Use of antibiotics leading the occurrence of Antibiotic Resistant Bacteria on hydroponically grown Mung bean sprouts.

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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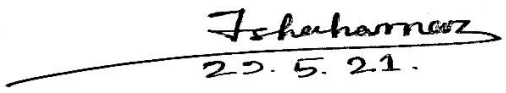

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Approval

The thesis/project titled “Follow-up of Practiced Treatment Regimens & Health Conditions of Recovered Patients of COVID-19 Residing in Dhaka City: A Survey-based Descriptive Cross-sectional Study” submitted by

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Abstract

Antibiotics are used to prevent the growth of harmful bacteria that can cause plant disease or associate to human disease during the development of food crops. The goal of this study was to observe the effect of antibiotics on the organisms of mung bean sprouts, also if organisms residing into sprouts achieve antibiotic resistance at different antibiotic concentration changes. Mung bean sprouts were harvested on day seven following the final treatment of antibiotics (amoxicillin, gentamicin, and ciprofloxacin) at concentrations of 100 ppm, 300 ppm, and 500 ppm) for one week. This investigation discovered a community of complete aerobic bacteria as well as bacteria that were resistant to antibiotics. Antibiotics had varying degrees of success in lowering organism size. At a minimum dose of 100 ppm, all three antibiotics exhibited no significant reduction in bacterial population on mung bean. Amoxicillin had little effect on microbial population, gentamicin and ciprofloxacin inhibited some specific organisms, but there was a significant reduction in total aerobic bacterial count, albeit it was not completely inhibited. Gentamicin and ciprofloxacin showed a 1.5 log reduction in microbial population at concentrations of 300 and 500 ppm. Amoxicillin and gentamicin resistance was found in all of the microorganisms examined. Ciprofloxacin suppressed some pathogens but did not completely suppress total aerobic bacterial count and had an effect on sprout germination. In conclusion, antibiotic use does not effectively suppress microbial populations, and a high percentage of microorganisms develop antibiotic resistance. This investigation focuses on the various effects of antibiotics on mung bean sprouts, as well as the necessity for effective antibiotic concentration management and data in order to prevent the development of multi-antibiotic resistance bacteria through the consumption of fresh mung bean sprout.

Dedication

To my parents, Dr. Md. Nazrul Islam & Shahina Nazrul.

Thank you, for being the anchors of my life.

To my long-term friend from university,

Hasnat Abdullah Omi,

Thank you for your enormous mental support.

Acknowledgement

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List of Acronyms

MDR	Multi drug resistant
Commensal organisms	Normal Flora
Mung bean	Serial seed
Sprouts	Edible seeds
CARS	Centre for advance research in science
Pathogens	Harmful organisms
Antibiogram	Antibiotic susceptibility testing
Germination	Rising of seeds to grow plants

Chapter 1: introduction and literature review

Mung bean sprouts are commonly eaten as a salad item in various countries in Europe and Asia such as Thailand, Malaysia, Italy, Spain and many other countries. These sprouts are delicious to eat and very convenient to grown with little processing and grow throughout the year. It is also rich in nutrition such as vitamin, C, fiber, low in calories and fat. (1) Freshly cut mung bean sprouts are stir-fried with other vegetables to increase the flavor of any meal in Malaysia and Japan. In Thailand, they are eaten as raw vegetables as complementary to other dishes. The increasing popularity and heavy demand leading people to grow it the way they can, without proper sanitation or maintaining any hygiene process. (2) Many foodborne outbreaks occurred linked to sprouts contamination with *Salmonella, E. coli* 0157:H7*, Enterobacter, listeria, Staphylococcus aureus, bacillus,* and many other foodborne pathogens. (3) The biggest outbreak linked to sprout contamination occurred in Ontario, Canada for salmonella enteritis. There are so many ways associate to sprout contamination from production, harvesting to storage and transport. (3)

During germination barrier of the seed coat is broken that is hazardous as nutrients from sprouted seed allow many bacterial pathogens to grow. As sprouts are eaten as salad items, raw or a very little touch of cooking, contaminated sprouts may find a potential route that can lead to human illness. (4) Sprouts are being grown in watery condition, high humid and moderate temperature and the whole process during growing sprout permit the growth of more pathogen. Seeds for home sprouting are usually soaked overnight prior to growth (1). The lack of proper recommendation for disinfection, sanitary condition, chemical decontaminants for home growing mung sprouts are at high risk for microbial contaminations. Regular irrigation is done in Commercially or hydroponically grown sprouts under the warm dark humid condition which also suitable for pathogen growth. (5)

Seeds are more likely to get cross-contaminated with fecal contaminants through the soil, seed farm located near the domestic animal farm, worker hygiene, distribution, and packaging. It is very hard to identify in which phase the seed can get contaminated. U.S food and drug administration (US FDA) in 1999, provided rules and regulations such as seeds must be decontaminated with calcium hypochlorite for at least 15 minutes before sprouting. (6) There were so many other methods have been invented for seed disinfecting process such as hot water treatment, acid treatment, irradiation, high, electrolyzed acidic water pressure that are effective against pathogen and reduce seed contamination. Canada has recommendations of 2,000 ppm calcium hypochlorite

or sodium hypochlorite for 15-20 minutes or 6- 10% hydrogen peroxide for 10 minutes as a decontamination treatment.(1). A good seed from a good source and a good harvesting procedure can potentially reduce the number of food-borne pathogens.

However, complete inhabitation of pathogens is not fully arisen from both raw seeds and sprouted mung seed. Sometimes, disinfectants and their concentration play an important role over germination and flourishing the sprout. The number of germinations that occur from raw mung seed can be inhibited or affected by various cleaning processes. It's also been discovered that *E. coli* and *salmonella* can live inside the seed or grow inner tissue coating. (7) Disinfectants only remove pathogens from the seed's outer layer, not from inside the seed. Unlike *salmonella and E. coli*, any disease that penetrates seed through any path and seed fissures is difficult to eliminate with disinfectants alone, as seed coating protects them. (7) If the pathogen survives the decontamination operation, it will in sprout at a rapid rate, eventually reaching high levels. Inhibiting infections from interior tissue is both a source of concern and a significant difficulty. (6) Many scientists are now recommending that pathogenic bacteria be inhibited or reduced by applying antibiotics to sprouts throughout the germination process and continuing to do so until the sprouts are ready to harvest. (8) Since many agricultural crops contain some common bacteria or normal flora that aids in the germination process, mung bean likewise contains some normal flora. Normally, natural plant flora is not hazardous to human health; but, using antibiotics excessively on them might develop them into antibiotic-resistant bacteria, which can have long-term consequences for human health and can cause damage to the environment.(9) The goal of this study was to determine the efficacy of various antibiotics in reducing organisms in hydroponically grown mung bean sprouts, as well as whether antibiotic use leading the persistence of antibiotic resistance gene in both commensal and pathogenic bacteria of mung bean sprouts.

1.1. Working with mung bean sprout:

Hydroponically grown crops and roof gardening becoming popular in this era in many countries. Our country also developed many crops under the hydroponic method. Mung bean is easy to grow under the hydroponic method. Mung bean sprout is a popular food item in countries like Thailand, Malaysia, Canada Germany, USA and many developed countries. (10) People of different countries consume them as their food culture. It can be easily grown in home and commercially

because of its availability the whole year-round. This contains high protein (7.02g), fiber (7.60), a rich source of calcium and iron. vegetarian people can consume it to cover up the need protein. (1) A large number of vitamins B, C and k are also found in the nutritional properties of mung bean. (1) For all these reasons, many companies and suppliers making mung bean sprouts commercially available. It's high demand around the world and many outbreaks and illnesses occurrence motivated to work on mung bean sprouts. Moreover, it's availability, easy maintenance and easy handling in the laboratory, rapid growth in a short time, implanting hydroponic method for growing are the reasons for choosing mung bean for this research among many other alternatives. (1)

1.2. Natural microflora of Mung bean:

From germination to sprouting seeds are very quick and trouble-free process can be done in home or at a commercial scale. All we need for germinating seeds is maintaining high level of moisture and soaking dry seeds to allow germination. It grows within 2-7 days depending on the type of seed. Seed contamination results in the growing many pathogenic bacteria which degrades shelf life of foods. (11) Food spoilage may be caused by both pathogenic and nonpathogenic normal flora inside a crop. Protein, minerals, lipids, and vitamins also contribute to a favorable environment that produces more bacteria and allows them to survive for extended periods of time.(1) Mung bean sprouts are reported to have a huge population of microbial flora, which includes coliforms (10^2 to 10^6 /g), *Enterobacter (*10^4/g), aerobic bacterial counts (10^7 to 10^9/g), *klebsiella* (11.1%), *Pseudomonas* (14.4 %) *Citrobacter , Pantoea* species, *Proteobacteria* (90.4 %), *Bacteroidetes* (8.8 %)(2). The 16S rRNA sequences from uncultured mungo bean sprout samples were divided into five phyla and 34 bacterial taxa (2). Microbial flora in mung bean sprouts is frequently found to be higher (2.0 -3.0 log CFU/g) than in raw seed. During the creation of sprouts, several environmental, chemical, and enzymatical nutritional elements play a part in the proliferation of a large number of organisms. (12) The presence of a large number of trypsin inhibitors in mung seed may operate as a defense mechanism against bacteria that contain the trypsin enzyme, which promotes microflora multiplication. (1). The nutritional characteristics of raw beans are poor, but soaking them for germination can enhance the nutrients by tenfold, providing more accessible substance for microbial growth. Finally, the

hydroponic environment, which is high in moisture and heat, results in a favorable temperature for commensal bacterial development of raw mung bean sprouts (9). Many studies say that, aside from harmful organisms, the majority of bacterial species detected in mung bean sprouts are more likely to have originated from raw seeds, which are commensal organisms or mung bean dwelling under the seed coating tissue, rather than sanitary conditions and environmental elements of sprout formation. (13).

1.3. Effectiveness of seed treatment:

Besides the common flora of mung seed, some pathogens may appear due to contamination. If they reside during germination and sprouting process they can amplify at a high number, can cause health hazard (10). Many studies suggested eliminating pathogens from the seed surface and also reducing the number of microbial flora of seeds. Sodium hypochlorite at concentration 0.5ppm, chlorine and heat treatment using mung seed significantly reduce the number of viable cell count of raw seed. Nevertheless, it is also found not efficient in eliminating the pathogens from seed. Likewise, if Hot water treatment for 10 minutes at 54^0 C is induced, microbial population reduces however, seeds lose their viability for germination. Hot water treatment at 85 ^{0}C for 40 sec followed by dipping in cold water for 30 sec and soaking into 1500 or 2000 ppm chlorine water for 2 h was performed, no viable pathogens were found and no survivors were found in the enrichment medium and during the sprouting process.(15) This finding suggests that the *E. coli O157:H7 and Salmonella* population was totally inactivated after the 40-sec treatments with hot water at 85^0C followed by dipping in cold water for 30 sec and soaking into chlorine water (2000 ppm) for 2 h, and the bacteria were unable to repair and grow even when the proper environment is provided.(15). Some studies suggested using ethanol and sodium hypochlorite (1000ppm) or hydrogen peroxide which reduce aerobic plate count by 2.3-3.3 logs CFU/g, besides these harsh chemicals such as ethanol inhibit germination of seeds. Soaking the seed under sodium hypochlorite for 5 minutes does not eliminate microflora.(14) so many studies and findings suggested various effective methods for eliminating the pathogens from raw seed without affecting the germination, therefore, if typical flora or pathogen numbers stay low, whether latent or

damaged, there is a good possibility they may reappear throughout the sprouting phase.(10) Pathogens and natural flora that sneak into the seed covering tissue or lay under cracks and crevices, they are inaccessible to all treatments.

