10<sup>-3</sup> + 5% GTP". 0.225 g of GTP were added to this test tube to create a 5% solution. 5 ml were pipetted out of "E 10<sup>-4</sup>" and transferred to a sterile test tube labeled "E 10<sup>-4</sup> + 5% GTP". 0.25 g of GTP was added to this test tube to produce a 5% solution. The four final tubes were: "E 10<sup>-3</sup>", "E 10<sup>-3</sup> + GTP", "E 10<sup>-4</sup>", "E 10<sup>-4</sup> + 5% GTP"

The above procedure was repeated with *Escherichia coli* containing a plasmid for ampicillin resistance. The resistant *Escherichia coli* were obtained from a stock



colony grown on nutrient agar plates containing ampicillin and L-arabinose. The test tubes were labeled: "E(R)" in place of "E".

From each of the eight test tubes 1 ml was transferred to a sterile 1.5 ml microfuge tube. The original test tubes were placed in a shaker at 37C. The microfuge tube was centrifuges at 14k rpm for 2 min. The supernatant was discarded. 1 ml of sterile DI H<sub>2</sub>O was added and the pellet was resuspended. The microfuge tube was centrifuged at 14k rpm for 2 min to wash the sample. The supernatant was discarded and the pellet was resuspended. 100 µl were pipetted out of the tube and transferred to a Nutrient Agar plate. For the ampicillin resistant *Escherichia coli* the plates also contained ampicillin and L-arabinose. The plates were incubated for 24 hours.

The above procedure was repeated for all eight test tubes at 1 hour and again and 2 hours. The plates were observed the following day to obtain a CFU count.

## Results

## 1. Microbial Profiling

### A. Microbial Profiling for Antibiotic Studies

Twelve different antibiotics were used in this study: Ampicillin (AM10), Bacitracin (B10), Cephalothin (CF30), Chloramphenicol (C30), Doxycycline (D30), Erythromycin (E15), Gentamicin (GM10), Penicillin (P10), Polymyxin (PB300), Rifampin (RA5), Streptomycin (S10), and Tetracycline (TE30). Eight different microorganisms were used in this study: *Escherichia coli*, *Bacillus megaterium*, *Enterococcus faecalis*, *Enterobacter aerogenes*, *Staphylococcus epidermidis*, *Mycobacterium smegmatis*, *Serratia marcescens*, and *Proteus vulgaris*. The Zones of Inhibition were measured in millimeters across their diameter of their clear region. This region included the antibiotic disc itself and utilized MDL (minimum-detection limit) as shown in Figure 1 below.



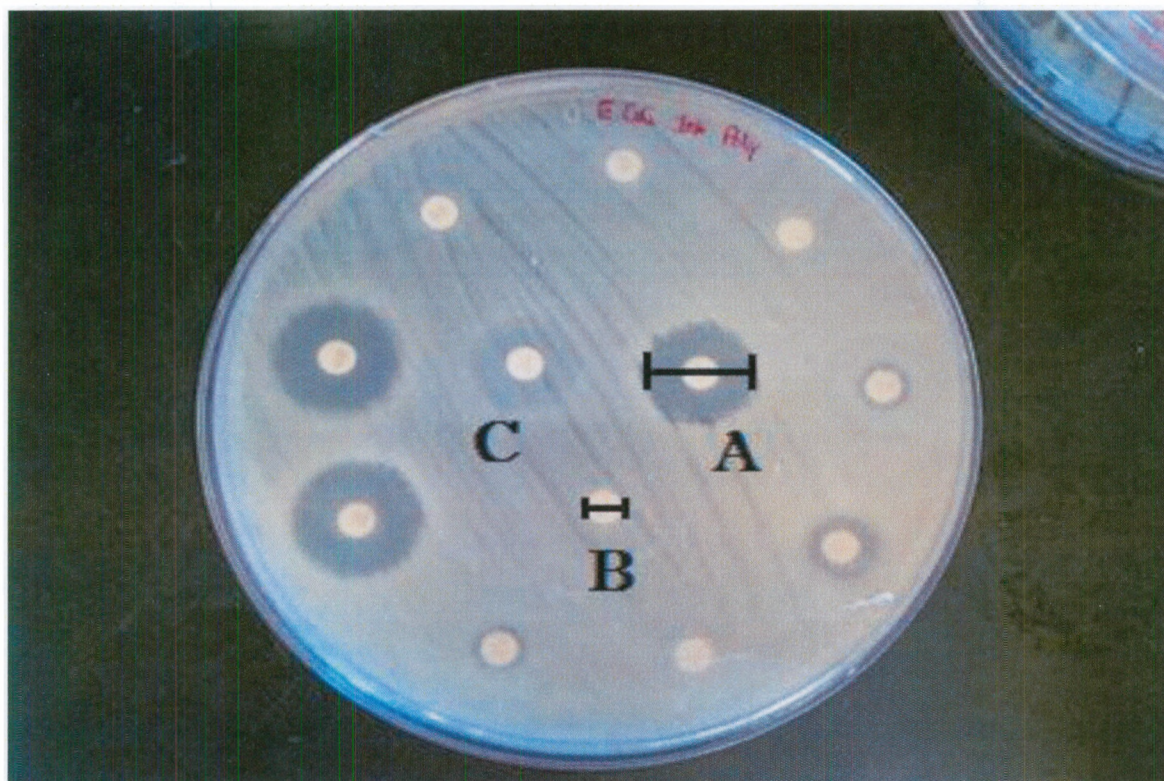


Figure 1: Disk Diffusion Susceptibility

- \*A = Complete Zone of Inhibition (ZOI),
- \*B = No Zone of Inhibition (6mm disk included),
- \*C = Partial/Weak Zone of Inhibition

The results of different microbial profiling are shown in Figure 2 to Figure 39. These results embody the normal conditions (control) of the antibiotic as well as the treated formulation of Green Tea Polyphenols, EGCG, and Curcuminoids. The interaction of antibiotic and polyphenol will be based upon observing the best interaction between antibiotic and polyphenol on the most susceptible and least susceptible microorganism to establish a novel and easy-to-read profile for the effects of combining natural derivatives with known antibiotics.



## 1. *Escherichia coli* (*E. coli*)

### a. Antibiotic profiling on *E. coli*

Three repeating of antibiotic disc diffusion test on *E. coli* were carried out on twelve different antibiotics and their results are shown below in Figure 2. In this figure the zone of inhibition (ZOI) is indicated under each antibiotics and the “S”, “I”, and “R” are marked on top of respective antibiotics. (put the fig after this sentence). The ZOI of three repeatings, mean and standard deviation (SD) are shown in Table 1.

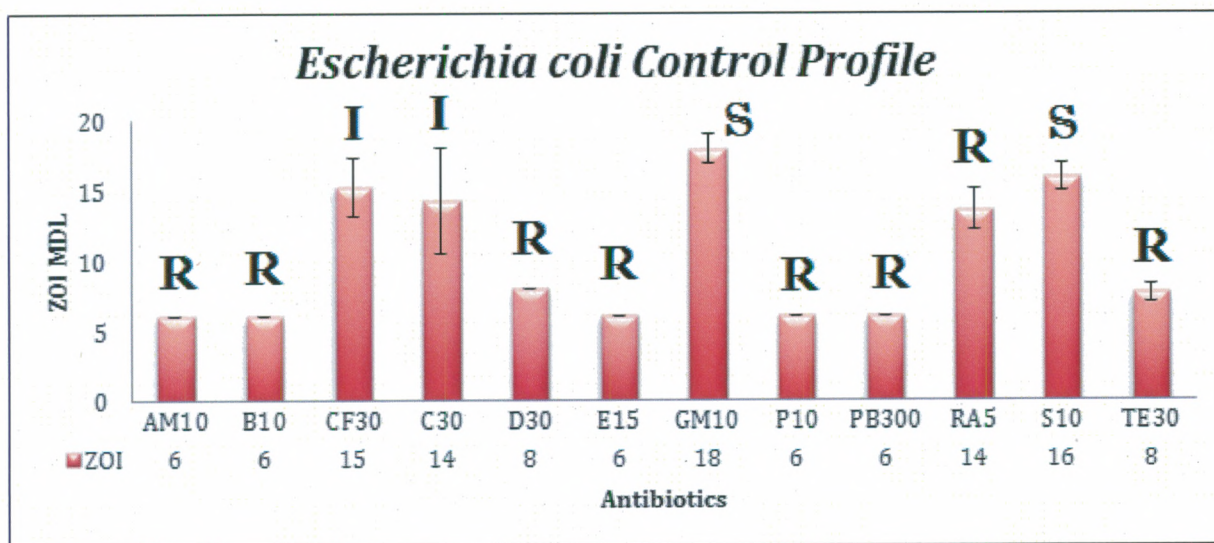


Figure 2. Zone of Inhibitions of 12 antibiotics on *E. coli*

The results indicated that *Escherichia coli* have resistance towards Ampicillin (AM10), Bacitracin (B10), Erythromycin (E15), Penicillin (P10), Polymyxin (PB300) and doxycycline (D30). The antibiotic Chloramphenicol (C30) and Cephalothin (CF30) is intermediate; between resistant and susceptible.

Antibiotics	Abbreviation	Reading 1	Reading 2	Reading 3	Average	Standard Deviation
Ampicillin	AM10	6	6	6	6	0.0
Bacitracin	B10	6	6	6	6	0.0
Cephalothin	CF30	16	17	13	15	2.1
Chloramphenicol	C30	16	10	17	14	3.8
Doxycycline	D30	8	8	8	8	0.0
Erythromycin	E15	6	6	6	6	0.0
Gentamicin	GM10	19	17	18	18	1.0
Penicillin	P10	6	6	6	6	0.0
Polymyxin	PB300	6	6	6	6	0.0
Rifampin	RA5	12	15	14	14	1.5
Streptomycin	S10	15	16	17	16	1.0
Tetracycline	TE30	7	8	8	8	0.6

Table 1: Antibiotics against *E. Coli*

Streptomycin (S10) and Gentamicin (GM10) have shown in inducing susceptibility for *Escherichia coli* the profile depicts the *E. coli* and its current status against different antibiotics. The strength of the antibiotic is determined by manufacturer settings and displayed after the antibiotic abbreviation Resistant, intermediate and susceptible antibiotics on *E. coli* is summarized in Table 2.

<u>Category</u>	<u>Antibiotic</u>
Resistant	Ampicillin, Bacitracin, Doxycycline, Erythromycin, Penicillin, Polymyxin, Rifampin, Tetracycline
Intermediate	Cephalothin, Chloramphenicol
Susceptible	Streptomycin, Gentamicin

Table 2: Summary of effectiveness of antibiotics on *Escherichia coli*



The resistance of gram negative *Escherichia coli* is high with a marked resistance towards 8 of the 12 antibiotics. There are 2 antibiotics that are intermediate in strength are cephalothin and chloramphenicol. The other 2 microorganisms, which make the organism susceptible, are streptomycin and gentamicin.

### **Combination study of EGCG and antibiotic Profiling on *E. coli***

The determination of combining EGCG with antibiotics against *Escherichia coli*, a gram-negative bacteria that has potential pathogenic properties, is organized into three different categories (S, I and R) using zones of inhibition measurements (Kirby Bauer Method).

Antibiotics combined with 1% of EGCG were used to study their effect on *Escherichia coli* (*E. coli*) by using the disc diffusion test to measure the zone of inhibition in mm. And the result is shown in Figure 3. The antibiotics study was kept at the same color (red) as the profiled screen in Figure 2 and the combination study was in blue and compared the data using 2D bar-graph analysis.



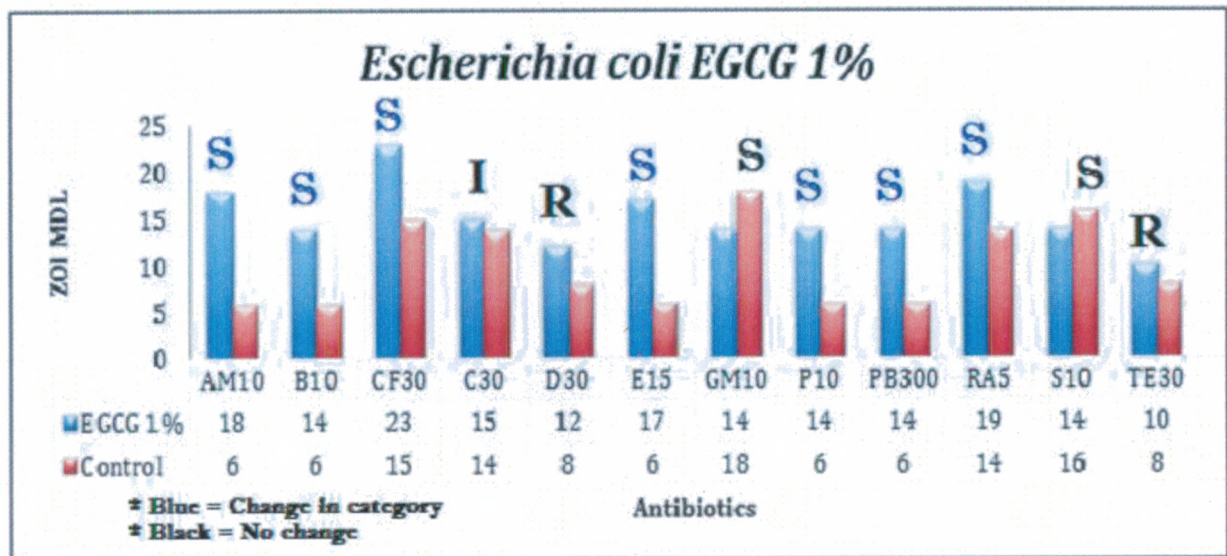


Figure 3: *Escherichia coli* against Twelve Antibiotics Supplemented with 1% EGCG

\*Blue indicates change from initial status \*Black indicates no change in current status

This study indicates that the antibiotics that were able to become susceptible from its initial resistant nature were ampicillin (AM10), bacitracin (B10), and polymyxin (PB300). The antibiotics that went from an intermediate phase to susceptibility were cephalothin (CF30), erythromycin (E15), penicillin (P10), and rifampin (RA5). The results suggested that 1% EGCG is able to illicit a response for the antibiotics to make them convert from resistance to intermediate/susceptible. Resistant, intermediate and susceptible antibiotics on *Escherichia coli* are summarized in Table 3, the antibiotics changed their effect are bracketed in the right column and their new category is displaced towards the left.

Category	Antibiotic [Original Category]
Resistant	Doxycycline [R], Tetracycline [R]

Intermediate	Chloramphenicol [I]
Susceptible	Ampicillin [R], Bacitracin [R], Cephalothin [I], Erythromycin [R], Penicillin [R], Polymyxin [R]

Table 3: Profile of Antibiotics and 1% EGCG combined on *Escherichia coli*

The effect was outstanding for the reversal of resistance in *Escherichia coli*. There was the ability to convert 5 of the 8 antibiotics into the susceptible range which was in the antibiotic resistant category). That means 62.5% of the original resistant antibiotics were converted to have susceptibility after treatment (5/8).

### C. The effect of 1% EGCG on the anti *E. coli* activity of antibiotics

Figure 4 below shows the percentage increase and/or decrease and the marked effect of 1% EGCG on the antibiotics. The results indicate that in the presence of EGCG, the percentage of inhibition for ampicillin, erythromycin, bacitracin, penicillin, polymyxin are 200%, 183%, and 133% (for the remaining 3) increased respectively compared with antibiotics alone. . Cephalothin, doxycycline, rifampin, and tetracycline had a lesser effect with percentage of inhibition of 53%, 50%, 36%, and 25% increased respectively compared with antibiotics alone.



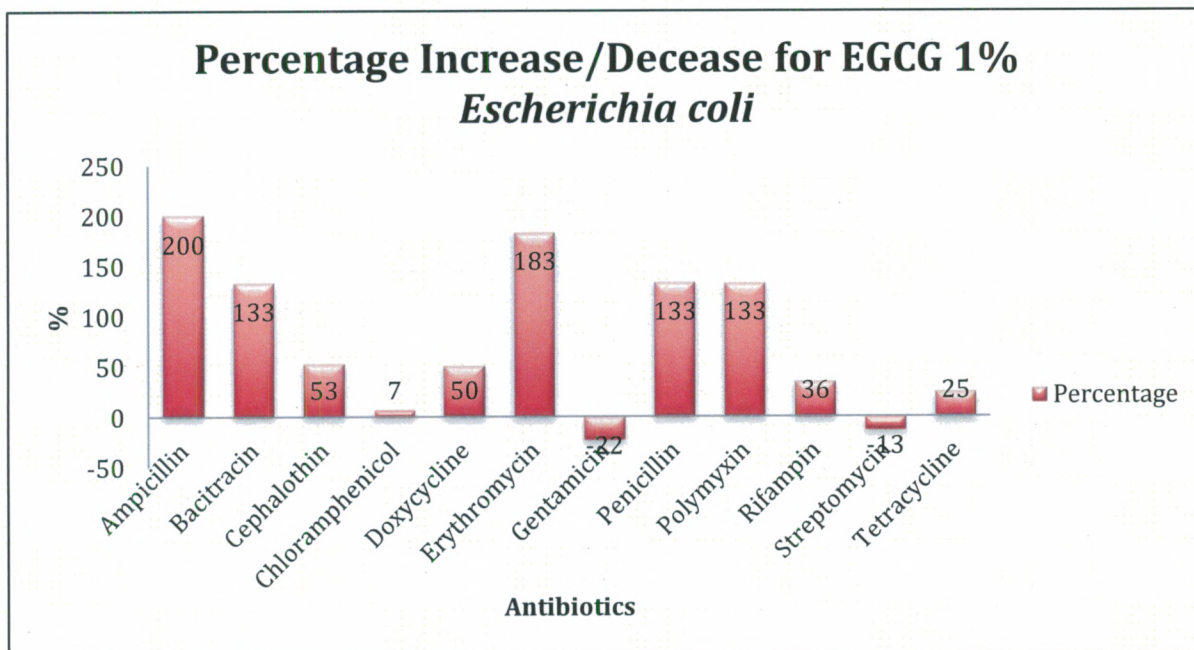


Figure 4: Percentage of efficiency for Twelve Antibiotics in *Escherichia coli* supplemented with 1% EGCG

In this study, from both zone of inhibition and percentage increase of inhibition, suggest that 1% EGCG can convert *Escherichia coli* resistance to susceptibility with the treatment of Ampicillin, Bacitracin, Cephalothin, and Polymyxin. The most apparent increase in percentage was founded in Ampicillin (AM10), Erythromycin (E15), Bacitracin (B10), Polymyxin (PB300), and Penicillin (P10). This result suggested that EGCG can be a synergistic agent to increase the antimicrobial activity of some antibiotics on *E. coli*. The rest of the antibiotics and their treatment rates were smaller or in the negative values. The negative values are suspected to have an effect that either interferes with the antibiotic mode of action or in the ability to read the correct zone of inhibition on a macroscopic ruler scale.