1.4. Difference between sprouting and germination:

The definition of seed is it is a biological structure with essential nutrients starch, fat, proteins and plant embryo encased by a protective outer shell. Embryo later develops through germination. (16). The seed has all the food sources of plant production. when a seed meets require factors like ambient temperature, water, the intensity of light, environment, oxygen to end the dormant period is known as germination. (10) Formation of plant from a seed after a period of dormancy is germination is, plant develops two structure plumule and radicle. Germination never occurs if seed does not have embryo in it. Dormancy of seed is followed by external environmental conditions. Following the imbibition, the process seed takes up water from the environment to germinate. Water breaks down the reserved food into chemicals form and starts metabolic process including aerobic respiration until the development of leaves of a plant. (10)

Sprouting on the other hand is the soaking of seeds after germination for several hours leading to the formation of a protrusion. After this process is complete, seeds are taken as a food source. (17) Some seeds are not edible lack of digestible properties, moreover, in raw condition seed also have harmful effects on living system. If seeds are sprouted, it converts those ingestible form of seeds into digestible form. Bioavailability of nutrients like zinc, calcium, iron within the seeds are increased during sprouting and anti-nutrient properties are decreased. (1)

Figure 1: Germination and sprouting.

1.5. Nutritional value, chemical constituents, and metabolite changes of mung bean:

Mung bean during germination faces more obvious biological activities and actives many biosynthetic enzymes useful for secondary metabolites. Mung bean contains 20-24% of protein. 50-60%of carbohydrates, twenty-one organic acids including phosphoric and citric acid and 16 lipids including gamma-tocopherol. (12) Important metabolites of mung bean found are flavone, isoflavone, flavonoids, and isoflavonoids. Most flavonoids have antioxidant activity, involved in stress protection like oxidative and temperature stress and signaling in legume nodule. phenolic acid is a secondary metabolite and twelve phenolic acids have been identified from mung bean sprouts and seeds. (12). Phenolic acids are considered as major bioactive phytochemical throughout the germination process Gentistic acid, cinnamic acid, and p-hydroxybenzoic acid are found. (12). Caffeic acid, ferulic acid, and shikimic acid levels are low in the seed phase of the mung bean, but these chemicals increase dramatically following germination. Moreover, the levels of gallic acid, chlorogenic acid, and coumarin increase dramatically in the germination material until day 3 or 4, and catechin levels increase during the final stage of mung bean development (4). There is a certain increase of organic acids like lactic acid, phosphoric acid during sprouting was noticed. Level of fatty acid methyl esters decreases after initial soaking and germination phase, throughout the development of sprouts gamma-aminobutyric acid enhances. (18). Protease inhibitors prevent proteolytic enzymes from catalyzing their functions during sprout creation, which is why defensive mechanisms against fungus and pathogenic organisms are reduced during

the first five days of sprout development (9). The rapid growth of pathogenic and commensal organisms is aided by a series of metabolic events and continuous changes in chemical contents from germination to sprouting.

1.6. *E. coli, and Salmonella* associated to mung bean sprouts:

Mung bean sprouts can be contaminated by primary contaminations during growing and harvesting and secondary contamination while washing, soaking, packaging and preparation. Sprout's contamination is mostly coming from contamination of seeds because bacteria reside outer surface of the seed and some bacteria may get internalized. (11) Previously it has been reported that *E. coli and salmonella* can get established into the internal structures of sprouting seeds. Biofilms present on the outer surface of sprouted seed also protects pathogens. Sprout associated outbreaks are mostly recognized due to seeds that are contaminated with pathogenic organisms other than post production contamination. (11) During sprouting microorganism load increase because of high volume of water for production and the temperature for sprouting. (11) Total microbial population of raw seed was found lower than that of sprouted seeds, which is why disinfecting seeds using sanitizer is a must. Internalized organisms are difficult to eradicate just by cleaning seeds; as a result, post-harvesting methods or measures used during sprouting production are essential to prevent pathogens from multiplying. (6) At least 40 outbreaks occurred by *Salmonella enterica* and Shiga toxin producing *E. coli*, both of these are common foodborne pathogen and cause numerous health hazard to human. Beside these two vital pathogens, many enteric gram-negative organisms were also isolated from mung bean sprouts (19).

1.7. Antibiotics selection for this study:

Hydroponically grown item in agriculture uses many pesticides to prevent insects and to prolong the shelf life of the crops. (20). This system requires water, sands, trays. beds, bags to support the growth of the crops. Sprouting and germinating seeds only need water and a pot, some may use cloths to cover the pot and make a dark environment to grow. All these items must be

contamination-free; however, it is not all time possible to check every single detail; as a result, seed gets contaminated. (20). To prevent contamination of seeds many findings are now suggesting disinfecting the seeds first by any method; sadly, pathogens may still stay at the time of sprouting of mung beans. Many scientists are proposing to use antibiotics to eradicate organisms from sprouts even after disinfecting seeds. They consider using antibiotics worthwhile to eliminate disease-causing pathogens from plants to reduce foodborne diseases. There are not many reports found previously of using antibiotics to grow mung bean sprouts, even antibiotic use on crops, vegetables, and high-value fruits. Few out of many countries have national legislation to use antibiotics in crop agriculture; therefore, many countries using antibiotics are not being monitored, and official information is unavailable.

The use of antibiotics is adversely being seen in farming and livestock agriculture, which has already faced issues over antibiotic resistance. (14). Although so many debates are still going on using antibiotics on crops which can develop antibiotic resistance in the organism, that is a significant global health problem now. The objective of this study was to see if mung bean sprouts are treated with specific concentrations of antibiotics, it has any effect of eliminating pathogens and commensal microorganisms. (18). Therefore, under-treatment of antibiotics in mung sprouts leading to persisting MDR commensal bacteria.

Antibiotic concentrations of 50-ppm to 300-ppm are used to remove pathogenic bacteria in vegetables such as tomato, cabbage, carrot, cucumber, mustard, brinjal, lettuce, and leaf-based crops in Thailand, Malaysia, and Japan, according to reports. (17). Antibiotics are used mostly on crops by spray treatments which typically formulated as powder. Water used to dissolve antibiotics to concentration between 50 to 300 ppm and sprayed over the top as a fine mist. Antibiotic over plants remains active for less than a week but bacteria present in the plants may get the antibiotics into their gene and become resistant. Countries like USA, India, Israel, Japan, EU/EEE, New Zland reported using antibiotics are streptomycin oxytetracycline, kasugamycin, gentamicin, oxalinic acid, streptomycin but in what amount they are being used it is not found. (21) Most of the countries restricts antibiotic use and those who use they do not have proper data on usage of antibiotics over crops. Misuse of antibiotics also been reported in India among farmers. Crops grown in aquas solution refers as hydroponic crops, antibiotic uptake is higher in plants grown hydroponically rather than those grown in soil and it is scientifically proven. (2). Soil absorbs

antibiotics mostly and reduce the amount for uptake in plants. Existing data of concentration of antibiotics using over plant is very low and unavailable, there is no related data found antibiotic use over mung bean. This experiment is totally new so; antibiotic concentration was fixed according to the previous studies of using antibiotic concentration over crops and feedback from growers of Thailand. (20). Deciding concentration was a minimum of 100 ppm to maximum concentration was 500ppm. Antibiotics was chosen from three different groups (aminopenicillin, quinolones, aminoglycosides) having three different kinds of mode of action Ampicillin, gentamicin and ciprofloxacin.

Amoxicillin:

by adding amino groups to penicillin, they are called aminopenicillin to battle antibiotic resistance. They are used to treat gram-negative organisms *as E. faecalis, E. coli, salmonella, clostridium*, as well as gram-positive organisms, mostly streptococcus species. It is a beta-lactam antibiotic that binds penicillin-binding protein that inhibits transpeptidation, a cross-linking process in cell wall synthesis. (19). It activates auto enzymes that leads to lysis of the cell wall and destruction of bacteria cell. It is under bactericidal killing of the bacterial cell. Bacteria would not be able to withstand any changes in osmotic pressure or any other stressors which would lead to bacterial lysis or the death of the bacteria. Beta-lactamase inhibitors such as clavulanic acid and sulbactam often combine with amoxicillin, which works by adding to the catalytic site of an organism's beta-lactamase enzyme irreversibly. (21)

Gentamicin:

It is under the aminoglycoside group of antibiotics. It is used against infections especially caused by gram negative bacteria including *pseudomonas, klebsiella and Serratia marcescens*. It is also bactericidal by inhibiting protein synthesis. this antibiotic binds to the bacterial 30s ribosomal subunit, inhibits the translocation of tRNA during translation. (21). This leads the bacteria unable to synthesis any protein and bacteria dies. Main mode of action of gentamicin is inhibiting protein synthesis by binding to ribosomal sites, however, it also creates hole in the cell wall of gram-negative bacteria before it reaches to the ribosome. It is a broad-spectrum antibiotic used to inhibit

both gram-negative and positive bacteria. This antibiotic also promotes codon misreading in vivo and invitro, cause membrane damage however, the mode of action is very different than other antibiotics such as of chloramphenicol, tetracyclines, macrolides, and other inhibitors of ribosomal functions. gentamicin binds more than one binding sites of the ribosome suggested by the triphasic concentrations effects on translation, elongation inhibited at low concentration and at higher concentration inhibition diminishes. (4) The resistance of bacteria to aminoglycoside antibiotics is mostly attributed to the production of drug-modifying enzymes or to ribosomal alterations. In addition, the drug resistance of some organisms is due to decreased permeability of antibiotics into bacterial cells(5).

Ciprofloxacin:

It is under fluoroquinolone group and a broad-spectrum antibiotic. It is most effective against gram negative bacilli (*E. coli, salmonella, shigella Neisseria, pseudomonas).* (22) This antibiotic inhibits the bacterial DNA topoisomerase (ii, iv) enzymes and DNA gyrase which results to stop DNA replication, transcription, rapier and recombination. this antibiotic mode of action is related to the inhibition of DNA. It prevents DNA from introducing a negative super helical twist into its strands and bacterial cell no longer replicate. (22). Alpha subunits of DNA gyrase are the target of ciprofloxacin. Spontaneous single step chromosomal mutation associated with alteration in the alpha subunits of gyrase leads bacterial resistance to ciprofloxacin. Ciprofloxacin works very well in problematic infections caused by pathogens resistance to other antimicrobial agents. As Ciprofloxacin is a very new kind of quinolones, sometimes works far better than other groups of antibiotics eliminating pathogens. In contrast to *E. coli* KL16, for other aerobic gram-negative bacilli of the genera *Klebsiella*, *Enterobacter, Serratia, Providencia, and Pseudomonas*, the frequency of spontaneous single-step resistance to ciprofloxacin is higher (6).

2.7. Objective of the study:

- Structuring raw data that can help in the future, before introducing antibiotics over any freshly eaten crops.
- Effectivity of antibiotics eliminating pathogenic organisms.
- Besides inhibiting pathogens, commensal and other bacteria get the resistant gene from the treatment of antibiotics, leading to health hazard in human.

Chapter 2: Methodology and Materials

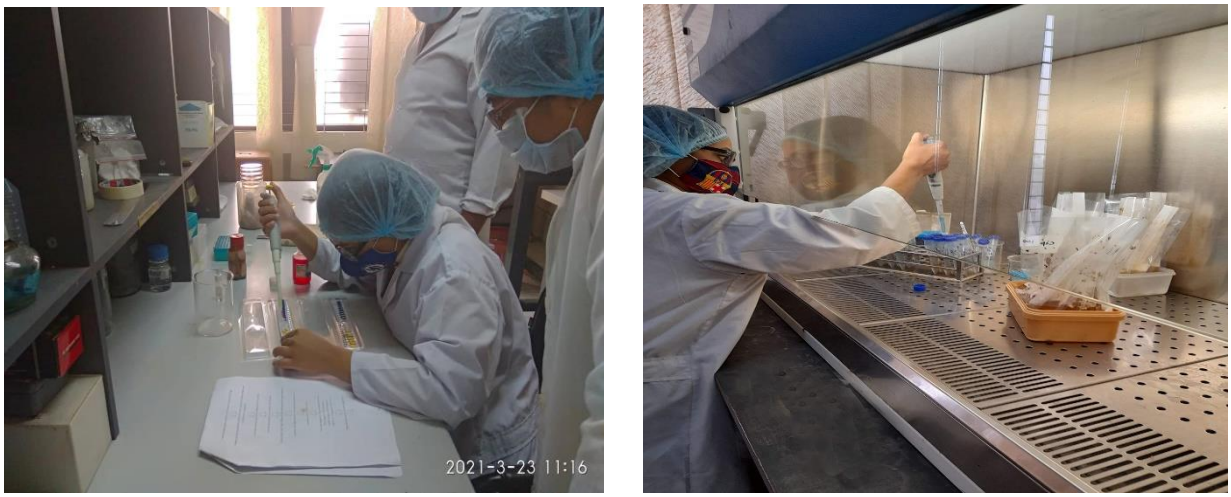


Figure 2: Working in Food microbiology lab, Centre for advance research in science, Dhaka University.

This experiment was originated in, Food analysis and research laboratory of the Centre for advance research, CARS, Dhaka University. Outline and the experiment were designed by the expertise of food analysis lab.

2.1 Outline of the Step-by-step procedure of the experiment:

1. 5 grams of Sample seed were washed properly and placed into a sterilized plastic glass. Amoxicillin, gentamicin and ciprofloxacin antibiotic liquid solutions were prepared from powder form at a concentration of 100,300 and 500 ppm.
2. 2 ml of each antibiotic of different concentrations were added over the seed. A sample of control was also made adding distilled water over the seed without adding any antibiotic solution. In this step, all the seeds must be drowned under liquid solutions.
3. All the nine glasses were covered and properly sealed with aluminum foil and kept into a dark environment where no light can come to contact for germination purposes.
4. After 24 hours, all glasses were observed with germinated seed, antibiotic solution and distilled water was fully soaked, this is why 1 ml of antibiotic solution and distilled water was added over the seed again. In this step seed needs a little amount of wetting for germination. Drowning it under a liquid solution may inhibit the growth of sprouts.
5. After 48 hours later, 2 cm growth of sprouts was seen by breaking the seed cover. All the seeds were added to 1 ml of antibiotic solution and control sample with distilled water. This process was repeated until the optimum 7-9 cm growth of sprouts was descried through seven days.
6. The growth of sprouts was monitored every day until day seven. Every day 1cm increased growth in sprouts were seen.
7. After seven days, all the sprouts were 7-9 cm in height and ready for further examination. Germination percentage was checked.
8. All the sprouts were taken into a stomaching bag to mash with saline water, and samples were plated with the desired dilution into three different growth media.

2.2. Materials used:

- Antibiotics: Powder form of Gentamicin, amoxicillin, ciprofloxacin, and antibiotic commercial disk for susceptibility test.
- API 20 E strip.
- Sorbitol MacConkey, bismuth sulfite, chromocult, tryptic soy agar medium
- Mung bean Sample from local bongo bazar.
- Different laboratory equipment on demand.

2.3. Table: The names and manufacturers of all Media that used.

Media/Broth	**Manufacturer**	**Country**
Chromocult Agar	Merck, KGaA	Germany
Bismuth Sulfite Agar	Himedia	India
Sorbitol- MacConkey agar	Liofilchem	Italy
Tryptone Soya Broth	AFC Agro Biotech Limited	Bangladesh

2.4: Sample processing:

Figure 3: Sample collected from local bongo Bazar.

- Mung bean samples were washed properly with distilled water and let dry under the biosafety cabinet, to prevent any contamination and fungal growth.
- Plastic glasses were sanitized with ethanol to prevent any cross-contamination through glasses.
- After drying, 3mg of seed (60seed in total) was taken into plastic glasses, were ready for further experiments.
- All samples were covered with clean aluminum foil after antibiotic treatment to prevent aerobic bacterial contamination and kept under dark environment that helps germinating the seeds.

2.5 Antibiotic preparation and treatment process

- Three antibiotics were chosen for treatment in different concentrations, 100, 300 and 500 ppm.
- 50 ml of Amoxicillin, gentamicin and ciprofloxacin solution were made from their powder form diluting them into water, it was done using the formula: "c1v1 = c2v2".
- Antibiotic solutions were stored in refrigerator, using them for one week.
- For germination, 3 ml of each antibiotic with different concentrations were added over the seed.
- After 24 hours, germination occurred, as sprouts need soaking and wetting reversely until their proper formation, 1 ml of each antibiotic solution was being added after every 24 hours until day six.

2.6 Table: Amount of antibiotic solution added:

(Day's count)

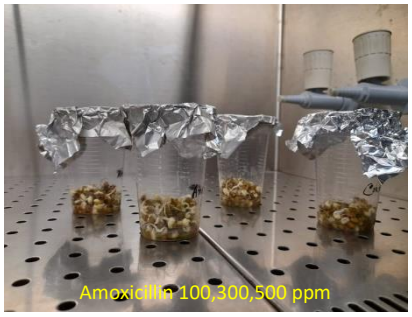
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
						
3ml	**1ml**	**1 ml**	**1 ml**	**1ml**	**1 ml**	**= Harvested**

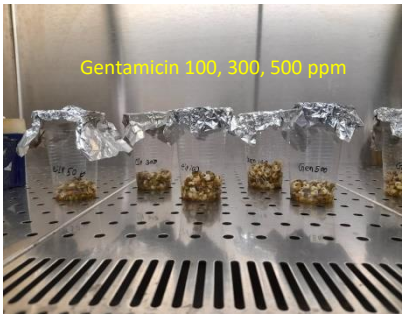
(Amount of antibiotic solution added)

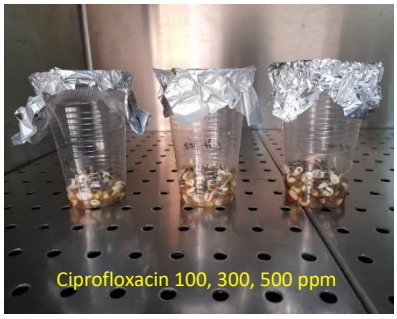
Total 9 ml of antibiotic solution was added throughout day six, and at day seven sprouts were harvested.

2.7. Pictures of Antibiotic treatment over mung bean sprouts throughout seven days:

Day 2:







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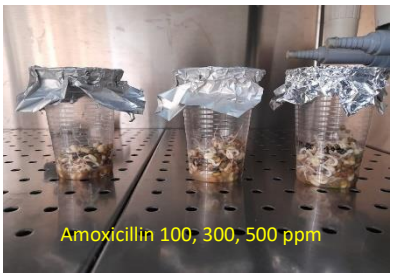
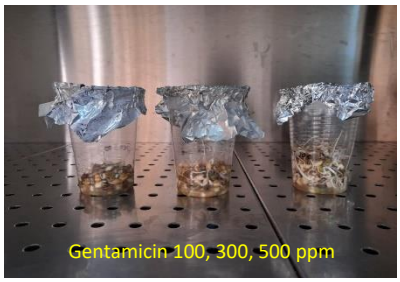
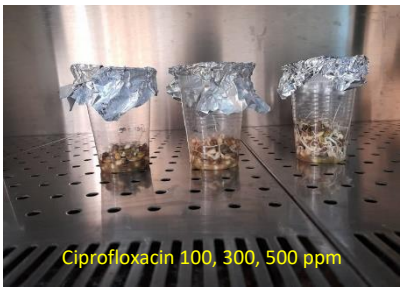
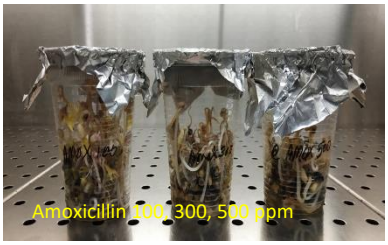
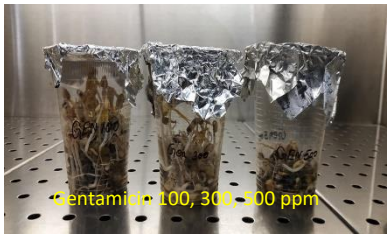


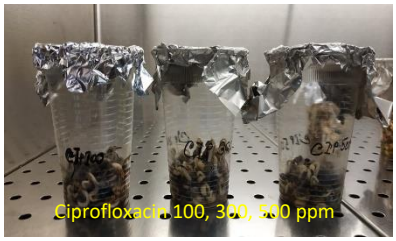




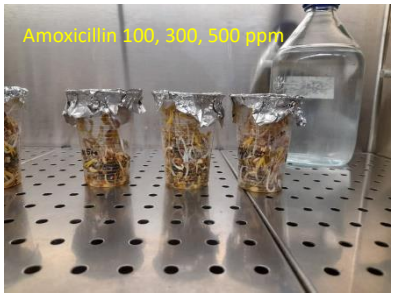
Day 5







Day 7



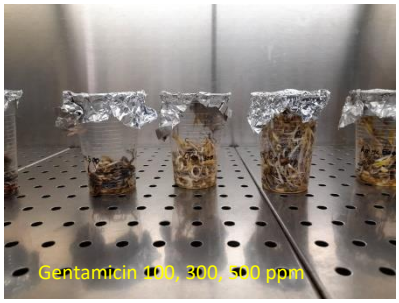


Figure 4: Germination and growth of mung bean sprout under various concentrations (100, 300 & 500 ppm) of antibiotic solution (amoxicillin, gentamycin and ciprofloxacin) up to 7 days of incubation at ambient temperature (25± 2^0C).

2.8. Microbial analysis:

- All samples (9 antibiotics treated samples and 1 sample control) were taken into stomacher bags carefully under the biosafety cabinet.
- After germination, sprouts weight was noted, it was approximately 20 grams of sprouts in per glasses (3g glass weight).
- Sterilized normal saline was measured 9 times with the weight and 180 ml of saline was added to stomacher bags for stomaching.
- All samples were evenly mashed using the stomaching machine.
- Serial dilution was prepared from the sample.
- All samples were plated according to the dilution factor

2.9. Spread plate method

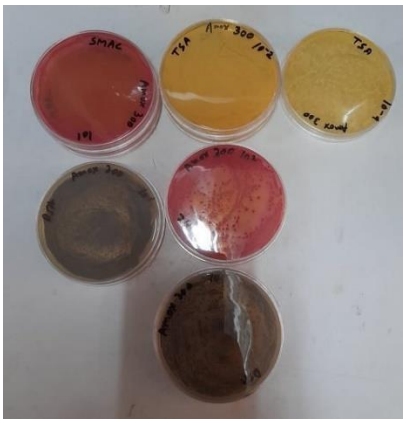
Appropriate dilutions of the samples were placed into the media and the surface spreading technique was followed. All selected dilution of the samples were plated two times to make copies for a getting a fair result from every sample.