The second natural product used in this study is Red Algae Polysaccharides. Red Algae Polysaccharides with 1% (originated from Israel) indicated that they have an effect on *Escherichia coli* as shown in Fig.5. Percentage Increase/Decrease for 1% Polysaccharides on antibiotic treated *Escherichia coli* is shown in Fig 7.

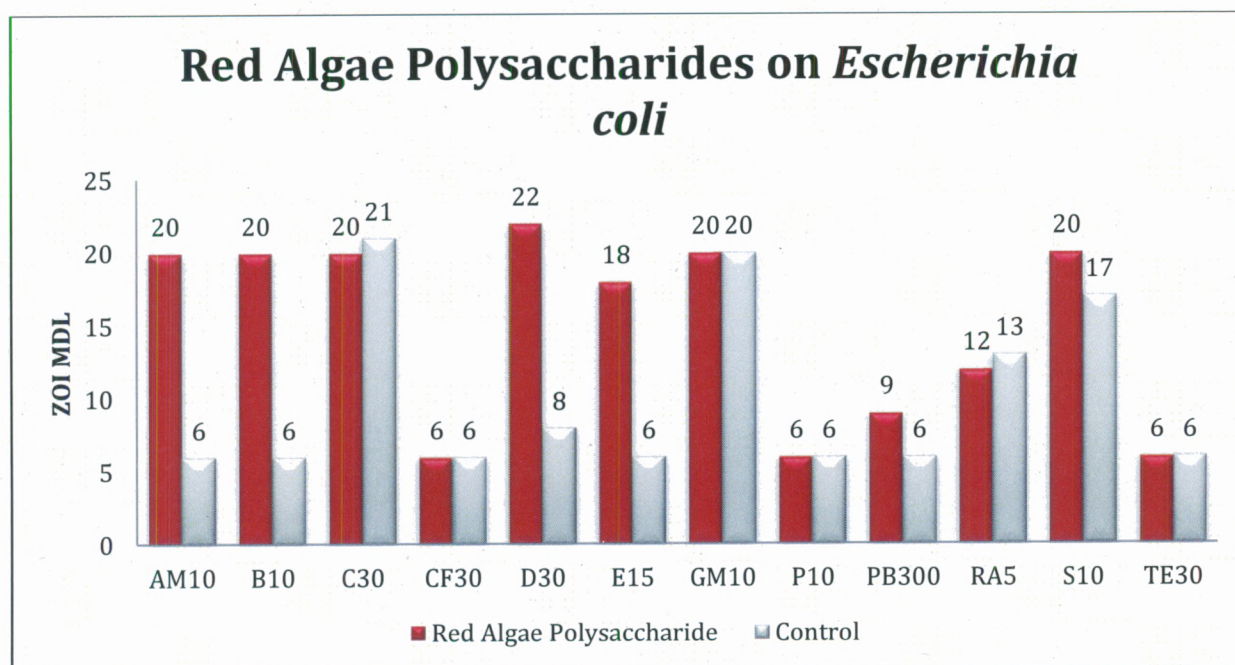


Figure 5: Israel Red Algae Polysaccharides on *E. coli*

The third compound used in this study is LTP, lipophilic green tea polyphenols. The results of the effect of LTP and the % of increase and/or decrease are shown in Fig 7 and 8.

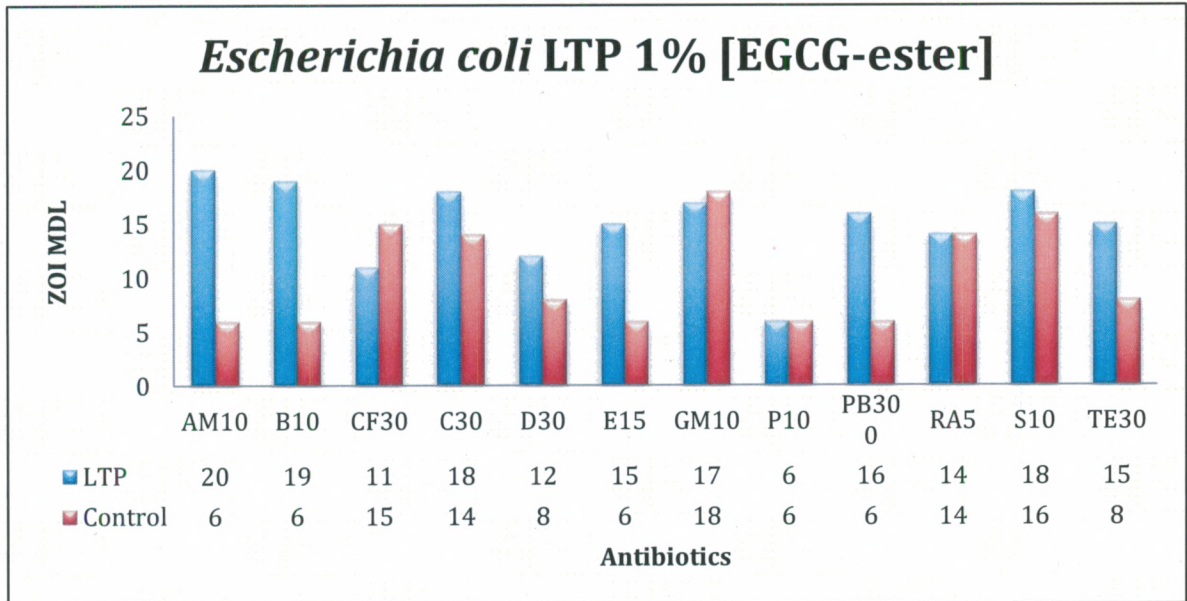


Figure 6: Increase/Decrease of Red Algae Polysaccharides on *Escherichia coli*

This data can be compared to that of EGCG, LTP (EGCG-ester), and GTP. The first comparison compares the Polysaccharides to EGCG by percentage. The result is shown in Fig 9.

When comparing the previous data for *Escherichia coli* for EGCG against Polysaccharides, the data indicates that polysaccharides from Israel's Red Algae have a slightly higher affinity for inducing antibiotic effectiveness for Ampicillin (AM10), Bacitracin (B10), Doxycycline



(D30), and Erythromycin (E15).

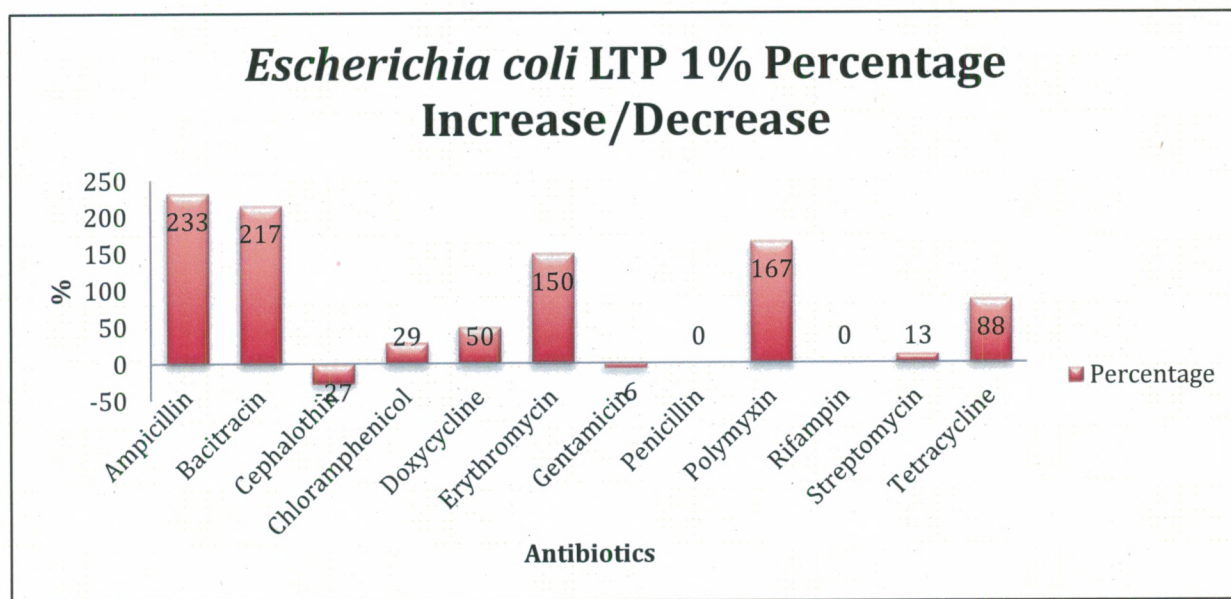


Figure 7: Increase/Decrease of EGCG-ester [LTP] on *E. coli*

The percentage increase and decrease for LTP in comparison to Red Algae Polysaccharides and EGCG are well established, yet similar. However, for instance, Penicillin (P10) had top priority with EGCG synergistic effect in comparison to the treated form with Polysaccharides and LTP. Based on these percentage data, one would conclude that Red Algae Polysaccharides were more fitting for this specific species of gram-negative bacteria.



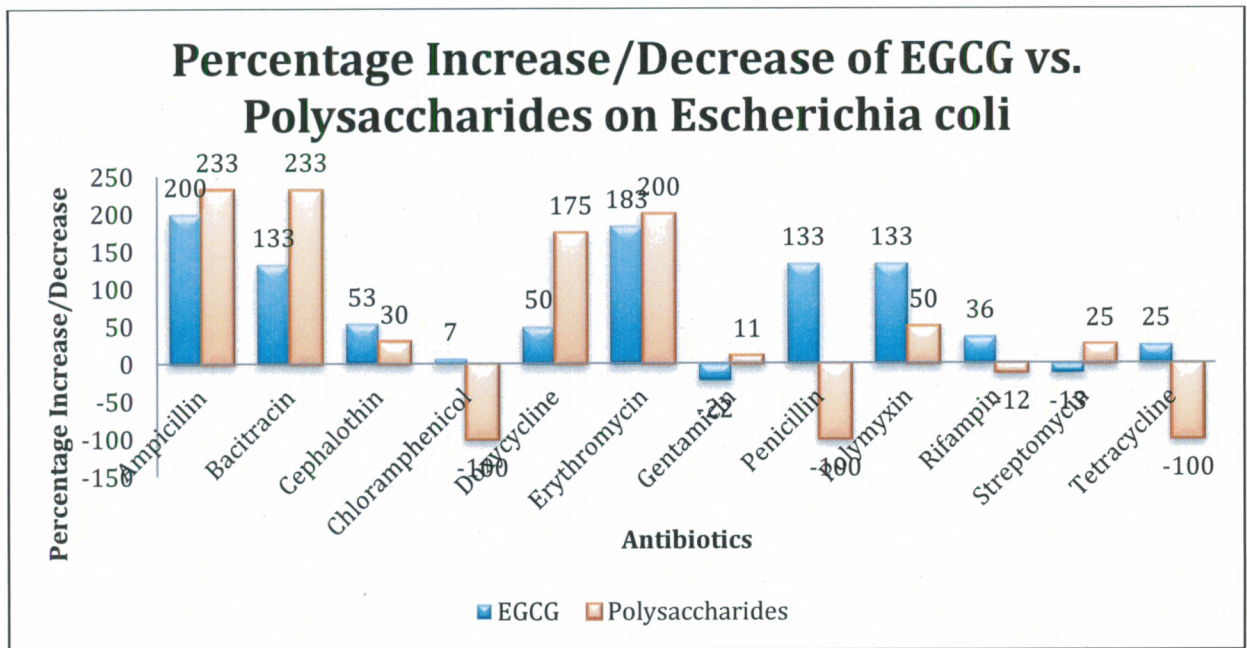


Figure 8: Increase/Decrease of EGCG vs. Polysaccharides

As easily observed above, the percentage change slightly varies for each of the compounds. However, there is a fundamental principle underlying both of the polyphenols in its interaction with the given antibiotics. They seem to have a correlation on the specific type of antibiotics which can be hypothesized to have a positive effect in that specific mode of action that the antibiotic exhibits on that particular type of bacteria. For instance, this gram-negative bacterium has increased susceptibility against the polyphenols for AM10, B10, D30, and E15. EGCG has increased percentages for P10 and PB300—while polysaccharides do not.

## 2. *Bacillus megaterium*

### a. Antibiotic profiling on *Bacillus megaterium*

*Bacillus megaterium* is a spore-producing microorganism and has severe implications in food microbiology. This microorganism was tested against twelve different antibiotics targeting different regions of the prokaryote (i.e. biosynthesis, cell wall formation, etc.). The results are shown in Fig. 9. The data displays three repeating with an average and standard deviation for the microorganism.

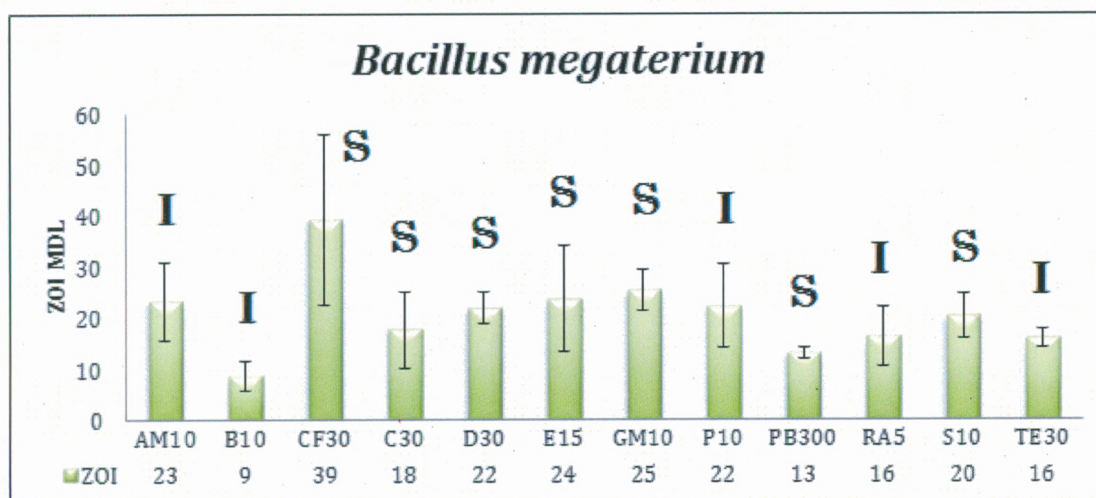


Figure 9: Zone of Inhibitions of 12 antibiotics on *Bacillus megaterium*

The antibiotics that were found to be susceptible (or very effective) against the microorganism were: Cephalothin (CF30), Chloramphenicol (C30), Doxycycline (D30), Erythromycin (E15), Gentamicin (GM10), Polymyxin (PB300), and Streptomycin (S10). The antibiotics that were found to have an intermediary effective strength were: Ampicillin (AM10), Bacitracin (B10), Penicillin (P10), Rifampin (RA5), and Tetracycline (TE30).



Antibiotics	Abbreviation	Reading 1	Reading 2	Reading 3	Average	Standard Deviation
Ampicillin	AM10	15	25	30	23	8
Bacitracin	B10	12	6	8	9	3
Cephalothin	CF30	20	50	48	39	17
Chloramphenicol	C30	9	23	21	18	8
Doxycycline	D30	19	22	25	22	3
Erythromycin	E15	12	27	32	24	10
Gentamicin	GM10	21	26	29	25	4
Penicillin	P10	13	25	29	22	8
Polymyxin	PB300	14	12	13	13	1
Rifampin	RA5	10	18	21	16	6
Streptomycin	S10	16	20	25	20	5
Tetracycline	TE30	15	15	18	16	2

Table 4: Antibiotic readings for *B. mega*

None of the antibiotics depicted proved to have a resistance by *Bacillus megaterium* indicating that this organism is quite sensitive by a broad spectrum of antibiotics are initially “weak” towards the modes of action of these antibiotics. The effect of antibiotics on *Bacillus megaterium* is summarized in table 6.

<u>Category</u>	<u>Antibiotic</u>
Resistant	
Intermediate	Chloramphenicol [R], Cephalothin [R], Polymyxin [R]
Susceptible	Bacitracin [R], Penicillin [R]

Table 5: Antibiotic categorization for *B. meg*

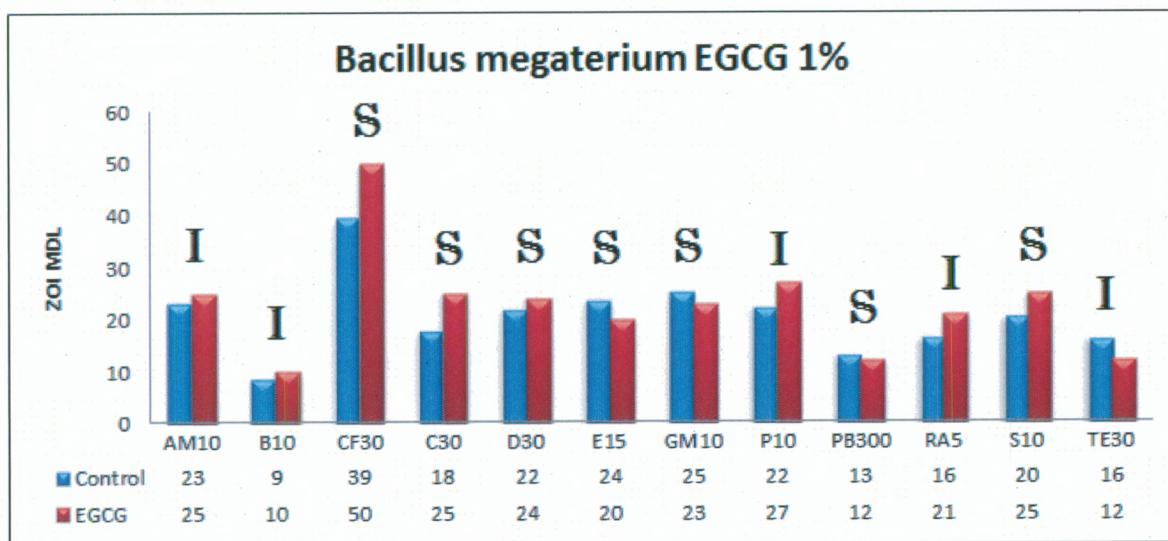
Gram positive *Bacillus megaterium* is extremely sensitive to antibiotic treatment with resistance towards 0 of the 12 antibiotics. There are 5 antibiotics that are intermediate in strength and another 7 which make the organism susceptible. The fact that they do not display any resistance does not indicate that the organism is easily killed or treated with



antibiotics. They are endospore formers and can survive harsh conditions while creating toxins that are able to contribute to human illnesses.