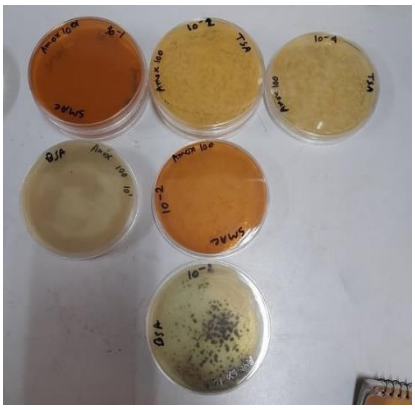
Both selective and nonselective agar media were used. Tryptic soy agar (Merck, Germany) medium was used for the total aerobic bacterial count and selective mediums were Chromocult Agar (Merck, Germany) for coliform, Sorbitol MacConkey for detecting *E. coli*, Bismuth Sulfite Agar (BSA; Himedia, India) for *Salmonella* spp.

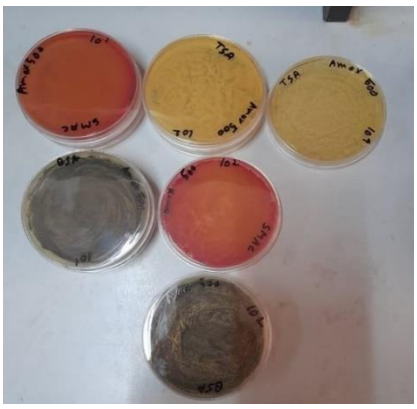
2.10. Observation and colony counting:

After 24 hours of 37^0C incubation in incubator, Microbial population was observed on non-selective TSA media and selective Chromocult, Bismuth sulfite and sorbitol MacConkey media. Plates were rearranged according to lower dilution 10^2 to higher dilution 10^4, all plates of various concentration of antibiotic treated samples were separated from each other.

plates of Amoxicillin:



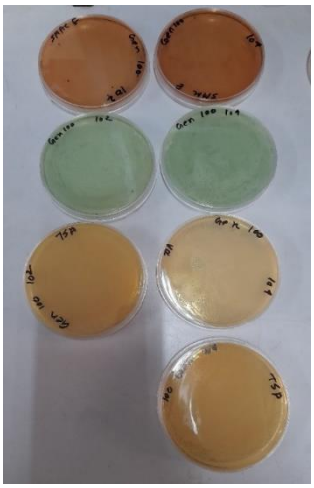


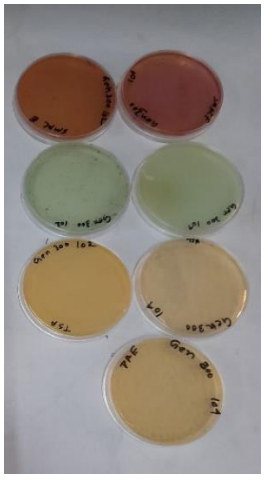


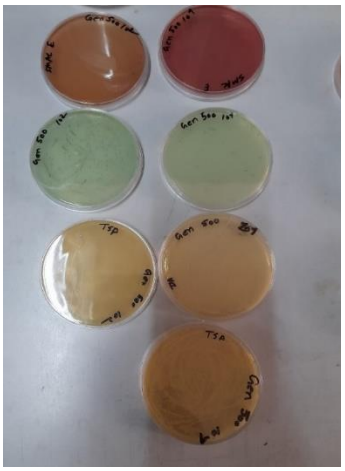
300ppm **100ppm** **500ppm**

Figure 6: Plates of Amoxicillin-treated sprouts (100,300 and 500 ppm antibiotic-treated sprouts were plated after seven days of germination to check the microbial population load).

Plates of Gentamicin:



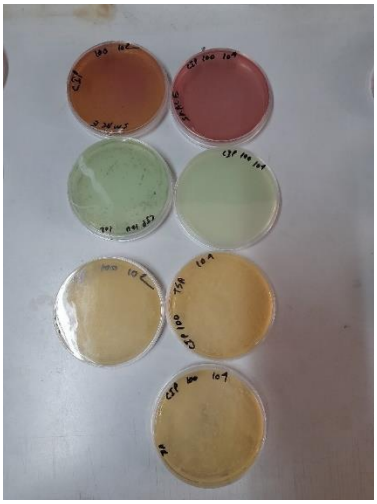




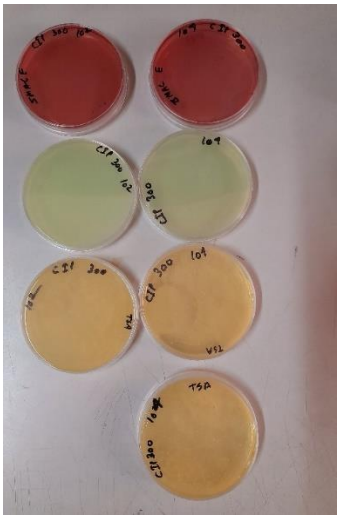
100ppm **300ppm** **500ppm**

Figure 7: Plates of Gentamicin treated sprouts (100,300,500 ppm antibiotic treated sprouts were plated after seven days of germination to check the microbial population load)

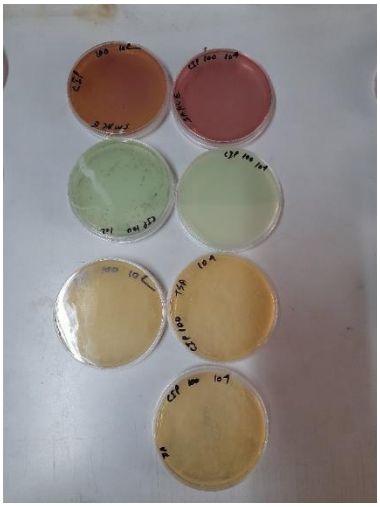
Plates of Ciprofloxacin:



100ppm



300ppm



500ppm

Figure 8 : Plates of Ciprofloxacin treated sprouts (100,300 and 500 ppm antibiotic treated sprouts were plated after seven days of germination to check the microbial population load)

2.11. Single colony isolation

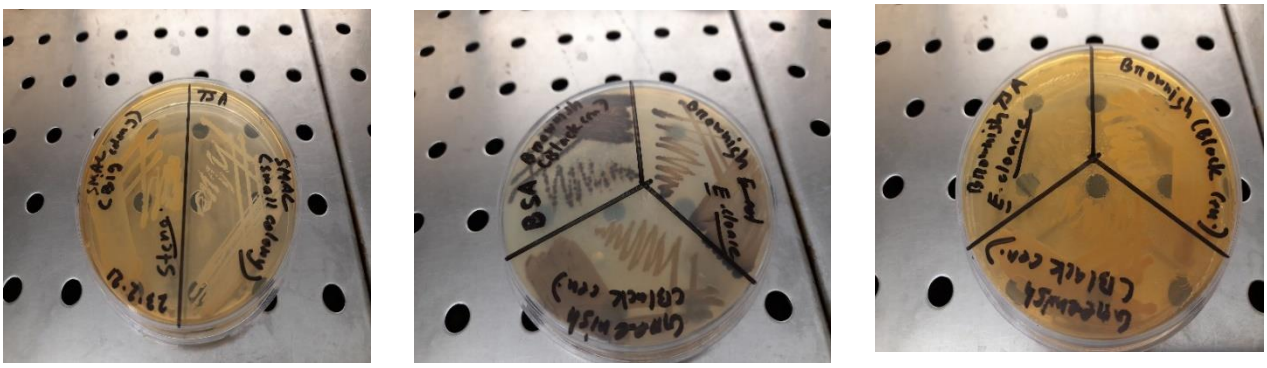


Figure 12: Single colony was isolated to perform API test and antibiogram.

Single colony was isolated on both selective and non-selective media to perform antibiogram and API 20E analytical profile index. Then streak plate method was performed. Plates incubated for 24-48 hours at 37°C until colonies are well formed.

2.12. Performing analytical profile Index (API 20E) biochemical test:

- At first microbial colonies from all selective and non-selective plates were isolated and plated into TSA agar to get pure culture.
- Bacterial suspension was made adding one or two colonies from isolated TSA agar into the 5 ml of normal saline.
- API 20e is a biochemical test strip holds 20 different bio-chemical reagents in 20 separate compartments.
- Using pipette, all the compartments are filled with bacterial suspension.
- ADH < LDC<ODC<H2S<URE compartments were covered with mineral oil.
- before putting the bacterial suspension, distilled water should be added to strip to avoid dehydration of the chambers.
- Trey was marked and incubated at 37°C for 18 to 24 hours.

2.13. Bacterial identification by biochemical test (API 20E):

Tests	Result	
	Positive	Negative
ONPG	Yellow[1]	Colorless
ADH	Red/Orange	Yellow
LDC	Red/Orange	Yellow
ODC	Red/Orange	Yellow
CIT	Blue-Green/Blue[2]	Pale Green/Yellow
H_2S	Black deposit/thin line	Colorless/Grayish
URE	Red/Orange	Colorless
TDA	Reddish Brown	Yellow
IND	Pink	Yellow
VP	Pink/Red[3]	Colorless/Pale Green/Yellow
GEL	Diffusion of black pigment	Colorless
GLU	Yellow/Grayish Yellow	No diffusion
MAN	Yellow	Blue/Blue-Green
INO	Yellow	Blue/Blue-Green
SOR	Yellow	Blue/Blue-Green
RHA	Yellow	Blue/Blue-Green
SAC	Yellow	Blue/Blue-Green
MEL	Yellow	Blue/Blue-Green
AMY	Yellow	Blue/Blue-Green
ARA	Yellow	Blue/Blue-Green
OX	See oxidase test pack direction	

1= A very pale yellow should also be considered positive
2= Reading made in the cupule aerobic
3= A slightly pink color after 10 minutes should be considered negative

2.14. Why API 20E used

Nonfastidious gram-negative rods and Enterobacteriaceae have a standardized identification system that is API 20E, which involves 21 miniaturized biochemical tests. Bacterial suspension was added to the chemicals and incubated, after incubation certain color changes occur spontaneously or addition of reagents. The chemical reactions are analyzing according to the reading table of the analytical profile index identification software. This study focuses on identifying organisms as *E. coli, salmonella, Enterobacter, klebsiella and* other possible disease-causing gram-negative bacteria that reside inside the mung bean tissue, this is why API 20 E was used for identification and confirmation of the organisms.

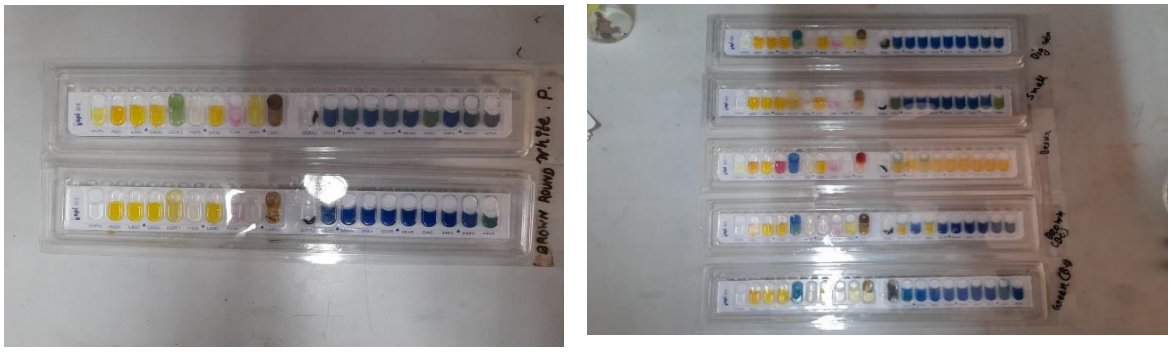


Figure14: Performing API 20E , inoculation or bacterial suspension into the API strip.

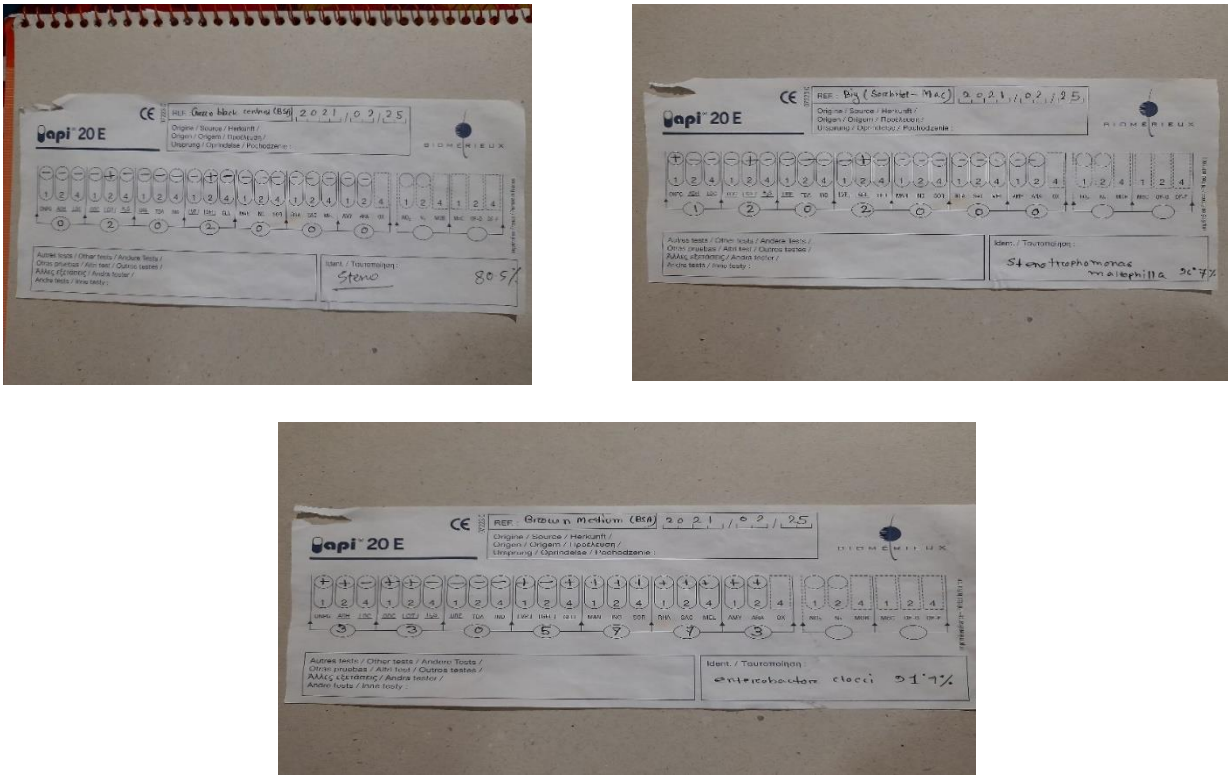


Figure15: After incubation of 24 hours, got the API result and API 20e checklist were filled up according to the result and matched the data with API website apiweb™ (biomerieux.com) , it can simply determine target microorganism.

2.15.API 20 E Biochemical Test Strip lay out

The lay out was as follows:

1. ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside

2. ADH: decarboxylation of the amino acid arginine by arginine Di hydrolase

3. LDC: decarboxylation of the amino acid lysine by lysine decarboxylase

4. ODC: decarboxylation of the amino acid ornithine by ornithine decarboxylase

5. CIT: utilization of citrate as only carbon source

6. H2S: production of hydrogen sulfide

7. URE: test for the enzyme urease

8. TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent to put-Ferric Chloride.

9. IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase. Reagent-Indole was detected by addition of Kovac's reagent.

10. VP: the Voges-Proskauer test for the detection of acetoin (acetyl methyl carbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway

11. GEL: test for the production of the enzyme gelatinase which liquefies gelatin

12. GLU: fermentation of glucose (hexose sugar)

13. MAN: fermentation of mannose (hexose sugar)

14. INO: fermentation of inositol (cyclic polyalcohol)

15. SOR: fermentation of sorbitol (alcohol sugar)

16. RHA: fermentation of rhamnose (methyl pentose sugar)

17. SAC: fermentation of sucrose (disaccharide)

18. MEL: fermentation of melibiose (disaccharide)

19. AMY: fermentation of amygdalin (glycoside)

20. ARA: fermentation of arabinose (pentose sugar)

(Tank Eshwar., 2015).

2.16. Antibiotic susceptibility testing

- Antibiotics are introduced against bacterial pure culture to see the viability of the organisms, weather the antibiotic can inhibit the growth of the organisms or organisms may achieve resistance and exhibit in the media.
- All the organisms from selective media were grown on TSA plate for pure culture.
- Organisms were taken from pure culture to TSB broth.
- 15 commercial antibiotic discs were taken.
- Two types of method were followed, disk diffusion where bacterial suspension was swabbed over the media than antibiotic disc was placed over it.
- Another is well diffusion, where organisms were swabbed over the media and media was welled with a tips and antibiotic of desired concentration (100,300,500ppm) were poured into the well.

3.16. List of Antibiotic discs used

- Chloramphenicol
- Erythromycin
- Kanamycin
- Ciprofloxacin
- Tetracycline
- Doxycycline
- Ampicillin
- Aztreonam
- Polymyxin B
- Amoxicillin

- Vancomycin
- Oxytetracycline
- Gentamicin
- Trimethoprim
- Nitrofurantoin
  sulphathiazole

2.17. Performing antibiotic susceptibility test:

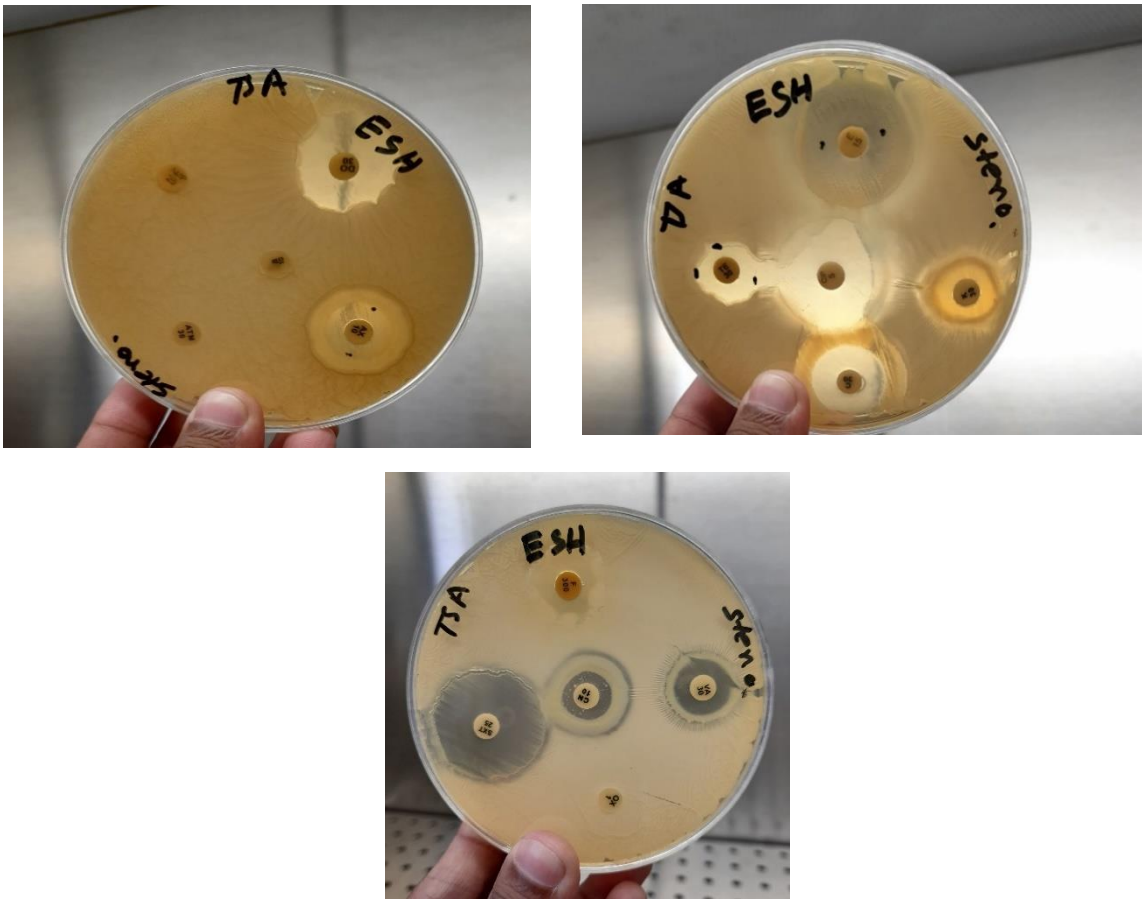


Figure 16: Disk diffusion method of antibiotic susceptibility testing performed for *Stenotrophomonas maltophilia.*

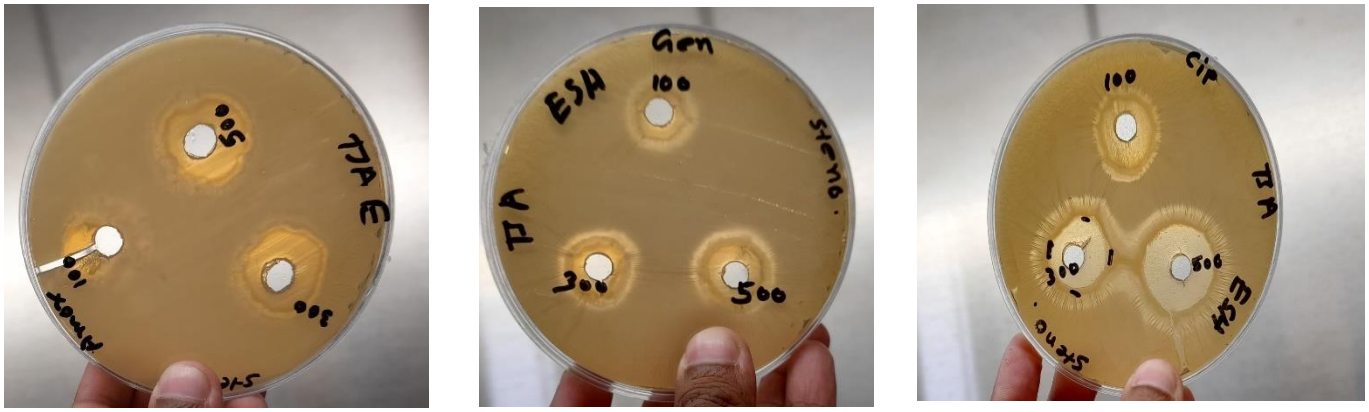


Figure 17: **Well diffusion method of antibiotic susceptibility testing performed for *Stenotrophomonas Maltophilia.***