**b. Combination study of EGCG and antibiotic Profiling on *Bacillus megaterium***

Combination study of 1% EGCG with all the antibiotics was also carried out to study the zone of inhibition using the disk diffusion methods and the results are shown in figure 10.

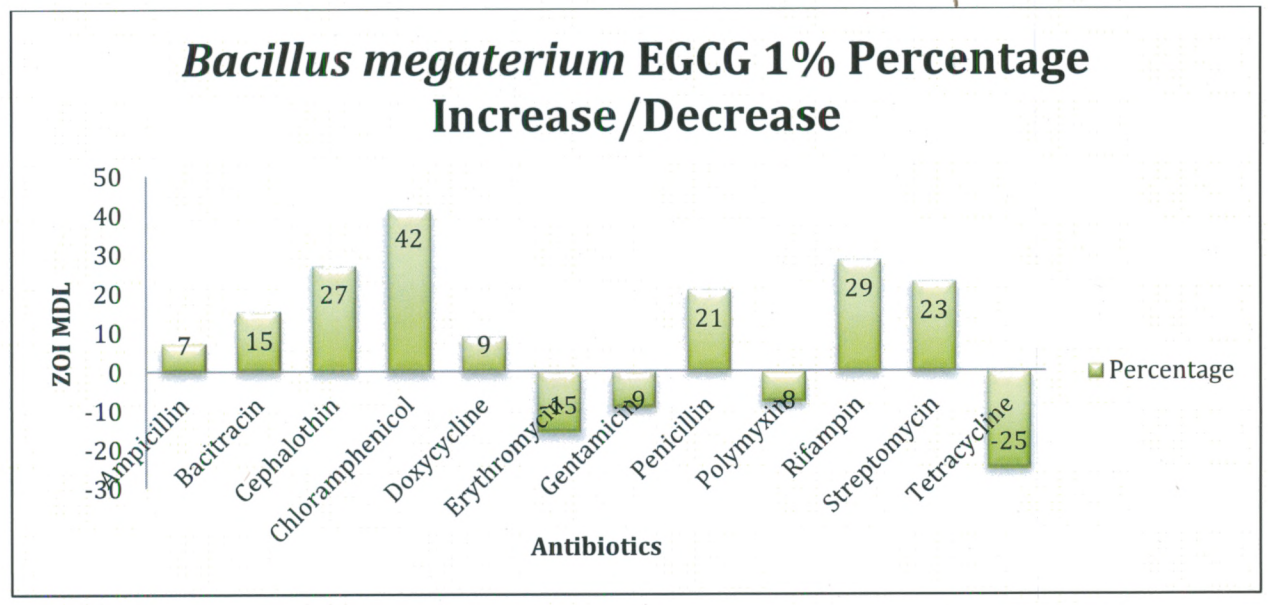


**Figure 10: *Bacillus megaterium* against Twelve Antibiotics Supplemented with Epigallocatechin-3-gallate (EGCG) at 1% Concentration**

**\*Blue indicates change from initial status \*Black indicates no change in current status**

The control were kept using the same color (red) as the profiled screen in Figure 2 and compared the data using 2D bar-graph analysis. There are no marked significant differences in the combination treatment versus antibiotic alone readings. The % of increase and/or decrease is shown in Fig. 12. This may be particularly due to the M.O.'s already susceptible nature and original zone of interference. There may also be speculation

that the spore-producing microorganism releases more spores and is able to counter-act the polyphenol-antibiotic interaction.



**Figure 11: Percentage of efficiency for Twelve Antibiotics in *Bacillus megaterium* supplemented with Epigallocatechin-3-gallate (EGCG) at 1% Concentration**

*Bacillus megaterium* shows that its susceptibility towards antibiotics is extremely high. Epigallocatechin-3-gallate (EGCG) at 1% concentration does not seem to have a very high effect compared to the previous studied microorganisms. However, when comparing and evaluating the percentage increase, it seems that there are some polyphenol-antibiotic interactions for Chloramphenicol (C30) at 42% increase. The following increases come at Rifampin (RA5), Cephalothin (CF30), Streptomycin (S10), and Penicillin (P10) in descending order.



### 3. *Staphylococcus epidermidis*

*S. Epi (Staphylococcus epidermidis)* is a common microorganism found to contribute to the normal flora of the human skin. It is an ideal model organism to use to study the more detrimental species of the same genus. This gives insight into figuring out whether or not the organism would be responsive to certain antibacterial treatments. It is a gram positive microorganism that contains a thick peptidoglycan layer that retains the crystal violet dye. The treatment against this organism gives direct insight into MRSA and MRSA-related species.

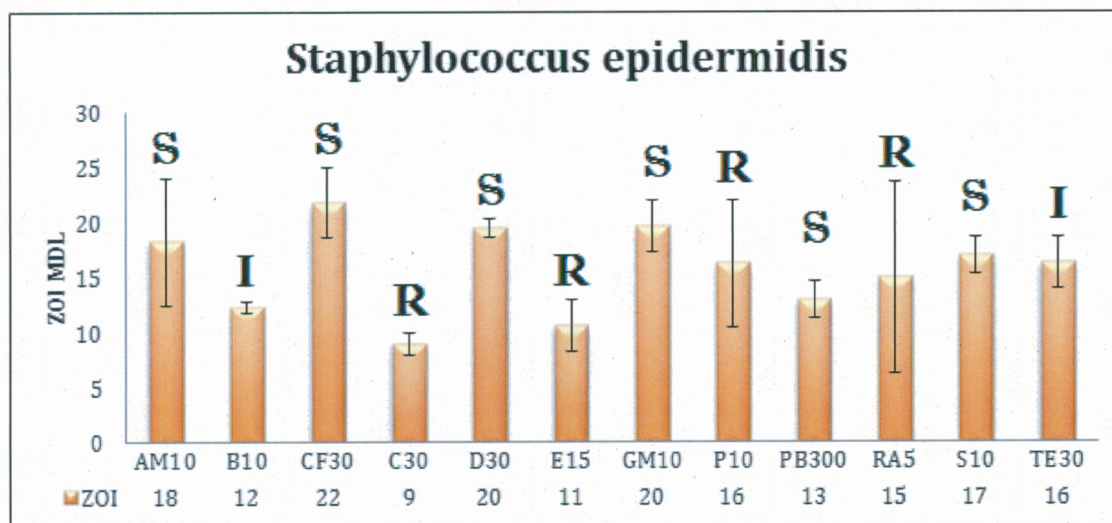


Figure 12: Summary of the microbial profile of *Staphylococcus epidermidis*

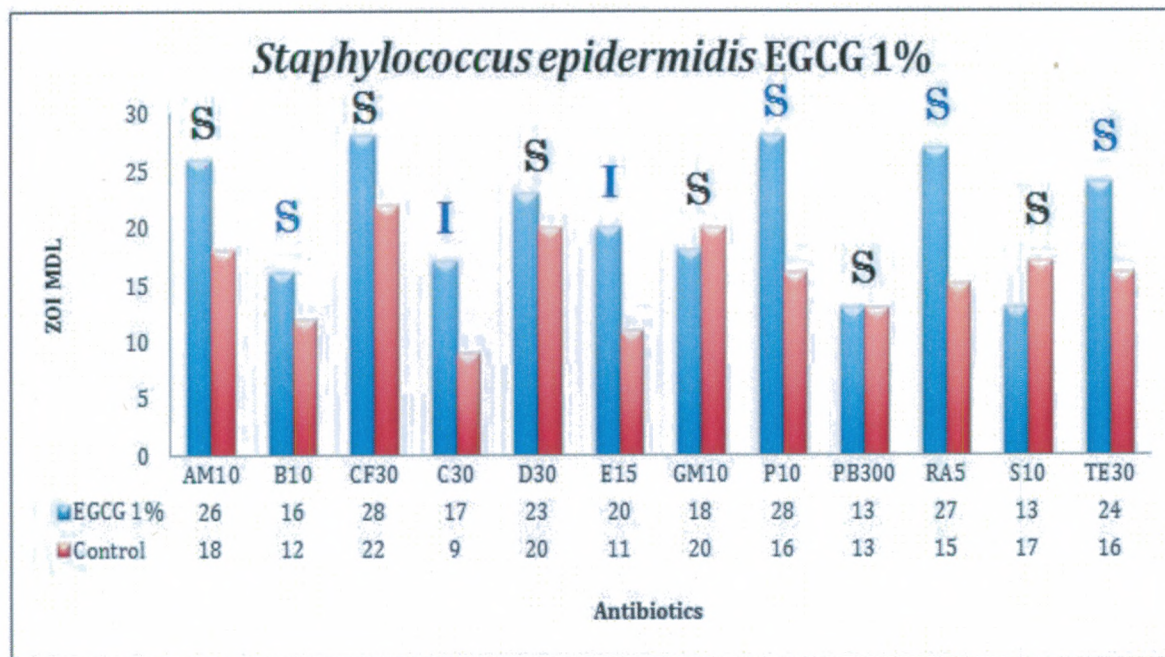
#### Against Twelve Antibiotics

Resistance	Chloramphenicol, Erythromycin, Penicillin, Rifampin
Intermediate	Bacitracin, Tetracycline
Susceptible	Ampicillin, Cephalothin, Doxycycline, Gentamicin, Polymyxin, Streptomycin

Table 6: Antibiotic categorization of *S. epi*



There are 4 antibiotics that are marked as being intermediate with 2 antibiotics in the intermediate strength. The remaining 8 antibiotics are in the susceptible range. The most desirable effect for this would be to evaluate the induction of reversing resistance in the remaining 2 antibiotics to categorize them into the susceptible range.



**Figure 13: *Staphylococcus epidermidis* against Twelve Antibiotics Supplemented with Epigallocatechin-3-gallate (EGCG) at 1% Concentration**

**\*Blue indicates change from initial status \*Black indicates no change in current status**

Category	Antibiotic [Original Category]
Resistant	
Intermediate	Chloramphenicol [R], Erythromycin [R]
Susceptible	Bacitracin [I], Penicillin [R], Rifampin [R], Tetracycline [I]

**Table 7: Antibiotic categorization for *S. epi* after treatment**

*Staphylococcus epidermidis* is an excellent model organism to study for the treatments of more serious infections such as *Staphylococcus aureus*. The antibiotics that were found to have initial inefficiency, meaning the microorganism displayed resistance, were listed as Chloamphenicol (C30), P10, E15, and RA5. Each of these antibiotics were effectively reversed and became into the intermediate or resistant ranges. The microorganism did not have any resistance towards of any of the antibiotics after the treatment of EGCG into the compound/mixture.

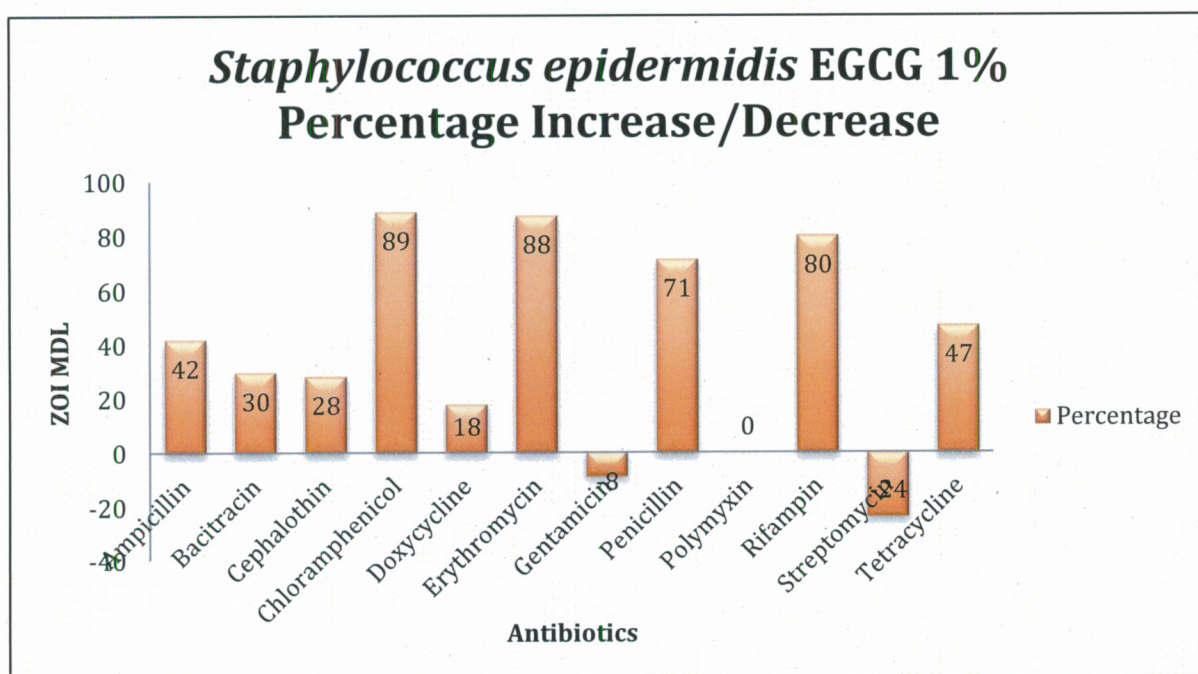


Figure 14: Increase/Decrease of *S. epi* with EGCG against Control

There are several marked increases in the zone of inhibition for each of the antibiotics. The highest marked increase is noted in Chloramphenicol (C30) followed by Erythromycin (E15) as 89% and 88% respectively. Polymyxin (PB300, did not show any significant changes.



Gentamicin (GM10) and Streptomycin (S10) showed the reverse effect and did not have any increase in percentage.



#### 4. Proteus vulgaris

*Proteus vulgaris* is a gram-negative microorganism that is commonly found in unsanitary living conditions. These microorganisms tend to plague bathroom stalls of Universities and Hospital settings that may cause students and patients to become ill. They are potential pathogens and can override the immune system to cause systemic damage. It is a bacillus shaped microorganism and found to an inhabitant of the gastrointestinal environment of the body. It is directly linked to the urinary tract infections and has indole production by the enzyme tryptophanase.

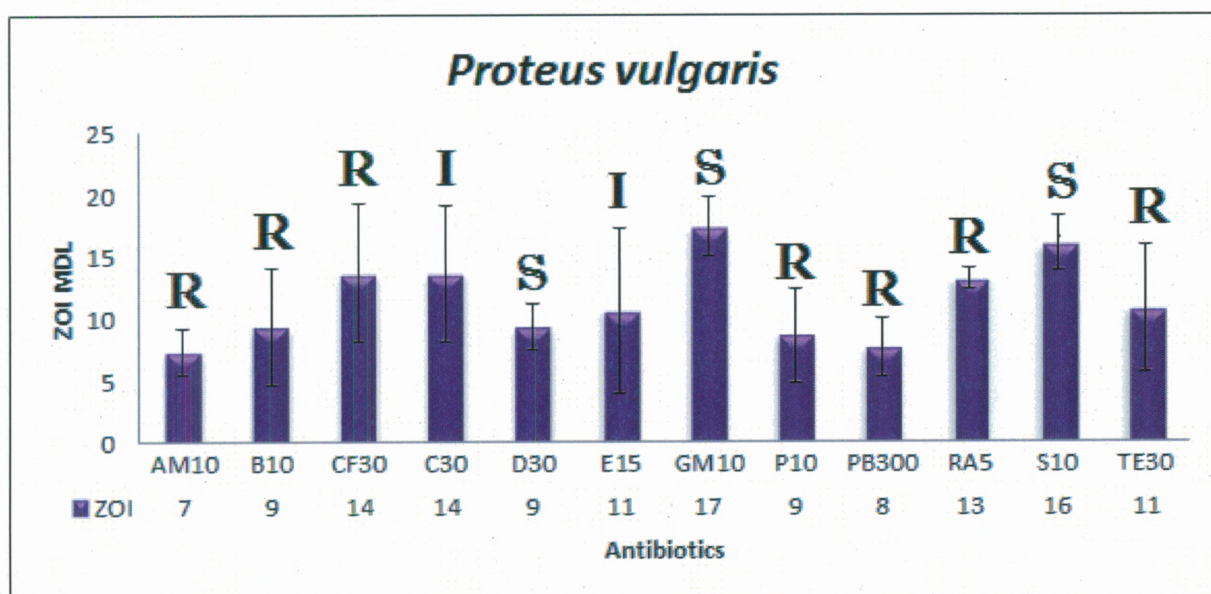


Figure 15: *Proteus vulgaris* against twelve antibiotics

		Reading 1	Reading 2	Reading 3	Average	Standard Deviation
Ampicillin	AM10	6	10	6	7	1.9
Bacitracin	B10	6	16	6	9	4.7
Cephalothin	CF30	6	16	19	14	5.6
Chloramphenicol	C30	18	17	6	14	5.4
Doxycycline	D30	8	12	8	9	1.9

Erythromycin	E15	6	20	6	11	6.6
Gentamicin	GM10	19	14	19	17	2.4
Penicillin	P10	6	14	6	9	3.8
Polymyxin	PB300	6	11	6	8	2.4
Rifampin	RA5	13.5	14	12	13	0.8
Streptomycin	S10	17	13	18	16	2.2
Tetracycline	TE30	6.5	18	8	11	5.1

Table 8: Antibiotic readings for *Proteus vulgaris*

The antibiotics that were resistant to the microorganism were founded to be: Ampicillin, Bacitracin, Doxycycline, Cephalothin, Penicillin, Polymyxin, Rifampin, and Tetracycline. The intermediate ranges were Chloramphenicol and Erythromycin. The susceptible category fell into Doxycycline, Gentamicin, and Streptomycin.