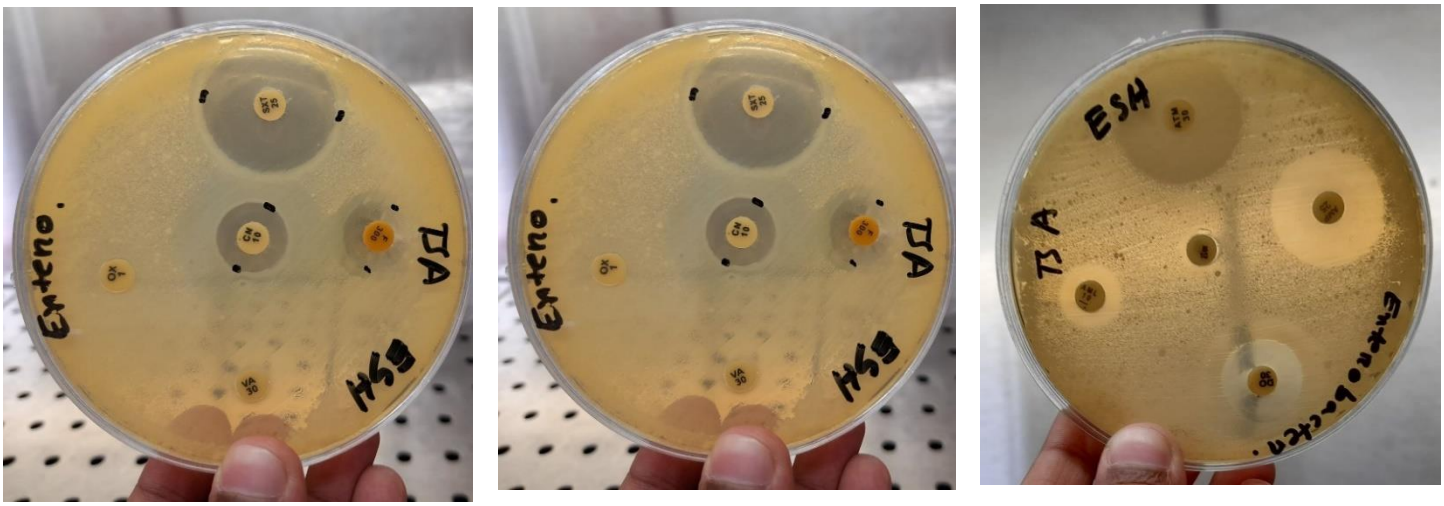


Figure18: Disk diffusion Antibiotic susceptibility testing method performed for *Enterobacter aerogenes.*

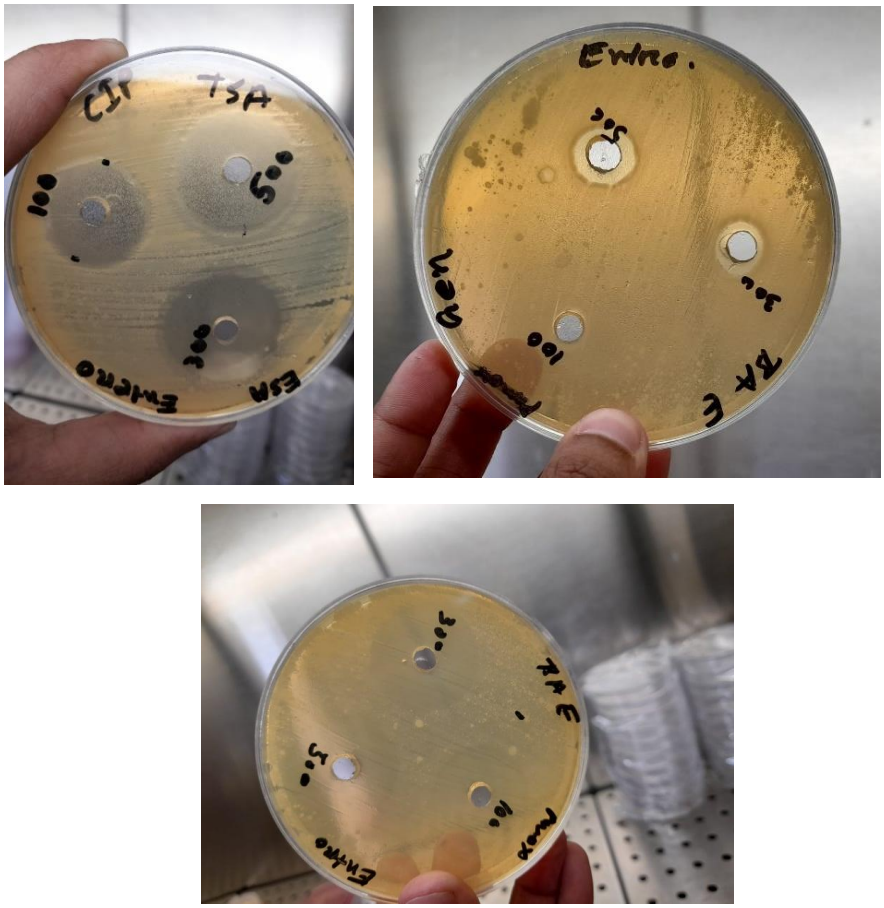


Figure19: Well diffusion Antibiotic susceptibility testing method performed for *Enterobacter aerogenes.*

Chapter 3. Result and Analysis:

Bacterial identification by biochemical test (API 20E):

Figure20: Positive *API 20E result of Stenotrophomonas maltophilia.*

Figure21: Positive API 20 E result of Enterobacter aerogenes.

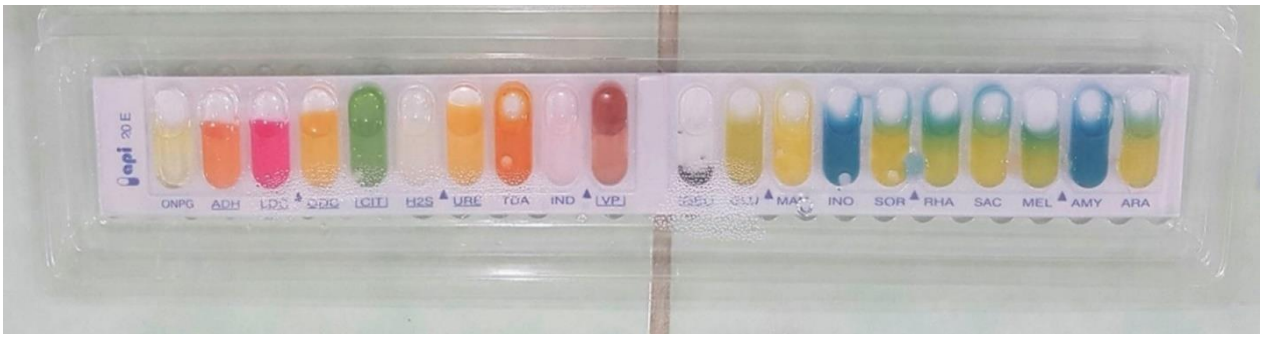


Figure22: Positive API 20E result of E. coli.

Figure 24: Chart of colony Characteristics on selective and non-selective media and API result:

Media	Colony characteristics	Organissms Identified
BSA	Greenish, black centred round and small (sometimes irregular)	*Stenotrophomonas maltophila* 80.5% on API
	Brownish , white peripheral around, medium colonies	*enterobacter aerogenes* 91.4% on API
Sorbitol MacConkey	Pink, big irregular colonies	*Stenotrophomonas maltophila* 96.7% on API
	Small colorless, opaque pink medium colonies	*E. coli*
TSA	White and yellowish colonies (small, Medium and irregular colony formation)	No API

Isolated organisms on Selective media (Bismuth sulfite and sorbitol MacConkey agar):

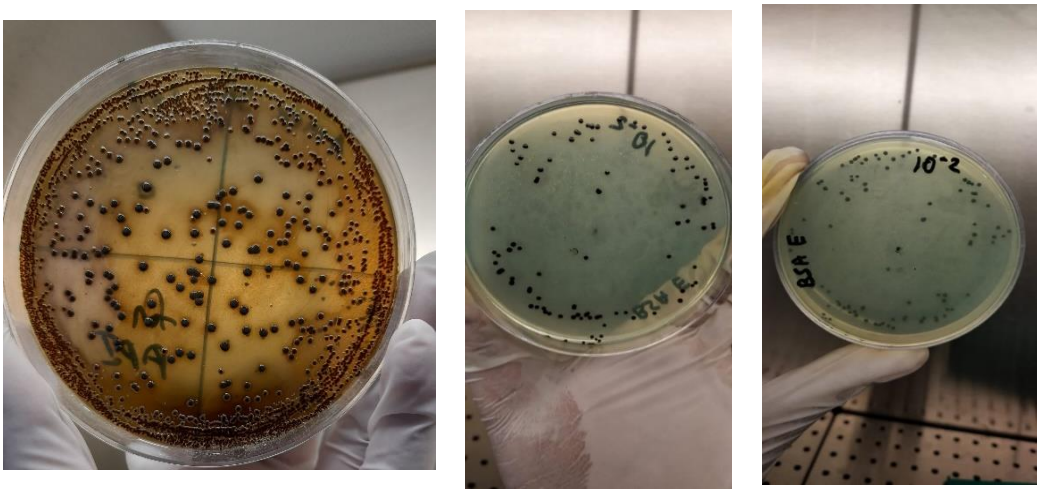


***Figure 23: Enterobacter aerogenes* on bismuth sulfite agar.**

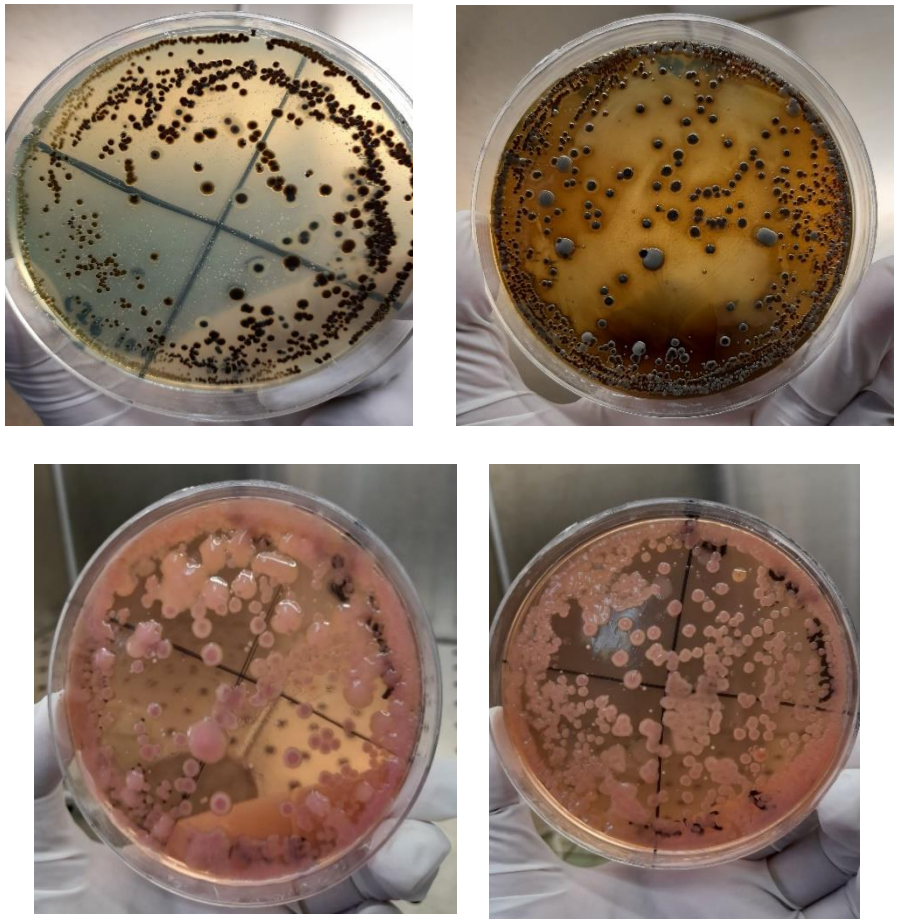


Figure 24: Stenotrophomonus maltophila on Bismuth sulfite and sorbitol MacConkey agar.

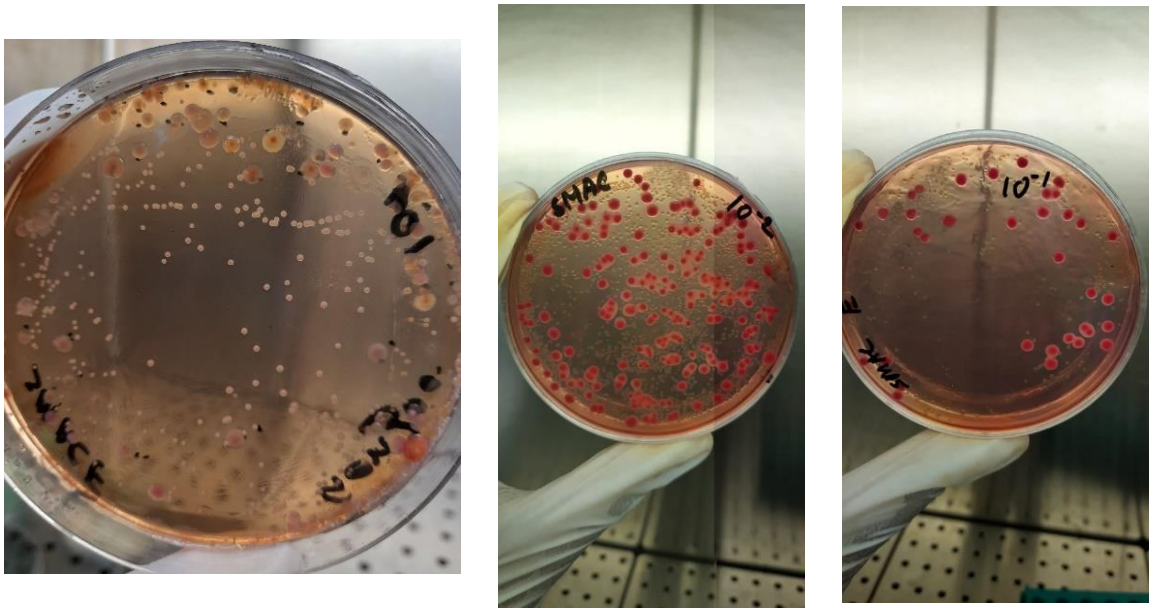


***Figure 25: E. coli* on Sorbitol MacConkey agar.**

3.1. Bacterial identification:

All bacterial colonies grew on selective (BSA and Sorbitol MacConkey, chromocult) and non-selective (TSA) media were observed accordingly, based on their characteristics and appearance on different media. (Figure:24). Colonies from selective media were isolated again and grew on nonselective trypticase soy agar, to perform analytical profile index (API 20 E). Our consideration was to detect gram-negative organisms since they are more frequently found in mung bean sprouts. Gram staining was done to confirm the presence of gram-negative organisms only. API 20 E was used, which is selective for gram- negative bacteria. Saline solution of 5.0 ml was made of each organism isolated from selective media to TSA agar, maintaining 0.5 MacFarland bacterial suspension. All organisms were inoculated into the API 20e strip and incubated for 24 hours. After 24 hours, API 20 e strip exhibited positive results. *Stenotrophomonas maltophili*a acquired from both sorbitol MacConkey agar and bismuth sulfite agar, *E. coli* identified from sorbitol MacConkey and *Enterobacter aerogenes* attained from bismuth sulfite agar.

3.2. The number of colonies in the Control sample of mung sprout:

Microorganisms	(CFU/ml)
TABC	TNTC
TCC	0
E. coli	TNTC
Stenotrophomonas maltophilia	TNTC
Enterobacter aerogenes	TNTC

3.3.Result of Amoxicillin treated mung sprouts: (CFU/ml)

			Amoxicillin (CFU/ml)		
			Antibiotic concentrations		
			100ppm	300ppm	500ppm
Microorganisms					
TABC			8.1×10^7	7.7×10^7	6.2×10^7
TCC			0	0	0
E. coli			7.8×10^7	5.4×10^7	3.9×10^7
Enterobacter aerogenes			6.9×10^7	3.4×10^7	2.7×10^7
Stenotrophomonas Maltophilia			7.3×10^7	3.9×10^7	3.6×10^7

Result of Amoxicillin treated mung sprouts: (LOG CFU/ml)

			Amoxicillin						
			Microorganisms Log (CFU/ml)						
Antibiotic concentration	TABC	TCC	*E. coli*	*Enterobacter aerogenes.*			*Stenotrophomonas maltophilia.*		
100ppm	7.9	0	7.89	7.84			7.86		
300ppm	7.88	0	7.73	7.59			7.65		
500ppm	7.79	0	7.59	7.34			7.48		

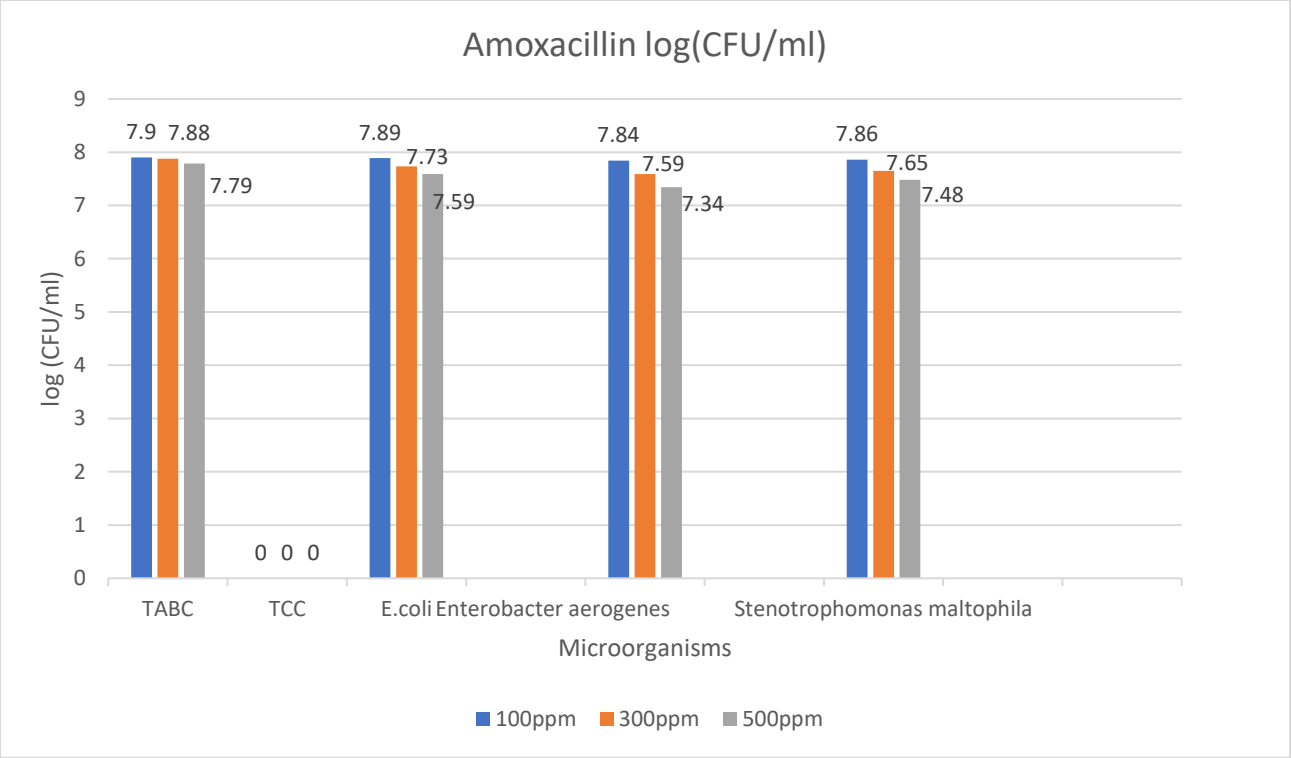


Figure 25: Bar diagram of microbial population of amoxicillin treated mung bean sprouts.

3.4. Result of Gentamicin treated mung sprouts: (CFU/ml)

			Gentamicin CFU/ml		
			Antibiotic Concentration		
			100ppm	300ppm	500ppm
Microorganisms					
TABC			6.8×10^{7}	4.7×10^{7}	8.4×10^{6}
TCC			0	0	0
E. coli			2.4×10^{7}	7.0×10^{6}	0
Enterobacter aerogenes			2.8×10^{7}	9.7×10^{6}	3.5×10^{6}
Stenotrophomonas Maltophila			3.4×10^{7}	1.2×10^{7}	8.8×10^{6}

Result of Gentamicin treated mung sprouts: (LOG CFU/ml)

					Gentamicin Log (CFU/ml)			
Antibiotic Concentration		*TABC*	*TCC*	*E. coli*	*Enterobacter aerogenes*		*Stenotrophomonas maltophilia*	
	100 ppm	7.83	0	7.38	7.45		7.54	
	300 ppm	7.68	0	6.84	6.98		7.07	
	500 ppm	6.92	0	0	6.54		6.94	

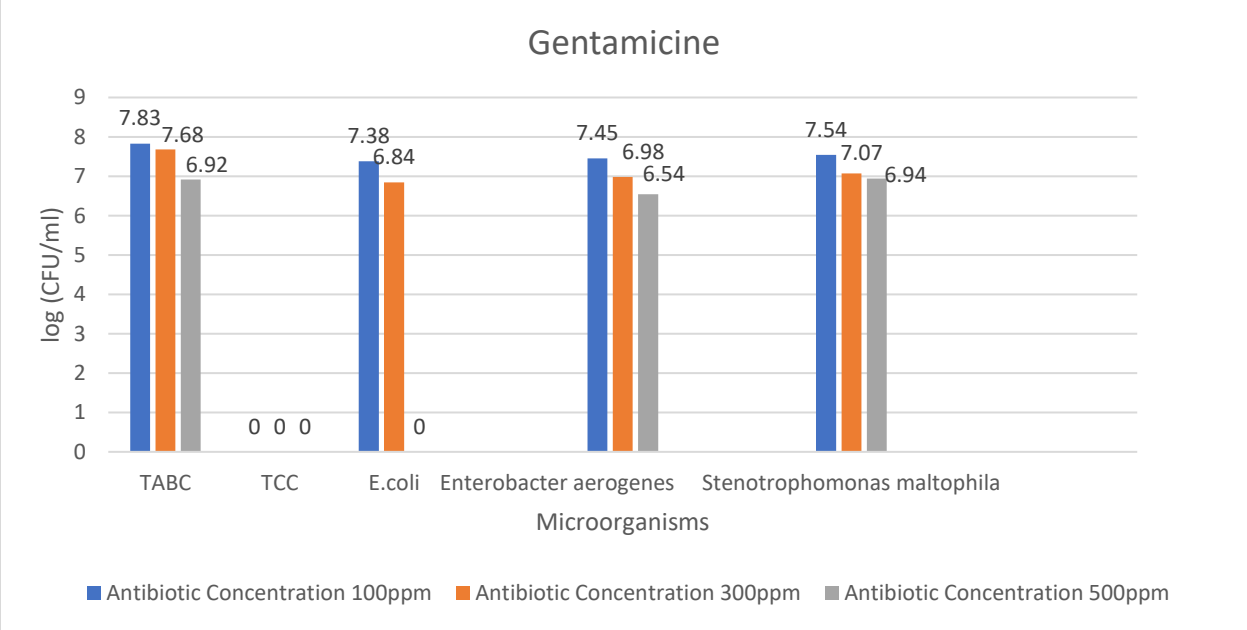


Figure 27: Bar diagram of microbial population of gentamicin treated mung bean sprouts.