<u>Category</u>	<u>Antibiotic</u>
Resistant	Ampicillin, Bacitracin, Doxycycline, Cephalothin, Penicillin, Polymyxin, Rifampin, Tetracycline
Intermediate	Erythromycin, Chloramphenicol
Susceptible	Streptomycin, Gentamicin

Table 9: Antibiotic categorization for *Proteus vulgaris*

These ranges mimicked the original antibiotic breakdown of *Escherichia coli* with the exception of the role reversal in category for Erythromycin and Cephalothin (changing from intermediate to resistant). Otherwise, this categorization is a class *E. Coli* classification and, as a matter of fact, makes sense due to its similar gram negative and entero-property nature.



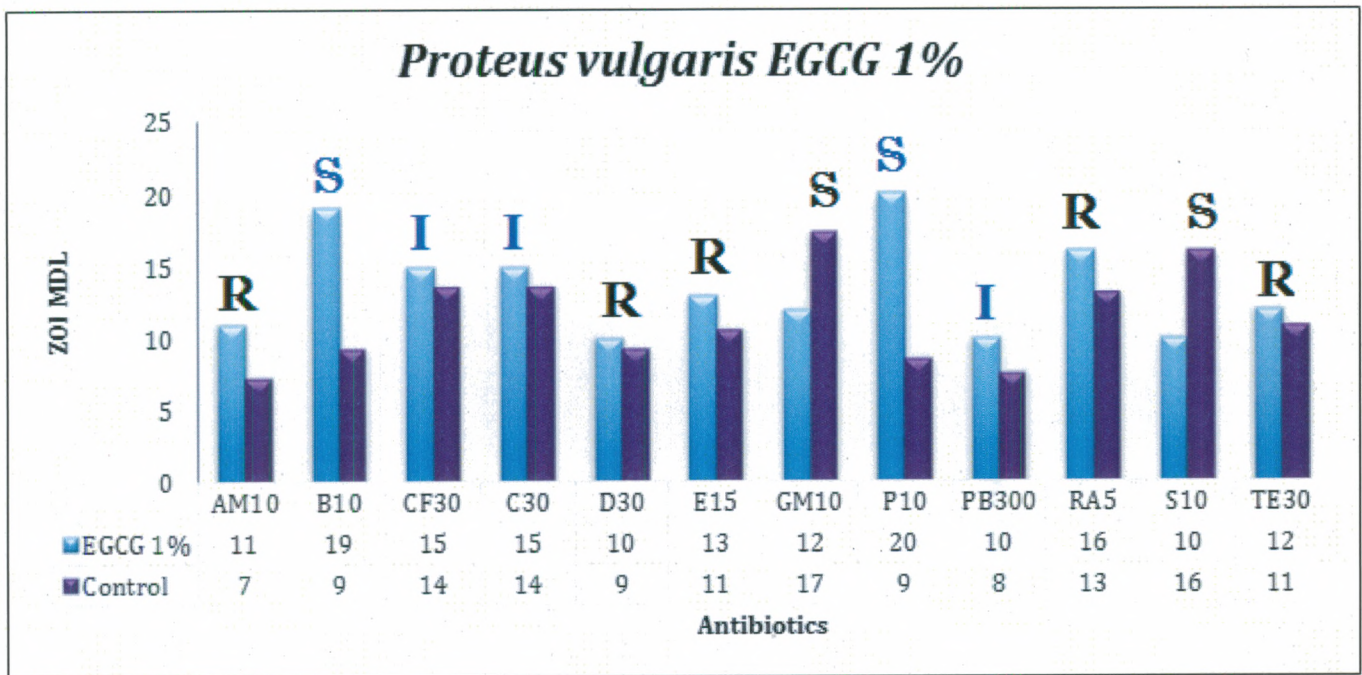


Figure 16: *Proteus vulgaris* treated with EGCGG

Bacitracin (B10) had a remarkable difference in its zone of inhibition. Cephalothin (CF30) and Chloramphenicol (C30) had a changed to an intermediate change. Penicillin had a very remarkable difference to susceptibility from an average of 9mm to 20mm. Polymyxin had a slight increase, but was enough to convert the antibiotic from having a resistant categorization to intermediacy.

There are two major significant increases with over 100% growth for *Proteus vulgaris*. Bacitracin (B10) had 104% and Penicillin (P10) had 131% in their increase of zone of inhibition. The rest had an increase, but nothing as significant as the aforementioned two. However, ampicillin did come in a close third with 50% increase in ZOI efficiency.



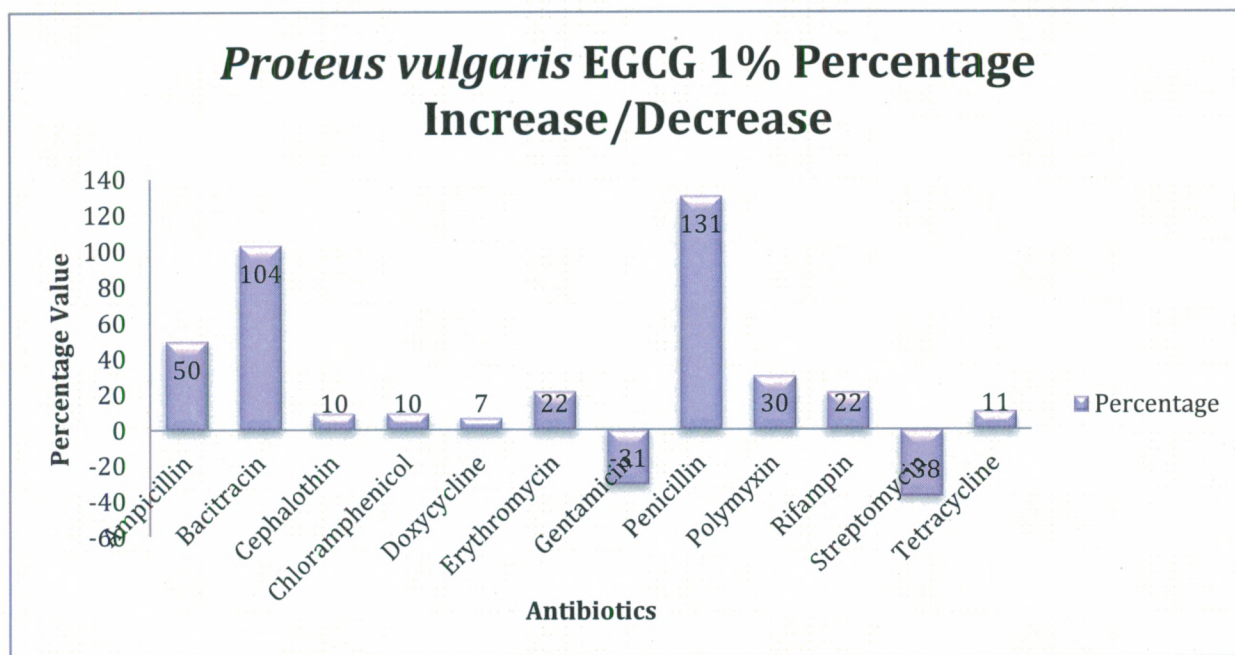


Figure 17: Increase/Decrease for EGCG on *Proteus*

<u>Category</u>	<u>Antibiotic [Original Category]</u>
Resistant	
Intermediate	Chloramphenicol [R], Cephalothin [R], Polymyxin [R]
Susceptible	Bacitracin [R], Penicillin [R]

Table 10: Antibiotic categorization for *Proteus vulgaris*

The figure above shows the treatment of the EGCG on 8 antibiotics and their resistance and reverting 5 of the 8 into an intermediate or susceptible category. The most successful antibiotics used in this treatment have been in the EGCG interaction of Bacitracin (B10) and Penicillin (P10) together in a polyphenol-antibiotic compound for disk diffusion testing.

## 5. *Serratia marcescens*

*Serratia marcescens* is a gram-negative microorganism that has a significant impact in the population. It is a very stubborn microorganism that displays many antibiotic resistant properties. It is considered a human pathogen and a nosocomial infection. Gastroenteritis is a common indicator and symptom of this pathogen. Mucous membranes seem to be a potential target for this microorganism and susceptibility increases as the strain resistance increases. Therefore, it is essentially to establish a sanitary condition in which inhibits the pathogenic capabilities of this microorganism at the same time decreasing resistant strains from proliferating.

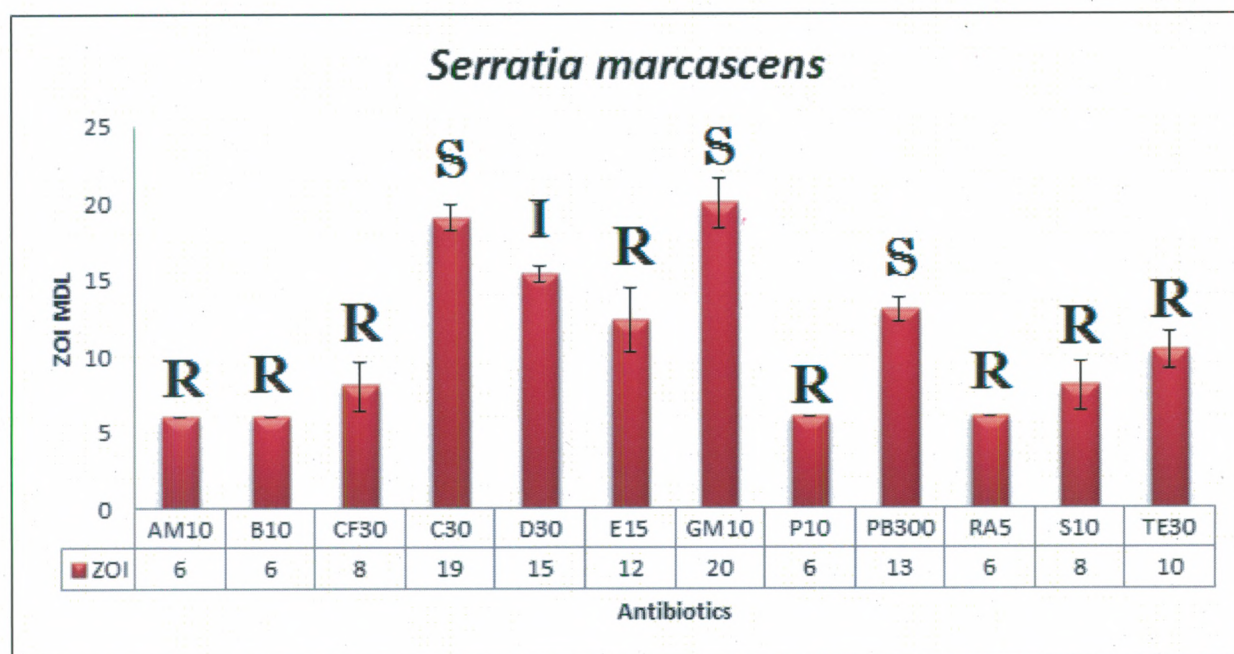


Figure 18: *Serratia marcescens* against twelve antibiotics

The averages and standard deviation of *Serratia marcescens* is shown above in Figure 19.

The numbers 6 represents zones of inhibition that were non-existent. Thus, this is accounting solely for the disk itself.



		Reading 1	Reading 2	Reading 3	Average	Standard Deviation
Ampicillin	AM10	6	6	6	6	0
Bacitracin	B10	6	6	6	6	0
Cephalothin	CF30	6	10	8	8	2
Chloramphenicol	C30	18	20	19	19	1
Doxycycline	D30	15	15	16	15	0
Erythromycin	E15	10	15	12	12	2
Gentamicin	GM10	18	22	20	20	2
Penicillin	P10	6	6	6	6	0
Polymyxin	PB300	12	13	14	13	1
Rifampin	RA5	6	6	6	6	0
Streptomycin	S10	6	10	8	8	2
Tetracycline	TE30	9	12	10	10	1

Table 11: Antibiotic readings for *Serratia marcescens*

*Serratia marcescens* is a gram-negative bacterium that displays high resistance to many of the traditional antibiotics. The above chart indicates a marked resistance for the control on AM10, B10, CF30, E15, P10, RA5, S10, and TE30. The table below organizes them into a resistant, intermediate, and susceptible classification. The resistant antibiotics dominate the table with 8 of the 12 antibiotics not having an effect—or displaying a very minimal, non-effective interaction. There is only one antibiotic that falls into an intermediate category and that is doxycycline. The susceptible antibiotics are C30, GM10, and PB300.

<u>Category</u>	<u>Antibiotic [Original Category]</u>
Resistant	Ampicillin, Bacitracin, Cephalothin, Erythromycin, Penicillin, Rifampin, Streptomycin, Tetracycline
Intermediate	Doxycycline
Susceptible	Chloramphenicol, Gentamicin, Polymyxin

Table 12: Antibiotic categorization for *Serratia marcescens*

The diagram below shows the effect that EGCG has on the antibiotics effects against the gram-negative bacteria. Unfortunately, EGCG had no intricate role in inducing susceptibility to the resistant antibiotics—as seen in the previous research with other microorganisms. In fact, the data would suggest that for this particular microorganism, the EGCG has some negative interaction that negatively affects the antibiotic mechanisms in creating zones of inhibition.

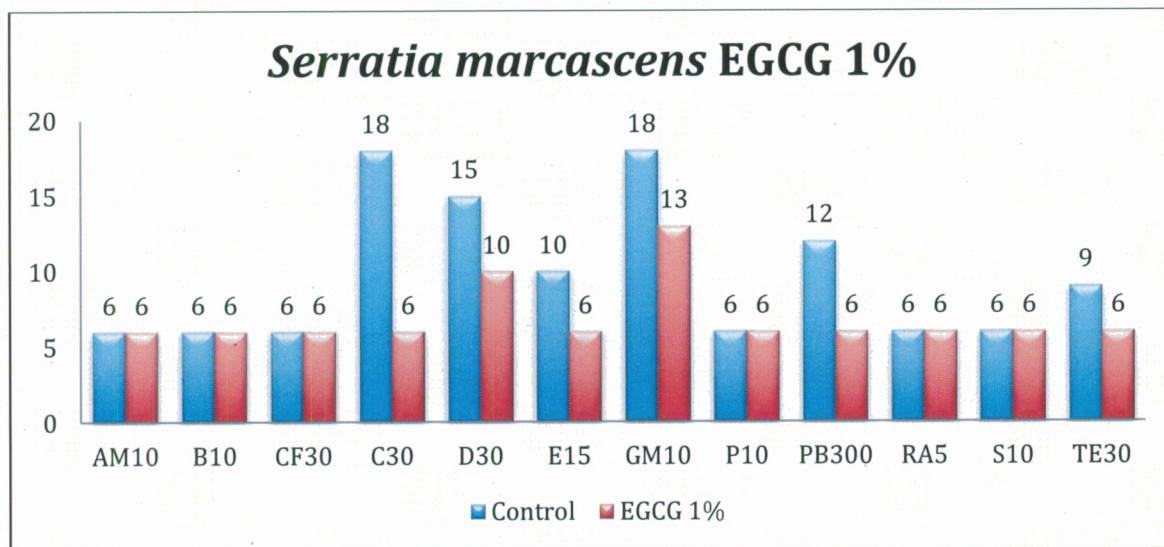


Figure 19: Antibiotic categorization for EGCG treatment for *Serratia marcescens*

Since EGCG had absolutely no impact that was expected as the other microorganisms, further testing was performed using a derivative of EGCG. This EGCG, also known as an EGCG-ester, was applied in the same format as EGCG with one unique exception. The solvent for this EGCG-ester (LTP) was a hydrophobic, organic solvent EtOH (ethanol) at 95% concentration. In Figure 22, there is a very indicative response for the way that the EGCG-ester interacts with the antibiotics to induce susceptibility in the antibiotics that were initially marked as being resistant.



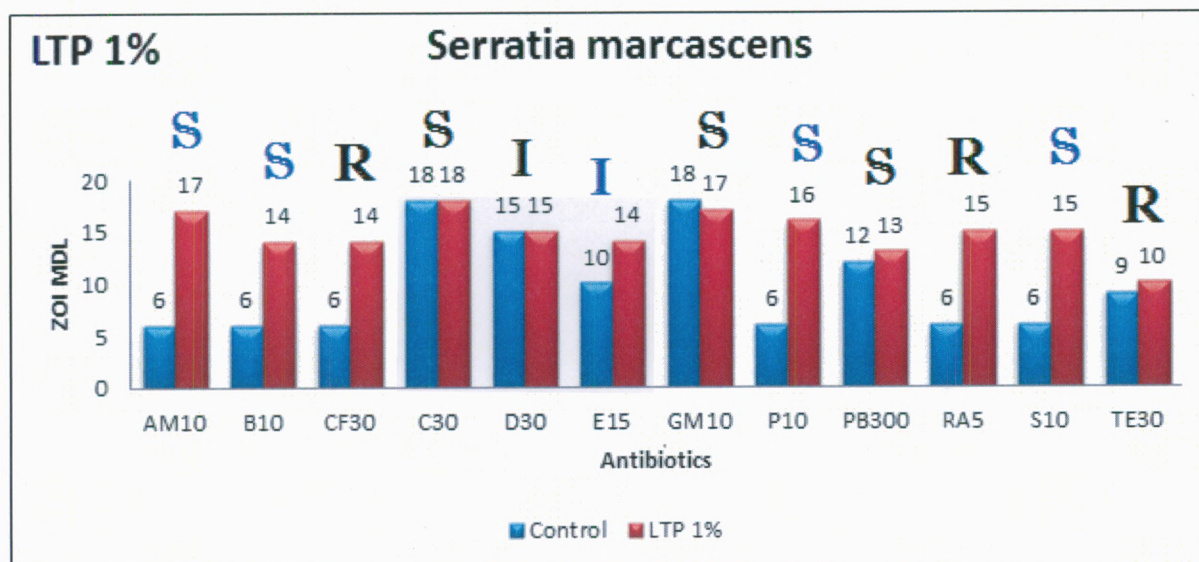


Figure 20: EGCG-ester [LTP] treated antibiotics against *Serratia marcescens*

In Figure 20, there is a clear indication that each of the antibiotics has successfully been positively enhanced using the EGCG-ester in lieu of the EGCG. This LTP, which only has an ester side chain attached, produces the desired effect initially expected of the stand-alone EGCG.

Category	Antibiotic [Original Category]
Resistant	Cephalothin [R], Rifampin [R], Tetracycline [R]
Intermediate	Doxycycline [I], Erythromycin [R]
Susceptible	Ampicillin [R], Bacitracin [R], Penicillin [R], Streptomycin [R]

Table 13: Antibiotic categorization of treatment with LTP for *Serratia marcescens*

The ensuing results of the antibiotic study for *Serratia marcescens* indicate that the most prominent antibiotic without treatment is Gentamicin (GM10) with an average ZOI of 20mm. The least effective antibiotic in the same category was Ampicillin (AM10), Bacitracin (B10),

Penicillin (P10), and Rifampin (RA5).

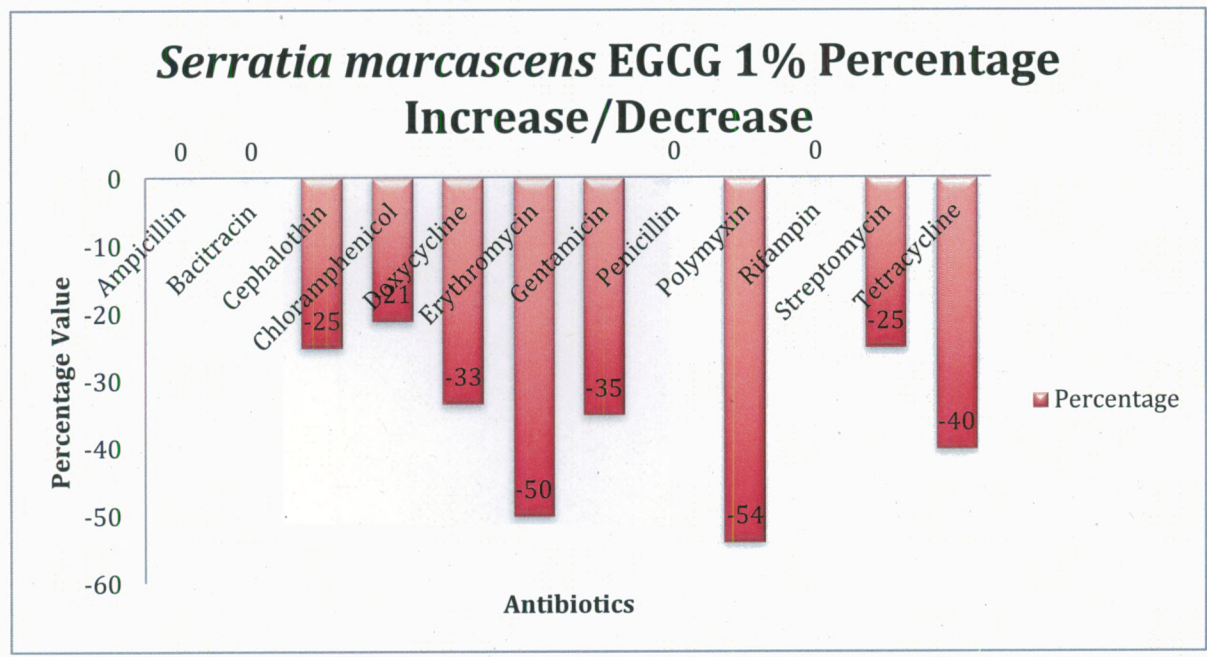


Figure 21: Increase/Decrease of EGCG treated versus Control for *Serratia marcescens*

The treatment with EGCG was not effective and thus does not give any results to correlate.

The treatment with EGCG-ester (LTP) was very effective in inducing susceptibility in the resistant antibiotics. In matter of fact, scientists can observe that there is a negative feedback towards supplementing the antibiotics with EGCG. It could be plausible that there is an inhibition mechanism occurring.



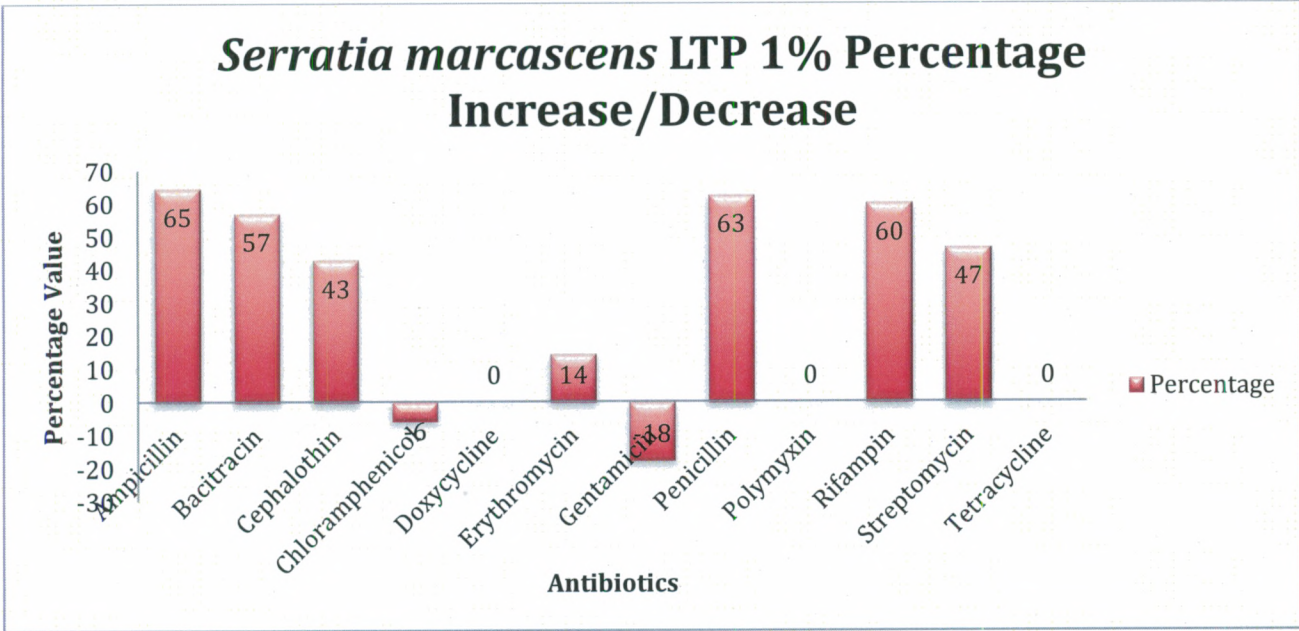


Figure 22: Increase/Decrease Percentage of EGCG-ester for *Serratia marcescens*

The most effective treatment with LTP on the antibiotic was Ampicillin (AM10) with an increase from 6mm (disk only) to 17mm (ZOI). This is followed by Penicillin (P10) with 16mm, Streptomycin (S10) with 15mm, and Bacitracin (B10) with 14mm.

## 6. Enterococcus faecalis

*Enterococcus faecalis* is the most stringent pathogenic strain of microorganism that was tested with the green tea treatments of GTP, EGCG, and LTP. These organisms have been speculated to account for more than 90% of clinical isolates causing human infection. They are also classified to group into vancomycin-resistant enterococci (VRE). This is a gram-positive microorganism that is also a classified nosocomial infection and potential pathogen. The extreme pathogenicity is correlated directly with the antibiotic resistant strains. It has a high-level vancomycin resistance and can cause meningitis.

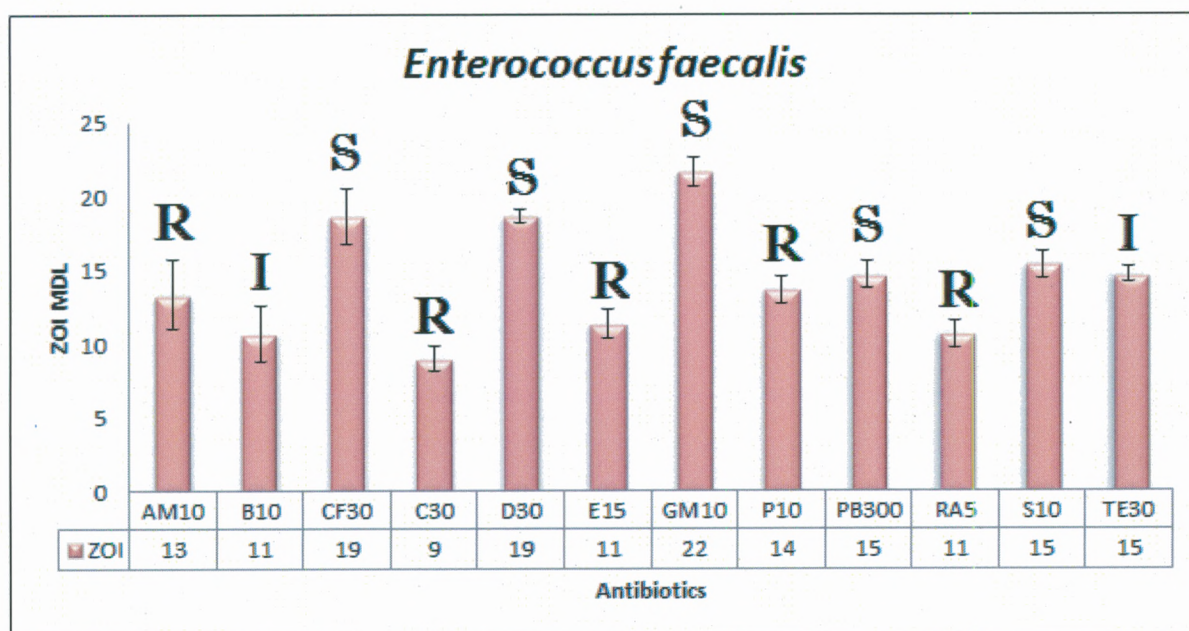


Figure 23: Control profile of *Enterococcus faecalis* with twelve antibiotics

*Enterococcus faecalis* is a gram-positive coccus microorganism that has a range of susceptible and resistant antibiotics associated with it. There are three readings, an average, and standard deviation associated with the findings.



		Reading 1	Reading 2	Reading 3	Average	Standard Deviation
Ampicillin	AM10	15	15	10	13	2
Bacitracin	B10	12	12	8	11	2
Cephalothin	CF30	20	20	16	19	2
Chloramphenicol	C30	9	10	8	9	1
Doxycycline	D30	19	19	18	19	0
Erythromycin	E15	12	12	10	11	1
Gentamicin	GM10	21	21	23	22	1
Penicillin	P10	13	13	15	14	1
Polymyxin	PB300	14	14	16	15	1
Rifampin	RA5	10	10	12	11	1
Streptomycin	S10	16	16	14	15	1
Tetracycline	TE30	15	15	14	15	0

Table 14: Antibiotic Readings for *Enterococcus faecalis*

The above figure displays that there are many resistant antibiotics to this bacteria. This strain of *Enterococcus faecalis* is actually a true pathogen and had to be carefully cultivated. The resistance the microorganism depicts against the antibiotics is a cause for concern. The table below shows the categorization.

Category	Antibiotic
Resistant	Ampicillin, Chloramphenicol, Erythromycin, Penicillin, Rifampin
Intermediate	Bacitracin, Tetracycline
Susceptible	Cephalothin, Doxycycline, Gentamicin, Polymyxin, Streptomycin

Table 15: Antibiotic categorization for *Enterococcus faecalis*

Currently, the resistant and susceptible antibiotics are equal in amount. There are two intermediate antibiotics that are heavily used on the market (i.e. Pharmaceuticals). It would

be advantageous to note any effect that EGCG or other polyphenolic compounds would have on the antibiotics and reversing resistance or intermediacy.

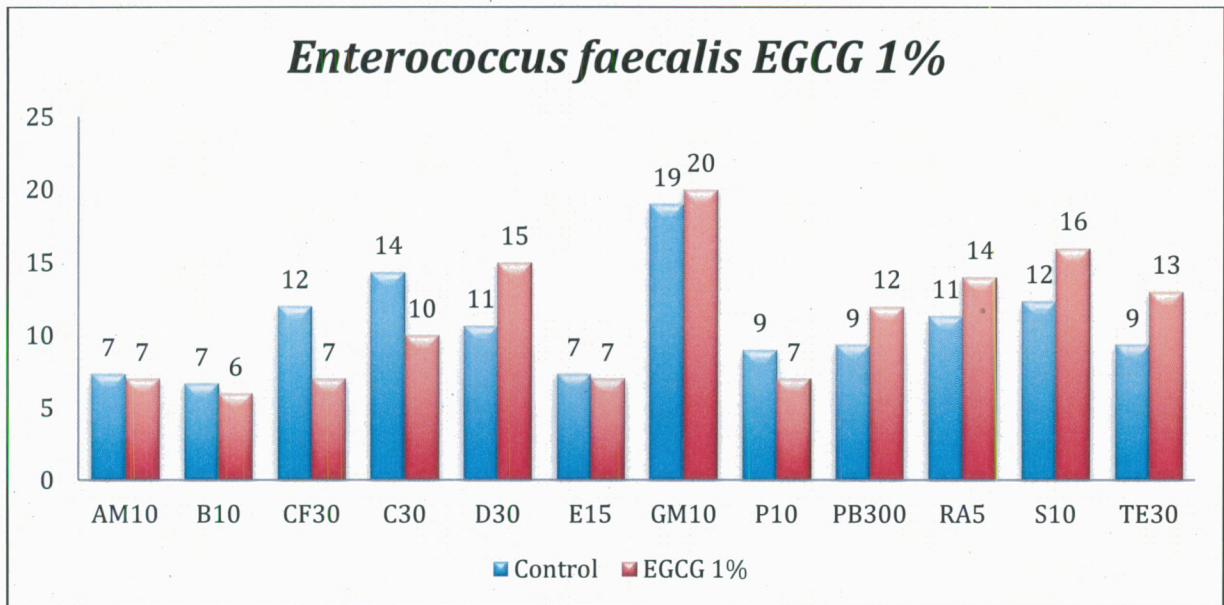


Figure 24: Antibiotic treated with EGCG against *Enterococcus faecalis*

The numbers that EGCG at 1% produced on this pathogenic strain of bacteria was not satisfactory. It would be deemed that the phenolic compound was not sufficient enough to either be quantified as having a great synergistic effect or somehow amputated the effects the original antibiotic had on the microorganism.

Category	Antibiotic
Resistant	Ampicillin, Chloramphenicol, Erythromycin, Penicillin, Rifampin
Intermediate	Bacitracin, Tetracycline
Susceptible	Cephalothin, Doxycycline, Gentamicin, Polymyxin, Streptomycin



Table 16: Antibiotic categorization for *Enterococcus faecalis*

Since EGCG did not have a satisfactory role in this bacteria. LTP at 1% was sought out. The LTP, EGCG-ester, is shown below in Figure 27.

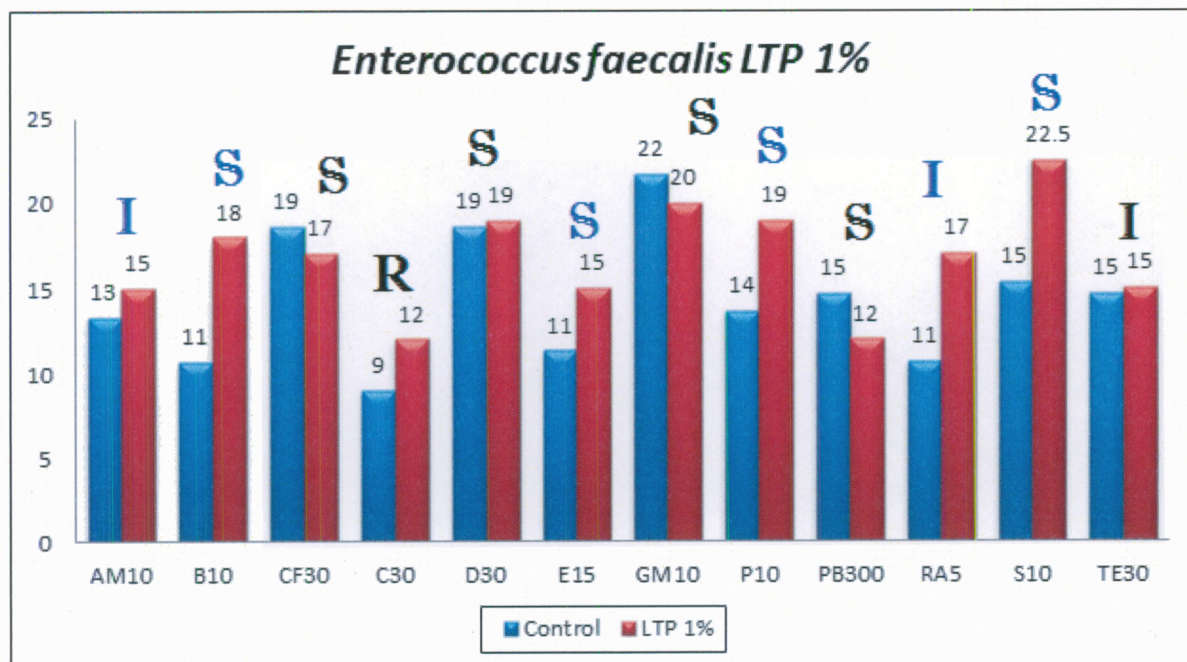


Figure 25: Antibiotics treated with EGCG-ester [LTP]

This Figure is able to portray the effective or inefficiency of the antibiotics in conjunction with the phenolic compounds as observed for synergistic properties and/or capabilities. The observation of a lot of the treated compounds does not have that much of a difference with

the antibiotic's original zone of inhibition.

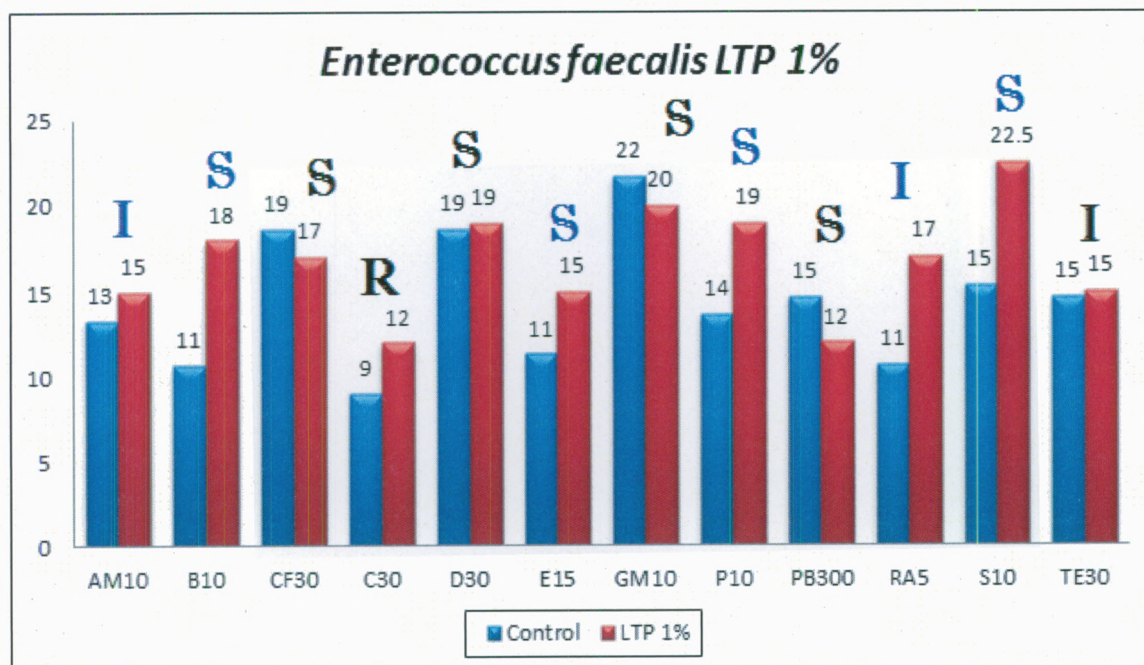


Figure 26: Antibiotic categorization for *Enterococcus faecalis*

The antibiotics AM10, B10, E15, P10, RA5, and S10 have changed in their categorization after the treatment of EGCG-ester (LTP @ 1%).

Category	Antibiotic [Original Category]
Resistant	Chloramphenicol [R]
Intermediate	Tetracycline [I], Ampicillin [R], Rifampin [R]
Susceptible	Bacitracin [I], Cephalothin [S], Doxycycline [S], Erythromycin [R], Penicillin [R], Polymyxin [S], Streptomycin [S]

Table 17: Antibiotic categorization after treatment with LTP



The EGCG-ester proves to be effective for Ampicillin (AM10), Rifampin (RA5), Bacitracin (B10), Erythromycin (E15), and Penicillin (P10). The AM10 was able to go from RESISTANCE to INTERMEDIACY; as well as RA5. B10 was able to from INTERMEDIACY to SUSCEPTIBILITY. The biggest role reversal for this microorganism was the antibiotics Erythromycin (E15) and Penicillin (P10) that was able to go from RESISTANCE to SUSCEPTIBILITY.

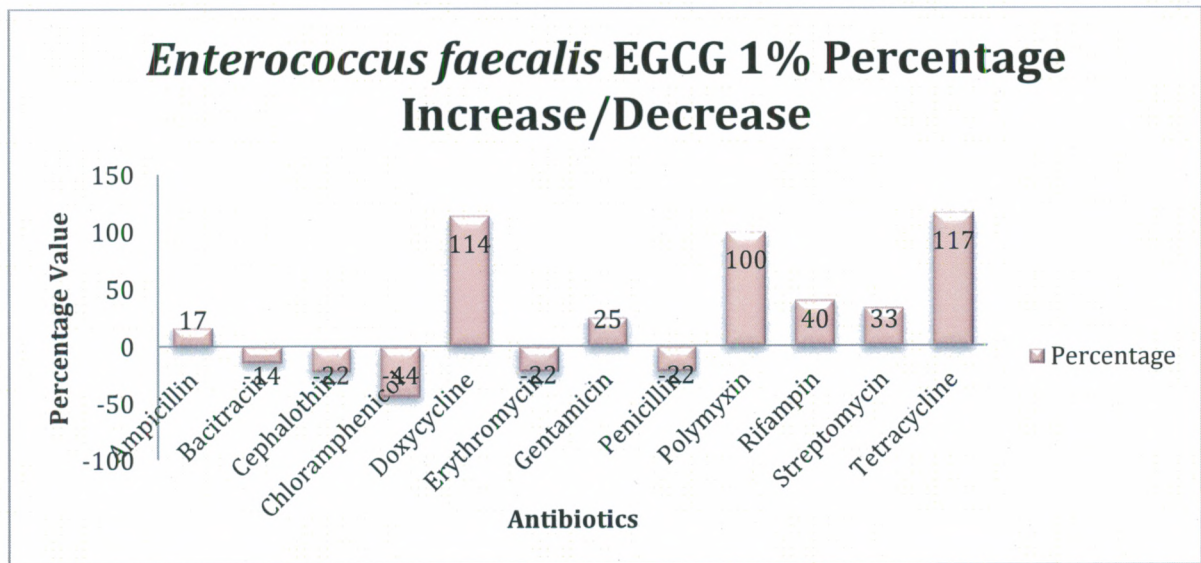


Figure 27: Increase/Decrease Percentage for EGCG

The highest average increase for EGCG treated antibiotics against *Enterococcus faecalis* was Tetracycline (TE30). The second and third were Doxycycline (D30) and Polymyxin (PB300) respectively.

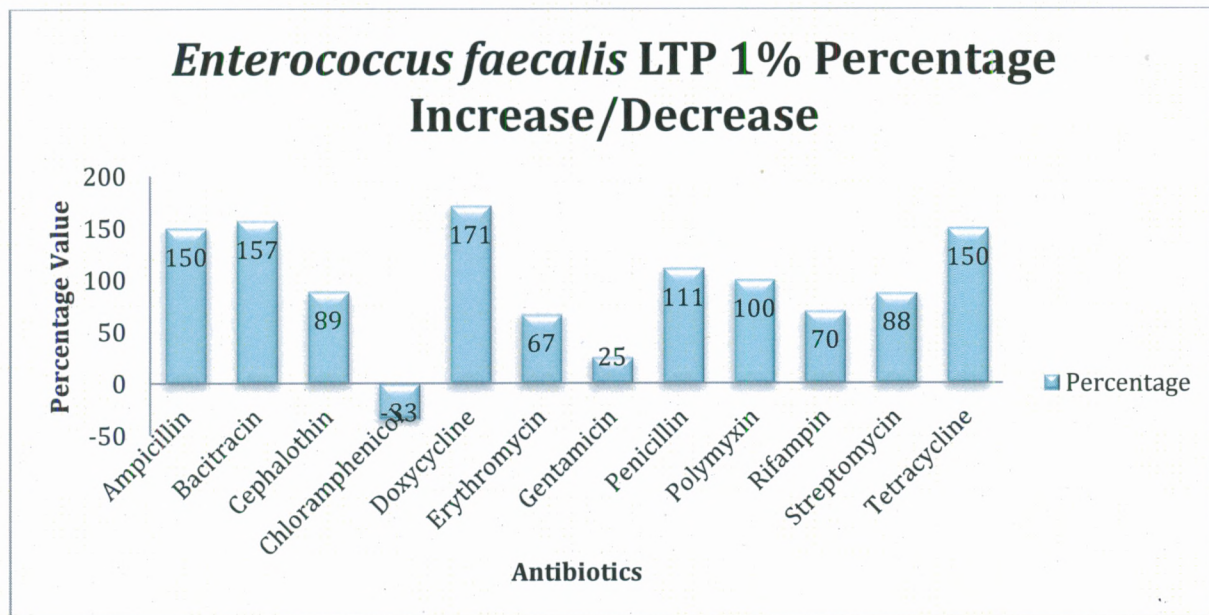


Figure 28: EGCG-ester [LTP] treated antibiotics

The LTP treated antibiotics seemed to be far superior in increasing percentage yield of zones of inhibition in comparison to EGCG. There seemed to be a commonality for D30 and TE30—which is in direct correlation with EGCG. However, AM10 and B10 showed a very great increase in percentage. This is favorable due to its common usage and topical properties. In summation, both EGCG and LTP had increases in D30 and B10, but LTP had increased in all, but one antibiotic (C30).



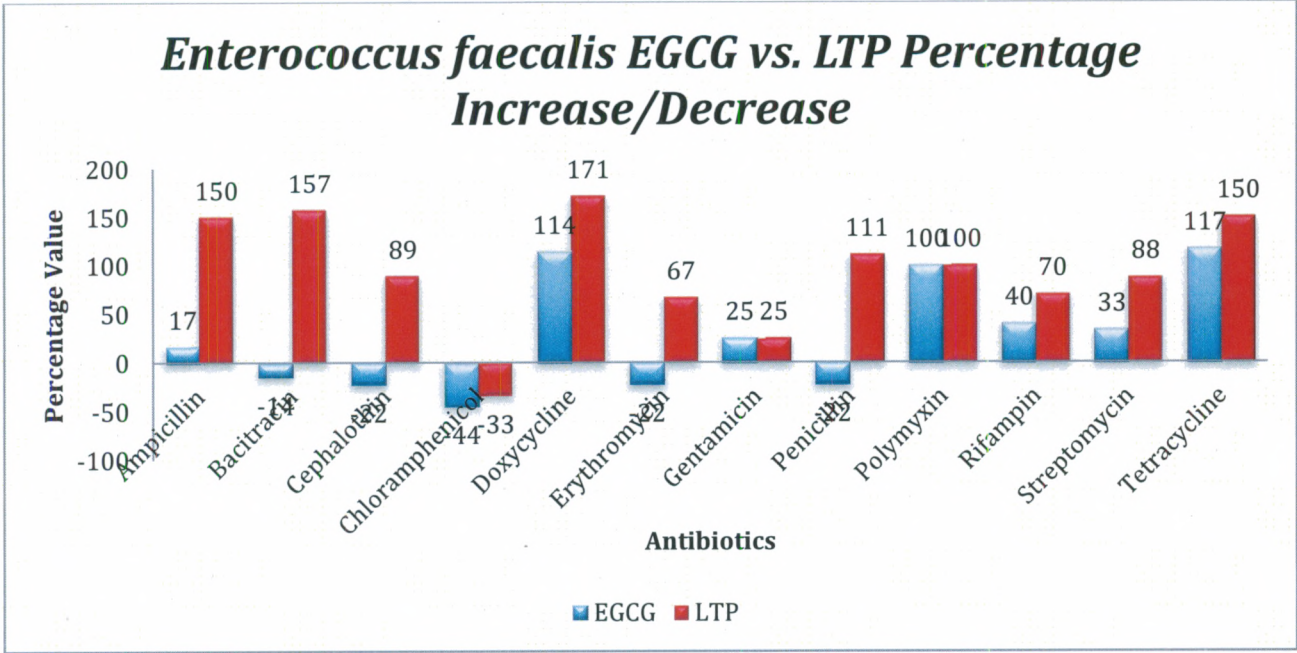


Figure 29: Percentage Increase/Decrease for EGCG vs. LTP *Enterococcus faecalis*

The chart above depicts the comparison of the percentages side-by-side. It is safe to conclude that LTP had a better result than EGCG for this pathogenic bacterium. Every single antibiotic was matched or increased by the presence of LTP in this scenario and a promising indicator of its beneficial properties.

## 7. Enterobacter aerogenes

*Enterobacter aerogenes* is a gram-negative microorganism that is a potential, nosocomial pathogen. It is an opportunistic infection that is studied extensively in the microbiology laboratories. The vast majority of these microorganisms are sensitive to antibiotics (opposite of enterococcus species) and do not cause extensive damage. The particular strain used in the laboratory indicated the full opposite and displayed a high resistance to many of the typical antibiotic regimes prescribed by healthcare professionals.

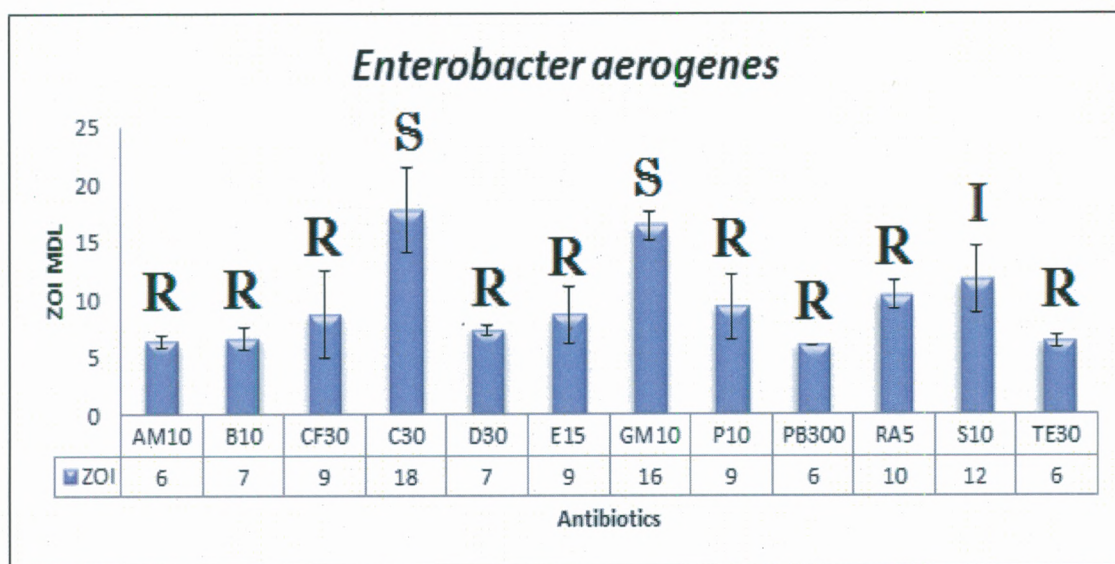


Figure 30: *Enterobacter aerogenes* against twelve microorganisms categorically

[S.I.R.]

*Enterobacter aerogenes* is a bacillus-shaped microorganism that is gram-negative. These microorganisms have the ability to become potentially pathogenic given the right conditions. The average total set for the antibiotics against the microorganism was 10 units using MDL.



		Reading 1	Reading 2	Reading 3	Average	Standard Deviation
Ampicillin	AM10	6	6	7	6	0
Bacitracin	B10	6	6	8	7	1
Cephalothin	CF30	14	6	6	9	4
Chloramphenicol	C30	22	13	18	18	4
Doxycycline	D30	7	7	8	7	0
Erythromycin	E15	6	12	8	9	2
Gentamicin	GM10	16	18	15	16	1
Penicillin	P10	6	13	9	9	3
Polymyxin	PB300	6	6	6	6	0
Rifampin	RA5	12	10	9	10	1
Streptomycin	S10	15	8	12	12	3
Tetracycline	TE30	7	6	6	6	0

Table 18: Antibiotic readings for *Enterobacter aerogenes*

Category	Antibiotic
Resistant	Ampicillin, Bacitracin, Cephalothin, Doxycycline, Erythromycin, Penicillin, Rifampin, Tetracycline
Intermediate	Streptomycin
Susceptible	Chloramphenicol, Gentamicin

Table 19: Antibiotic categorization for *E. aerogenes*

This microorganism had a very surprising result. It was not expected that this strain have such high yielded resistance towards many of the antibiotics. 9 of the 12 antibiotics had resistance with very little effect against the microbe. There was only 1 antibiotic that fit into the Intermediate category and 2 antibiotics falling into the susceptibility category. So far, a common occurrence of ampicillin and penicillin has been a common theme of having microbial resistance.

## 8. *Mycobacterium smegmatis*

*Mycobacterium smegmatis* is an acid-fast microorganism that contains waxy, lipid membrane that contains mycolic acids. These mycolic acids give the organism the unusual characteristic that it displays in clinical settings. The standard microbial staining procedures cannot be utilized on these. A technique performed called Acid-Fast Staining by Zehl-Neelson must be used to help the acidic dye to adsorb into the membrane layer. These microorganisms are commonly found on the genitalia of both male and female as normal sebaceous gland excretion after a periodic cycle containing a lack of typical hygienic regime. The microorganism itself is not harmful or disease-causing, but can become a pathogenic and potential invader in opportunistic infection if it travels into the blood stream. It is also the model organism of study for *Mycobacterium tuberculosis* and *mycobacterium leprae* which both causes tuberculosis and leprosis respectively.

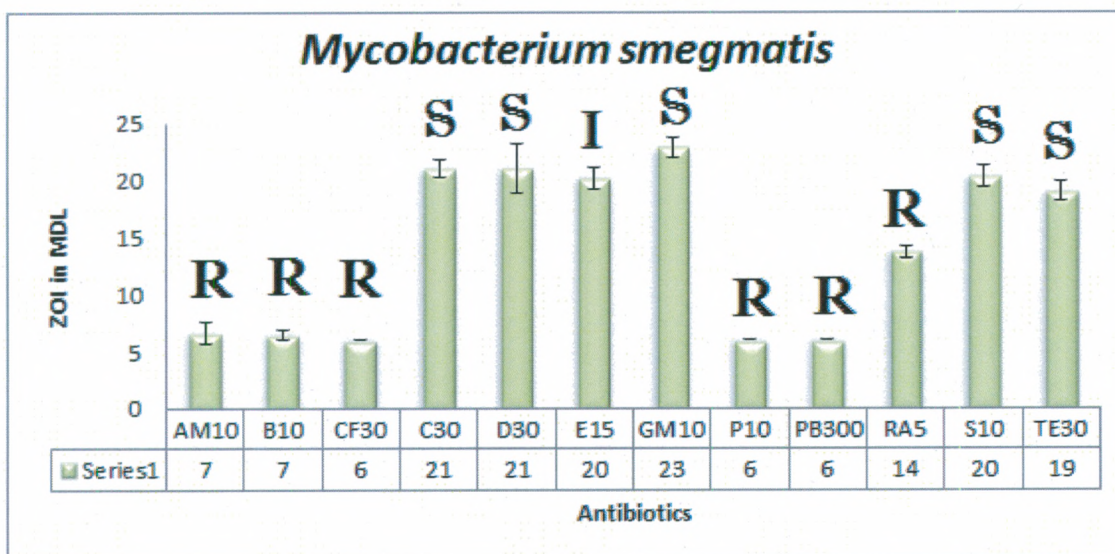


Figure 31: *Mycobacterium smegmatis* against twelve antibiotics



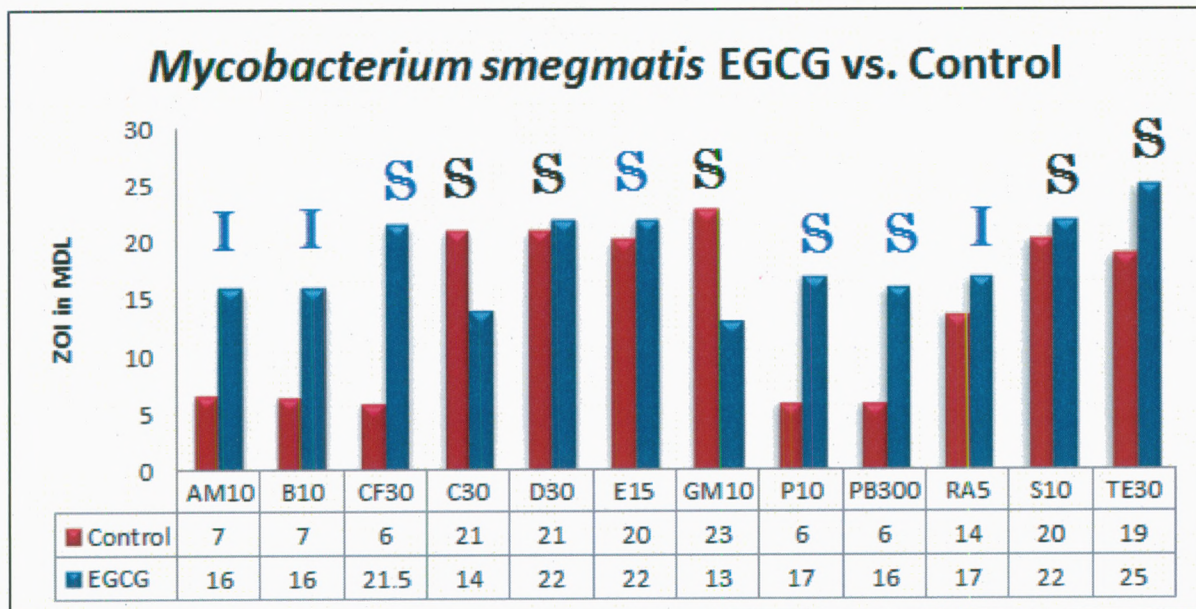


Figure 32: Smegma against Control and EGCG treated antibiotics

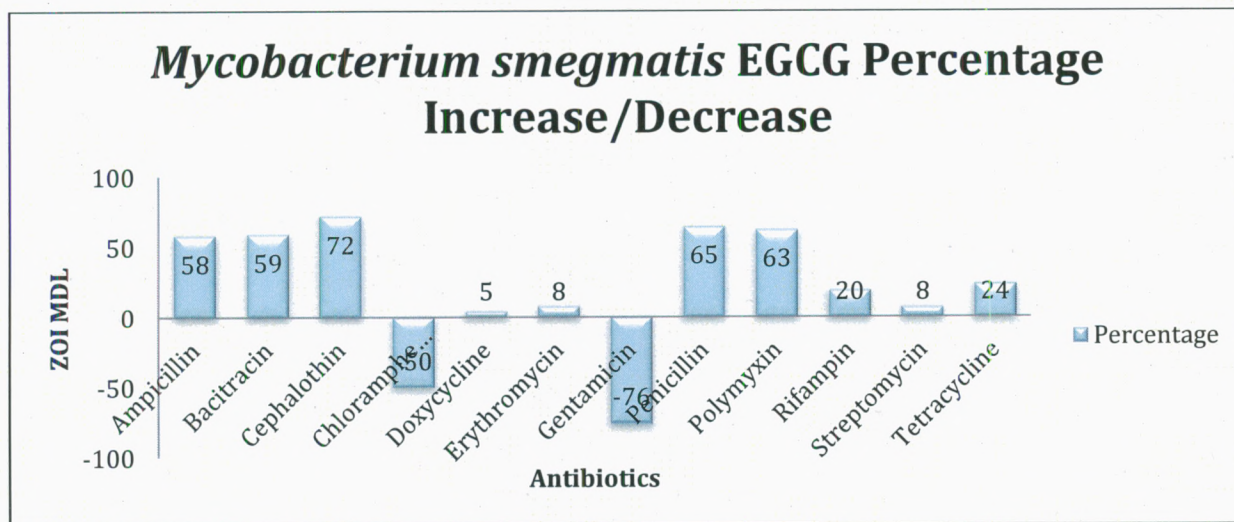


Figure 33: Increase/Decrease of EGCG against Control

<u>Category</u>	<u>Antibiotic [Original Category]</u>
Resistant	
Intermediate	Tetracycline [I], Ampicillin [R], Rifampin [R]



Susceptible	Bacitracin [I], Cephalothin [S], Doxycycline [S], Erythromycin [R], Penicillin [R], Polymyxin [S], Streptomycin [S], Chloramphenicol [R]
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Table 20: Antibiotic categorization of EGCG treated antibiotics

### III. Antiseptic Study

#### a. Green Tea Polyphenol (GTP)

GTP was added to filter disks to have the polyphenol adhere to the matrix membrane. The diffusion of the polyphenol set out and gave rise to the ability for one to measure out the effectiveness of the compound against the microorganism.

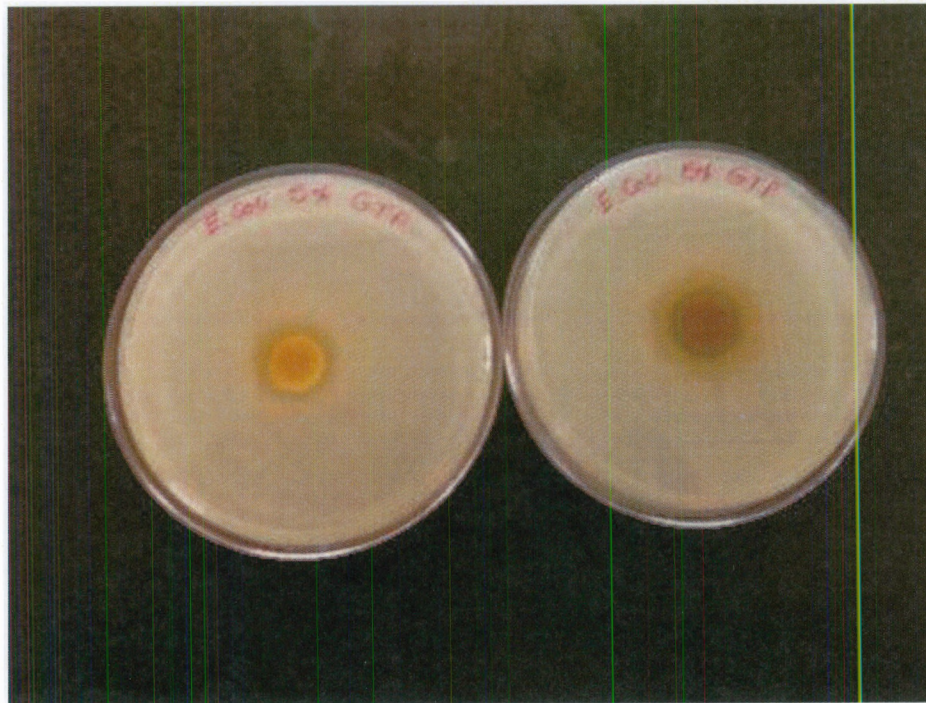


Figure 34: 5% GTP as an Antiseptic Left / 10% GTP on the Right

GTP was used as an antiseptic and disinfectant. The performing of this was done using disk filter paper and aseptically soaked into the membranous filter paper for duration of five



minutes. This duration ensured that proper time was allotted for the compound to adhere to the paper and then study its effect on bactericidal or bacteriostatic properties. The results are shown in figure 31.

b. Curcumin

Curcuminoids (derived from Turmeric) had semi-promising polyphenolic properties used antiseptically in the lab. The biggest determining factor was creating a solubility product that was ideal (ksp value). In order to achieve this, heat was used instead of dissolving in DMSO—since DMSO is toxic to cells and would not create an ideal drinking or topical solution. *Serratia marcescens*, *Enterobacter aerogenes*, and *Proteus vulgaris* seemed to have a positive response to the treatment.

## Curcumin as Antiseptic

Microorganism Strain	Zone of Inhibition in MDL
<i>Escherichia coli</i>	0mm
<i>Staphylococcus epidermidis</i>	0mm
<i>Bacillus megaterium</i>	0mm
<i>Mycobacterium smegmatis</i>	0mm
<i>Serratia marcescens</i>	8mm
<i>Enterobacter aerogenes</i>	10mm
<i>Enterococcus faecalis</i>	0mm
<i>Proteus vulgaris</i>	4mm

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Figure 35: Curcumin as antiseptic on 8 microorganisms



c. Pathogenic Growth Study (Time-Kill Curve of *Escherichia coli*)

This important piece of study indicates that as the time progresses for the bacteria to colonize and utilize its source of nutrients in the agar, the organism will flourish and reproduce. As the time increases, the colonies increase in its surface area and becomes abundant at which point can no longer be counted into groups of colony forming units. This behavior is known as becoming “confluent” or creating a lawn of bacteria.



Figure 36: *Escherichia coli* Time-Kill CFU count Study



*Escherichia coli* resistant cells were prepared according to the materials and methods section on resistant plasmid cells without the green fluorescent protein (GFP). The intervals were every half hour wash, re-suspend, centrifuge, and incubate. After an hour, the pathogen was inoculated onto the nutrient agar media and allowed to incubate at 37°C for 24 hours. The results indicate that the longer the duration of incubation, the more confluent and abundant the bacterium becomes indicating a healthy growth response and no effect of inhibition or bactericidal activity.

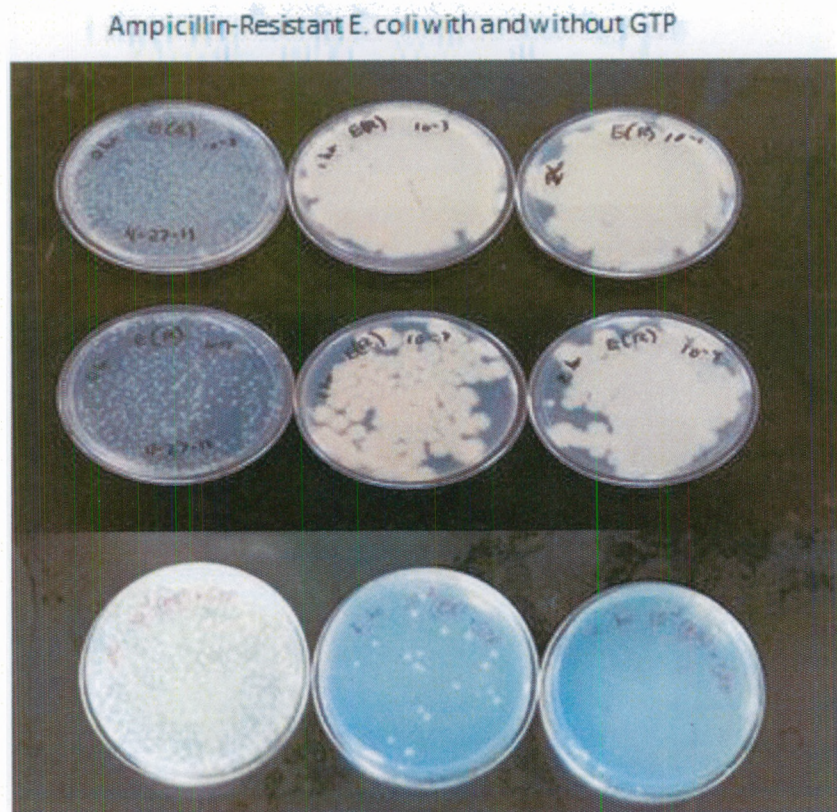


Figure 37: Time-Kill Growth Study of *Escherichia coli*

The above figure 37 shows that the microorganism is able to thrive and reproduce in the nutrient-rich medium. There are no bactericidal properties until GFP is added and the growth



of the microorganism is heavily reduced. After 2 hours of GTP treatment, there is 99.99999% reduction in the growth of the microorganism. There are no longer any visible colonies on the plate.

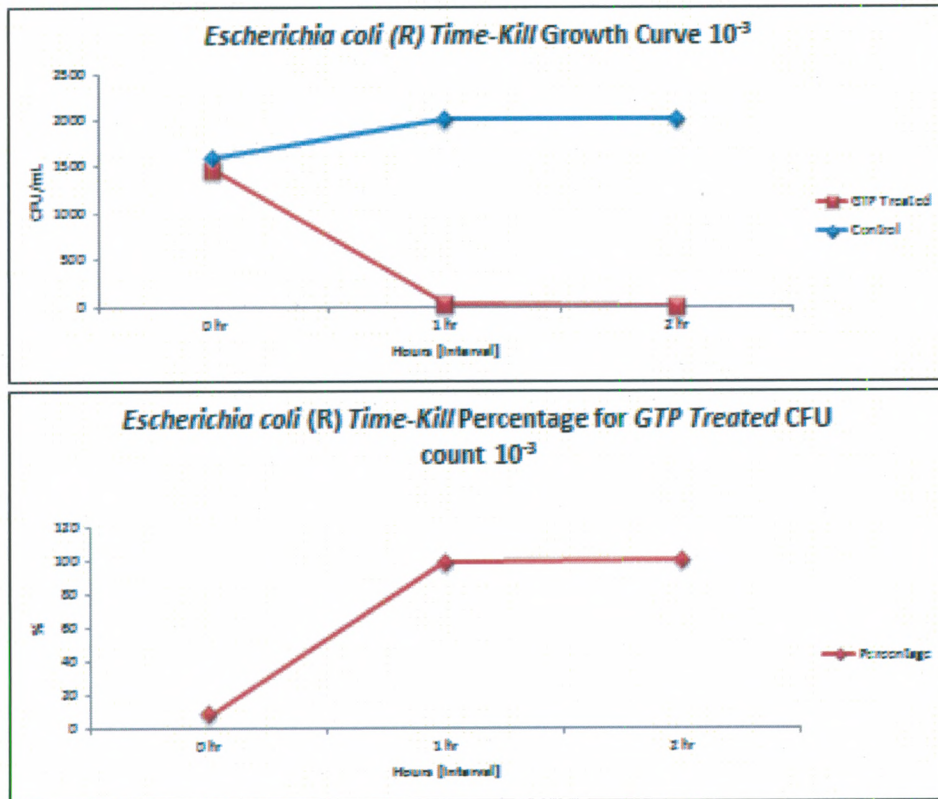


Figure 38: *Escherichia coli* Time-Kill Curve

The graph above shows the trend line for the growth and bactericidal activity of GTP against the pathogenic microorganism. The CFU/mL of the GTP treated microorganism is close to 0 after 2 hours and the time-kill percentage reaches almost a 100% approximation at the same interval/time and/or duration.

The spore-producing microorganism *Bacillus megaterium* is treated in the same manner as the potential pathogenic *Escherichia coli*. The induction of heat and heat with GTP was



utilized to establish an efficacy of diminishing the ability of the microorganism to proliferate and cause potential damage. In the above diagram, it shows that without the usage of heat to destroy the vegetative cells, the organism is able to reproduce and produce viable cells. In the presence of heat, the vegetative cells are killed, however, the spores are able to survive and produce a progeny. In the presence of heat and GTP, the vegetative cells are killed and many of the spores are damaged or killed giving the ability to visually observe a low CFU/mL count as shown in figure 39.

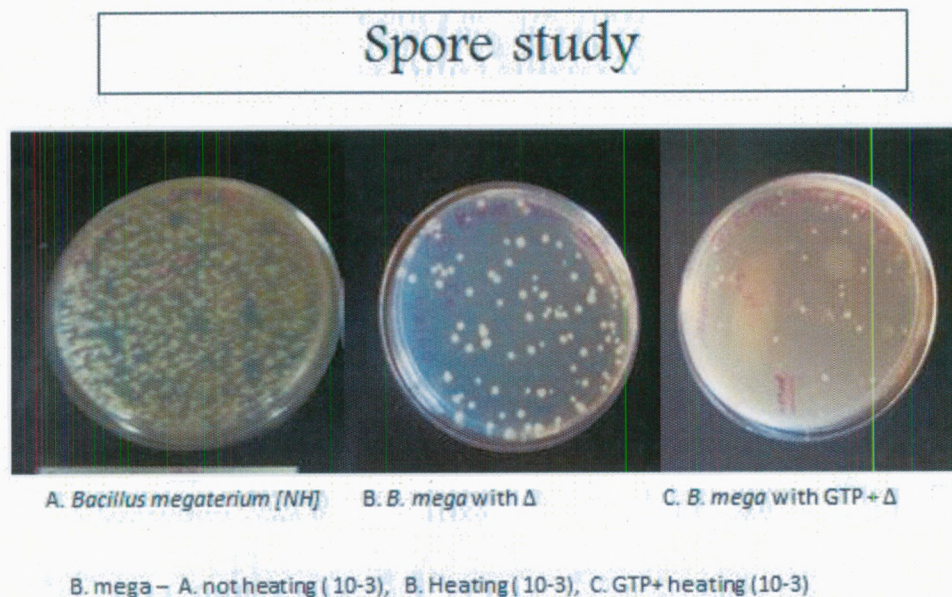


Figure 39: Spore Study of *Bacillus megaterium*

The spore-producing microorganism *Bacillus megaterium* is treated in the same manner as the potential pathogenic *Escherichia coli*. The induction of heat and heat with GTP was utilized to establish an efficacy of diminishing the ability of the microorganism to proliferate and cause potential damage. In the above diagram, it shows that without the usage of heat to

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## Discussion

Enzymatic proteins that are able to catalyze cyclic amides belong to a special group of molecules referred to as  $\beta$ -lactamases (Enzyme Commission # 3.5.2.6). These specialized proteins are secreted by various microorganisms that are competitively active against each other in any given environment. This process of “natural selection” that drives evolution for these prokaryotes play an intricate role in the secretion of antibiotics and their counterdisplay of resistance through the secretion of catalytic proteins that possess the properties necessary to hydrolyze the lactam ring of certain antibiotics. This has subsequently led to revolutionary studies involving the enzymatic protein and the abundant variation that consequently occurs from the causative factors that delegates the molecular interaction of  $\beta$ -lactamases and its affinity for antibiotics.

The classification system for  $\beta$ -lactamases is considered upon its functional properties and based upon a molecular mechanism that takes into consideration the amino acid and nucleotide sequence of the enzymatic proteins. The establishment of the molecular portion has a direct correlation to the functional system that is given in an alphanumerical ordering. The molecular class ranges from A through D and is indicative of their specific amino acid utilization in their mode of action—thus their mechanism of action through the utilization of mutations. The atomic displacement and charges of these proteins are important components and stressed further as this paper proceeds.

Group one  $\beta$ -lactamases belong to cephalosporinases that are part of molecular class C. This group is important due to its ability to avoid the inhibition given by the addition of clavulanic acid—inadvertently giving it tremendous clinical significance. Group two  $\beta$ -

lactamases encompass group one and goes into a tremendous amount of greater detail. These groups embody penicillinases, which is the main focus of our discussion, and cephalosporinases that are inhibited or anti-inhibited by the clavulanic acid. The correlative molecular classes to this group are A and D. Additionally, there are subgroups attached to this group as of recent due to the nature of the mechanisms that are displayed in the enzymes.

Group three  $\beta$ -lactamases are not inhibited by clavulanic acid and are known as metalloenzymes. The metal ion is typically zinc-based and provides the ability to hydrolyze several  $\beta$ -lactam drugs. Group four  $\beta$ -lactamases are known as specific penicillinases that are not inhibited by the clavulanic acid and thus one of the major pending threats for many species. These resistant enzymes hydrolyze the  $\beta$ -lactam drug and any combination derivatives given in conjunction to deflect the initial resistance have portrayed little to no significant impact. The majority of current research has been directed towards groups that depict this behavior.

There are many major considerations for these enzymes, their catalytic activity, hydrolyzation, and specific substrate binding affinity. These traits have been found in Gram negative bacteria due to being one of the forefront runners, category-wise dependent on peptidoglycan layer, for antibiotic resistance. Penicillinase, from the previous mentioned Group II and IV, have a specific mode of action when given in antibiotic regimens and in their developed antibiotic resistance. The resistant to penicillin has been one of the first mentioned resistant issues to become documented due to its abundant usage and the duration of years it has been administered orally and intravenously—Penicillin V and G respectively.



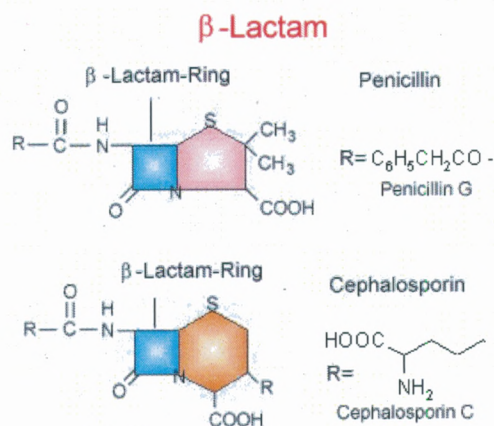
Veterinary Microbiology has also been producing concurrent and novel research on specific microorganisms that have developed such resistance. The issues extend to human and bovine isolates of strains that cause severe illnesses and damage. One of the prime examples for severe disease-causing bacteria is *Staphylococcus aureus*<sup>21</sup>. Despite the fact that *Staphylococcus aureus* is gram positive and should have increased susceptibility, these species have demonstrated an ability to undermine  $\beta$ -lactam drugs. Upon further research, it was founded that the BlaZ gene of *Staphylococcus aureus* was solely responsible for the hydrolyzation of methicillin and oxacillin by 2-D Gel Electrophoresis and Mass Spectrophotometer<sup>21</sup>. There is heavy basis of genetic elements that come into play for these enzyme production and *Enterococci* and *Staphylococci* share these similar proteins<sup>39</sup>. The difference in the aforementioned genera is the production of the  $\beta$ -lactamase protein and its observation in constitutive or inducible production—respectively in order to the previous sentence.

Enzymological assays have identified two genes responsible for the majority of  $\beta$ -lactamases: SHV-1 and TEM-1. These genes have undergone X-ray crystallography to determine the structure

Specifically, SHV-1 established by Rutgers implemented into the (Entry 1SHV) with the displayed indicating involved in enzyme

were to utilize kinetic equation ratios<sup>14</sup>. The relevancy of this is only in terms of

of these proteins. structure has been University<sup>24</sup> and Protein Data Bank catalytic center that it must be efficiency if one



mathematically quantifying the rates of kinesis for enzymatic reactions—nothing that delve into motif structure and function—but still important in regards to enzymology and their outstanding catalytic processing ability. <http://www.biokurs.de/skripten/bilder/blactam.GIF>

The occurrence of penicillin and ampicillin resistance in *Esherichia coli* is plasmid-mediated and constrains heavily upon the TEM-1  $\beta$ -lactamases. The crystal structure of Penicillin-Binding Proteins (PBPs) that are involved in resistance with TEM-1 and  $\beta$ -lactam inhibitors of important pathogens such as *Pseudomonas aeruginosa* have recently been demonstrated and published<sup>31</sup>. PBPs are the greatest and most integral portion of  $\beta$ -lactam target acquisition as they function to serve as “suicide substrates” by burlesquing as peptidoglycan precursors<sup>31</sup>. There are many types of penicillin-binding proteins and all are involved heavily in cell wall/peptidoglycan biosynthesis. The PBP mentioned in this research involved PBP3 and its active site located in the left cleft parallel with the  $\beta$ 3 strand of the transpeptidase doman<sup>31</sup>. It comprises as a two-domain protein resembling other class B PBPs lacking an  $\alpha$ -helical “head” domain. Figure 1 displays the “head” subdomain and motif.



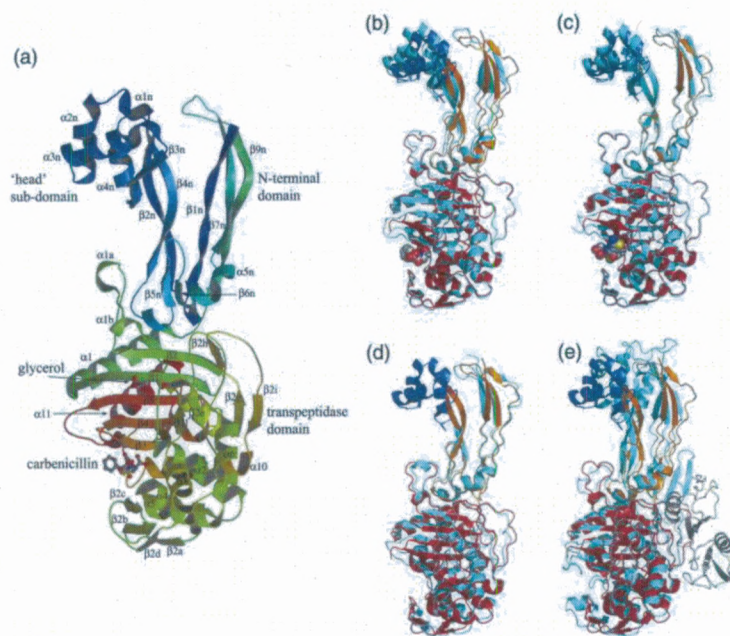


Figure 40:  $\beta$ -lactamase 3-Dimensional Protein Fold Image

One of the most common upper respiratory tracts infectious to children is *Haemophilus influenzae* and has the potential to become a deadly pathogen. Two of the low-molecular weight (LMW), as opposed to the high-molecular weight (HMW) class category for PBPs, was crystallized and imaged in high-resolution. This correlated to PBP4 and PBP5 of *Haemophilus influenzae*. The model organism for the development of novel  $\beta$ -lactams against this pathogen was *Pseudomonas aeruginosa* and determined if the active site disturbs the deacetylation step similarly<sup>20</sup>. One of the other speculations that dimerization might affect catalytic activity and appearance of protein surface in the rich solvent might perturb some medication or interaction. The results showed that between *Haemophilus influenzae* and *Escherichia coli* that there are conserved regions with only two residues per chain appearing in the outskirts of the Ramachandran plot—a useful plot determination of phi ( $\Phi$ ) and psi ( $\Psi$ )

angles in amino acid protein structure. This position is the Ala68 which is next to the active-site Ser69 and Ser151 <sup>11</sup>. The protein atoms are not displaced which suggests a rigid structure—in contrast to more easily adopted, flexible catalytic enzymes.

Synthetic models and constructs of penicillinases have been created using plasmid vectors. The synthetic counterpart contrasts the typically created protein in nature in relative size. For instance, utilizing PubMed resources in their protein database and conducting a search for “penicillinase” provides 28 amino acid synthetic constructs for the first few hits. Afterwards, the genus of *Bacilli* present themselves and dependent upon the species, users observe that there are penicillinases that present themselves in the quantity of 15 amino acids to 306 aa. The Figure on the right is a snapshot of an outcome of a search result using this method. The most common reasons for this occurrence is due to nature’s conservation of specific sequences in a given genome for the proper functioning of that protein. As long as the peptide is able to become utilized and perform its action in a necessary function without being easily debilitated by change in pH, temperature, or other various factors, then essentially the amount of amino acids it takes to produce the same effects is not that important. It however, becomes important if there are catalytic sites and arms that extend becoming important for binding and/or manipulating reactions.

- 3. [penicillinase \[synthetic construct\]](#)  
31 aa protein  
Accession: AAA72626.1 Gt: 209046  
[GenPept](#) [FASTA](#) [Graphics](#) [Related Sequences](#) [Identical Proteins](#)
  
- 4. [penicillinase \[unidentified cloning vector\]](#)  
28 aa protein  
Accession: AAA72473.1 Gt: 207780  
[GenPept](#) [FASTA](#) [Graphics](#) [Related Sequences](#)
  
- 5. [penicillinase \[Bacillus licheniformis\]](#)  
15 aa protein  
Accession: AAA22649.2 Gt: 7019625  
[GenPept](#) [FASTA](#) [Graphics](#)
  
- 6. [penicillinase \[Bacillus licheniformis 9945A\]](#)  
14 aa protein  
Accession: AAA22647.2 Gt: 7019624  
[GenPept](#) [FASTA](#) [Graphics](#) [Related Sequences](#) [Identical Proteins](#)
  
- 7. [penicillinase \[Bacillus subtilis\]](#)  
306 aa protein  
Accession: CAA84711.1 Gt: 522118  
[GenPept](#) [FASTA](#) [Graphics](#) [Related Sequences](#)
  
- 8. [penicillinase \[Bacillus amyloliquefaciens\]](#)  
306 aa protein  
Accession: CAA84712.1 Gt: 522112



The two most important microorganisms, today, that pose a significant threat to human population is resistant *Esheria coli* and *Klebsiella pneumoniae*. These microorganisms are able to invade the host body and cause severe destruction of internal organs. The effectiveness of antibiotics that are  $\beta$ -lactams have been rendered useless due to their evolved  $\beta$ -lactamases that evade inhibition allowing their penicillin-binding proteins to focus on cell wall biosynthesis instead of binding to penicillin. The PBPs, as previously mentioned, are followed by a number that is directly associated with their molecular weight. The penicillinase protein found in *Esheria coli* has approximately 377 aa—18.8% increase in linear sequence compared to the penicillinase protein found in *Bacillus subtilis* shown above (Accession # CAA84711.1). This relative difference of 18.8% roughly estimates to 19.5% difference in weight since *Bacillus subtilis* has 33.46kD and *Esheria coli* have 41.56kD for their protein sequence.

*Klebsiella pneumonia* is a gram-negative microorganism that has recently been given much needed attention. Its pandemic-like rise and increase in frequency for detrimental damage and nosocomial infections has been reported worldwide. Countries such as India are reporting the microorganism to flourish in their water systems and causing a paralysis of their entire irrigation system. *Klebsiella pneumonia* utilizes SHV-5—one of the genes marked for the production of  $\beta$ -lactamases mentioned previously in this article—and completely new strains have been emerging in 2011 known as the New Delhi variant. These variants render carbapenams completely and utterly ineffective leaving the most clinically significant member of the Enterobacteriaceae family to lead to host death<sup>39</sup>. This outbreak has occurred in Israel and other portions of the world as well<sup>10</sup> and is reportedly increasing in the United

States due to travelers that go to foreign countries such as India, drink their water, or divulge in unnecessary plastic surgery, and return back to the states.

*Klebsiella pneumonia* has been associated with several diseases at different stages of infection. Opportunistic infection can lead to endophthalmitis<sup>10</sup> and septicemia in newborn children<sup>5</sup>. The microorganism secretes  $\beta$ -lactamase SHV-1 that has an amino acid sequence of 286. This length conforms to an approximate value of 31.23kD. The penicillinase enzyme of these species share homology to that of *Esherichia coli* depicting a similar mechanism of action. Medical hospitals are currently experiencing outbreaks of *Klebsiella* that are resistant to the typically given treatments of ESBLs (Extended-Spectrum Beta-Lactamases). The carbapenem classes of antibiotics are a recent group of drugs that are given for resistant-strain of microorganisms. Most of these synthetic derivatives have become FDA-approved in the mid-to-late 2000's—meaning that if these fail to address the issues, then the last-resort has failed.

Since penicillin is one of the first compounds discovered, one of the major areas of research is to manipulate the protein in a manner that can establish its one-given efficiency. The proteins ( $\beta$ -lactamases), as previously mentioned, have catalytic ions that are important for the reactionsto occur. Penicillinase isolated and purified from *Streptococcal* to compare to *Bacillus* was performed in the addition to Michaelis constant and maximum velocities<sup>11</sup>. This study was able to determine the pH acidity in the binding sites of the penicillin to the protein after administration of phenyl penicillins. The pH activity curves helped determine the amino acid histidine affiliation with the active site of the penicillinase<sup>11</sup>.



The class D  $\beta$ -lactamases, which belong to the serine activity group, have a lesser degree of sensitivity to  $\beta$ -lactamase inhibitors. The crystalline structure of this protein coupled with a carbapenem meropenem to pave better insight into new drug inhibitor design. Structures in the form showed that there is a flexibility region on the serine 115 residue of the  $\beta$ -lactamase OXA-13—which is directly related to activity of these classes<sup>9,30</sup>. Dimerization traits have been tested discerning  $K_m$  values and amount of substrates.

There are several current challenges that scientists face in understanding the mechanism, structure, and function of the interaction between penicillinases and  $\beta$ -lactams in-vitro and in-vivo. The patient outcome and therapeutic choices for combating resistant-microorganisms are at an increasing rate for intervention, in-depth analysis, and further studies<sup>17</sup>. Most of the bacteria depict monomeric enzymes that range in their molecular weight of 23kDa to 44kDa. Oxa-enzymes are the ones regarded as dimers<sup>30</sup>. This further constitutes complexity that arises on the molecular scheme as protein formation is one of the most integral processes in relation to ligands, ligand-binding, and activity. There is a diminished longevity of creating novel synthetic compounds against microorganisms<sup>6</sup>. The manufacturing of these compounds are time-consuming and costly. The ability to create compounds that counter-act penicillinase and render penicillin groups once again useful is the best method of approach for current and future generations.

## Conclusion

The synergistic effects of EGCG varied dependent upon microorganism, strain, classification, and antibiotic used against certain strains. The EGCG compound was very effective against gram-negative bacteria *Escherichia coli*. It was not found to be effective against gram-negative *Serratia marcescens*. However, the usage of the esterified EGCG, also known as LTP [Lipophilic Tea Polyphenol] was found to have a significant impact against the previously mentioned microorganism. The effectiveness of EGCG and LTP was found to work prominently against *Escherichia coli* and *Staphylococcus epidermidis*—to which both serve as models for severe pathogenic microorganisms that plague clinics worldwide. These organisms serve as an essential model for potentially pathogenic strains that become pathogenic either due to plasmid exchange or developing other mechanisms to evade antibiotic resistance. It was also found that LTP had a greater synergistic effect with the antibiotics against the growth of the microorganism in direct comparison to EGCG. The ampicillin-resistant strains of *Escherichia coli* were also focused upon to determine if strains that develop antibiotic resistance can still become susceptible to treatment of the antibiotic in conjunction with the polyphenol to inhibit the mechanism of antibiotic resistance. The pathogenic growth study proved that EGCG and LTP may be used to reverse the antibiotic-resistance to ampicillin. This is a significant finding and proves that it may be possible to use the natural derivative(s) found in Green Tea in a similar manner as clavulanic acid—a  $\beta$ -lactamase inhibitor. The Red Algae Polysaccharides has also shown promise to inhibit the growth of many of these potentially pathogenic microorganism models. The spore study utilized crude GTP [extract] and indicated that there was a certain extent of inhibition—up to 64%.



### **Future study**

The future of this research project should be aimed at understanding and establishing microorganism antibiotic-resistance and the properties thereof. The identification of any proteins involved in these pathways or identifying if any proteins are involved that may have roles in this resistance. This can lead into a molecular mechanism study that may find out if genes are actually involved. The bacillus strains of microorganisms that produce spore and spoilage causing an abundance of health-related problems should also be established. Further testing, repeat studies, and identification of mechanisms provide a route for future studies and establishment for possible methods to avoid phenomena that contribute to food spoilage. Testing methods for LTP, soluble in organic solvents, should also be established to determine if any topical solutions may have a beneficial effect against the growth of microorganisms in an application that could be considered as an antiseptic (non-toxic to healthy-living tissue). The increase or combination of other polyphenolic compounds should also be established to understand other methods of application can be sought.

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## Appendix

### DRUG ANTIBIOTIC SHEET FOR SUSCEPTIBILITY, INTERMEDIACY, AND RESISTANCE COMPARISON

Drugs	Resistant	Intermediate	Susceptible
Ampicillin	Varies	Varies	Varies
Bacitracin	8	9-12	13
Cephalothin	14	15-17	18
Chloramphenicol	12	13-17	18
Doxycycline	12	13-15	16
Erythromycin	13	14-22	23
Gentamicin	12	13-14	15
Penicillin	Varies	Varies	Varies
Polymyxin	8	9-11	12
Rifampin	16	17-19	20
Streptomycin	11	12-14	15
Tetracycline	14	15-18	19

Table A.1: SIR Chart for Susceptibility (ZOI)

This is the drug diagram which was used to identify the correct categorizations of susceptibility, intermediacy, and resistivity of each of the antibiotics against the certain strain of microorganisms. Each of these measurements and numbers are in MDL and thus have units of mm.





Figure A.1: Procedure for testing substances antiseptically





Figure A.2: Kirby-Bauer Method for Antibiotic Disk Diffusion Testing