3.5. Result of Ciprofloxacin treated mung sprouts: (CFU/ml)

				Ciprofloxacin			
				Antibiotic Concentration			
Microorganisms (CFU/ml)				100ppm	300ppm	500ppm	
TABC				5.5×10^{7}	1.1×10^{7}	4.9×10^{6}	
TCC				0	0	0	
E. coli				8.5×10^{6}	0	0	
Enterobacter aerogenes				9.3×10^{6}	0	0	
Stenotrophomonas Maltophilia				4.5×10^{7}	1.7×10^{7}	9.1×10^{6}	

Result of Ciprofloxacin treated mung sprouts: (log CFU/ml)

				Ciprofloxacin LOG (cfu/ml)					
Antibiotic concentration		*TABC*	*TCC*	*E. coli*	*Enterobacter aerogenes*		*Stenotrophomonas maltophila*		
100 ppm		7.74	0	6.92	6.96		7.65		
300 ppm		7.04	0	0	0		7.23		
500 ppm		6.69	0	0	0		6.95		

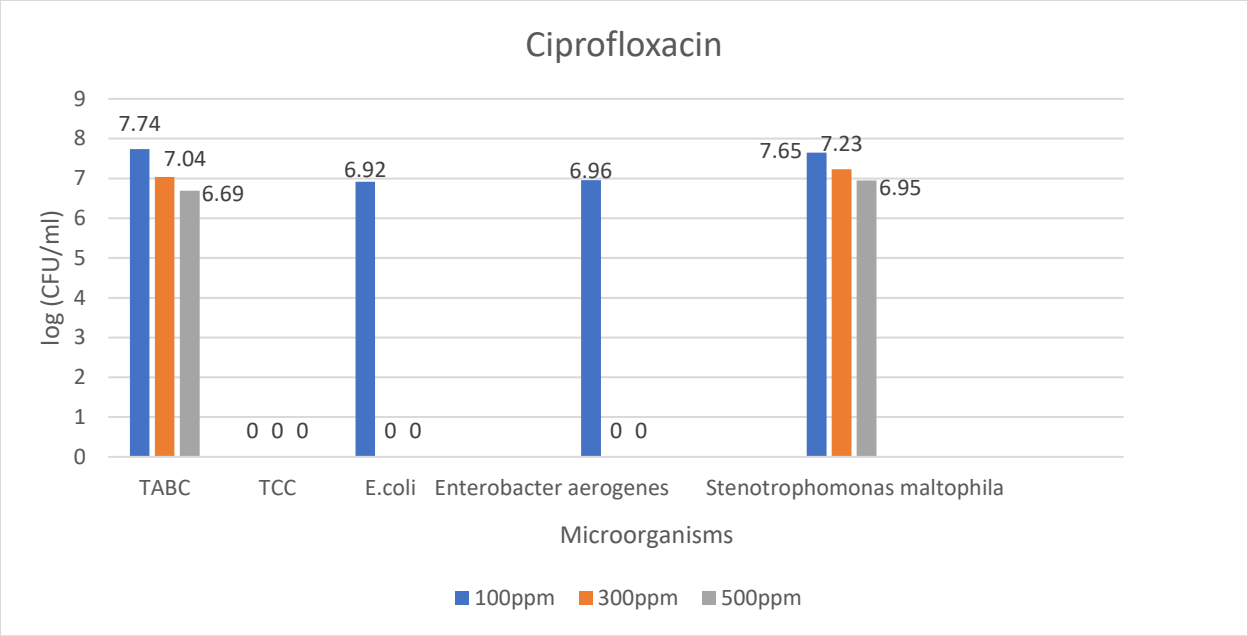


FIGURE 28: Bar diagram of microbial population of ciprofloxacin s treated mung bean sprouts.

3.6. Microbial population screening after treating sprouts with different concentrations of different antibiotics:

The initial total bacterial count of the control sample was too numerous to count.

Sprouts treated with Amoxicillin with three different concentrations did not show any log (CFU/ml) parameter count changes. There was no significant logarithmic decrease found in any concentrations of this antibiotic. All plates showed a high number of growths in both selective and non-selective media. Total bacterial populations on Amoxicillin treated mung bean was approximately 7 log (CFU/ml), in all the antibiotic concentration. (100,300 and 500ppm)

At the concentration of 100 ppm, gentamicin and ciprofloxacin had bacterial population of an average of 7 log (CFU/ml). A gradual decrease of the microbial population was demonstrated after increasing the concentration of both antibiotics at 300ppm to 500ppm. At 500 ppm concentration of gentamicin and ciprofloxacin, the population declined 1.5 log (CFU/ml) on non-selective media;

however, the microbial population was absent on two selective media that had *E. coli* and *Enterobacter aerogenes*. At 300 ppm concentration of ciprofloxacin, there was no microbial growth observed on selective media for *E. coli* and *Enterobacter aerogenes*, and 1 log decrease was noted for gentamicin.

The growth declination of *Stenotrophomonas Maltophila* on selective media was observed similar to microbial colonies on non-selective media. There was no significant declination noted at the concentration of 100 and 300 ppm of gentamicin and ciprofloxacin. When the antibiotic concentration was increased to 500 ppm, 1.0 log (CFU/ml) declination was demonstrated on the selective media plate of *Stenotrophomonas Maltophila* for both antibiotics.

3.7. **Antibiotic susceptibility testing result by disk diffusion method (mung bean sprouts were treated with amoxicillin, gentamicin, ciprofloxacin at 100,300 and 50ppm)**

		Bacteria isolated from antibiotic treated mung bean.			
Antibiotic Names and concentration		Resistance pattern of *Stenotrophomonas Maltophilia*		Resistance pattern of *Enterobacter aerogenes*	
		(diameter =mm)	R>I>S	(diameter =mm)	R>I>S
C	30 µg	18	I	16	I
E	15 µg	14	I	6	R
K	30 µg	6	R	6	R
CIP	5 µg	12	I	6	R
TE	30 µg	6	R	18	S
DO	30 µg	8	R	17	S
AMP	25 µg	6	S	20	S
ATM	30 µg	6	R	6	R

B	10 µg		6		R	6		R
AML	10 µg		6		R	6		R
VA	30 µg		12		R	6		R
OX	10 µg		13		R	6		R
CN	10 µg		6		R	6		R
SXT	25 µg		27		S	16		I
F	30 µg		6		R	6		R

R=resistant, I= intermediate, S= sensitive

The zones of inhibition were measured and recorded and interpreted as susceptible (S), intermediate (I) or resistant (R) based on Clinical and Laboratory Standards Institute (CLSI) (CLSI,2016).

3.8. Antibiotic susceptibility testing result by well diffusion method (mung bean sprouts were treated with amoxicillin, gentamicin, ciprofloxacin at 100,300 and 500ppm)

				Bacteria isolates treated with antibiotic					
				Resistance pattern of			Resistance pattern of		
Antibiotic Names		Concentration		*Stenotrphomonas maltophila.*			*Enterobacter aerogenes*		
		100 ppm		6	(mm)	R	6	(mm)	R
Amoxicillin		300 ppm		6	(mm)	R	6	(mm)	R
		500 ppm		6	(mm)	R	6	(mm)	R
		100 ppm		6	(mm)	R	6	(mm)	R
Gentamicin		300 ppm		6	(mm)	R	6	(mm)	R
		500 ppm		6	(mm)	R	6	(mm)	R
		100 ppm		6	(mm)	R	11	(mm)	I
Ciprofloxacin		300 ppm		16	(mm)	I	17	(mm)	S
		500 ppm		21	(mm)	S	25	(mm)	S

R=resistant, I= intermediate, S= sensitive

3.9. Antibiotic susceptibility result by disk diffusion method (used commercial antibiotic disks) of antibiotic untreated mung bean sprouts sample:

					Bacteria isolates without antibiotic treatment			
Antibiotic Names and concentration				Sample control		Sample Control		
				Stenotrophomonas maltophilia		*Enterobacter aerogenes*		
				(diameter=mm)	R>I>S	(diameter =mm)		R>I>S
C	30 µg			25	S	22		S
E	15 µg			10	R	9.5		R
K	30 µg			6	R	8		R
CIP	5 µg			18	I	24		S
TE	30 µg			12	I	21		S
DO	30 µg			13.8	I	19		S
AMP	25 µg			26	S	23		S
ATM	30 µg			6	R	8		R
B	10 µg			17	S	6		R
AML	10 µg			10	R	6		R
VA	30 µg			15	S	13		R
OX	10 µg			11	R	16		I
CN	10 µg			9	R	20		S
SXT	25 µg			6	R	15		S
F	30 µg			6	R	13		I

R=resistant, I= intermediate, S= sensitive

The zones of inhibition were measured and recorded and interpreted as susceptible (S), intermediate (I) or resistant (R) based on Clinical and Laboratory Standards Institute (CLSI) (CLSI,2016).

3.10. Antibiotic susceptibility result of antibiotic untreated mung bean sprouts (Concentration used for treatment of mung bean sprouts):

					Bacteria isolates without antibiotic treatment				
Antibiotic Names		Concentration		Resistance pattern of *Stenotrophomonas maltophilia*			Resistance pattern of *Enterobacter aerogenes.*		
		100ppm		10	(mm)	R	8	(mm)	R
Amoxicillin		300ppm		11.5	(mm)	R	13	(mm)	I
		500ppm		16.4	(mm)	I	22	(mm)	S
		100ppm		11	(mm)	R	12.8	(mm)	I
Gentamicin		300ppm		14	(mm)	I	17	(mm)	S
		500ppm		19	(mm)	S	24	(mm)	S
		100ppm		14.7	(mm)	I	17	(mm)	S
Ciprofloxacin		300ppm		20	(mm)	S	19	(mm)	S
		500ppm		23	(mm)	S	27	(mm)	S

R=resistant, I= intermediate, S= sensitive

3.11. Result of antibiotic susceptibility of treated and non-treated mung bean sprouts:

Antibiotic susceptibly check was done in two methods, disk diffusion and well diffusion, using fifteen commercially available antibiotic disks of different concentrations and the concentration we treated the mung bean sprouts.

Untreated sample control showed greater sensitivity of *S.maltophila* and *Enterobacter* towards ciprofloxacin at the concentrations we used to treat mung bean sprouts. Untreated *S.maltophila* showed resistance towards Amoxicillin at 100 ppm and 300ppm, also gentamicin at 100ppm; it may be because of its inherited history of great multidrug resistance capacity. *Enterobacter aerogenes* had intermediate growth at the concentration of 300ppm and sensitive at 500ppm of gentamicin, including amoxicillin. It was resistant at 100ppm of amoxicillin.

Untreated mung bean sample showed mixed result against commercially available antibiotic discs. *Stenotrophomonas maltophila* showed resistance against seven antibiotic discs and *Enterobacter* showed antibiotic resistance against five antibiotics.

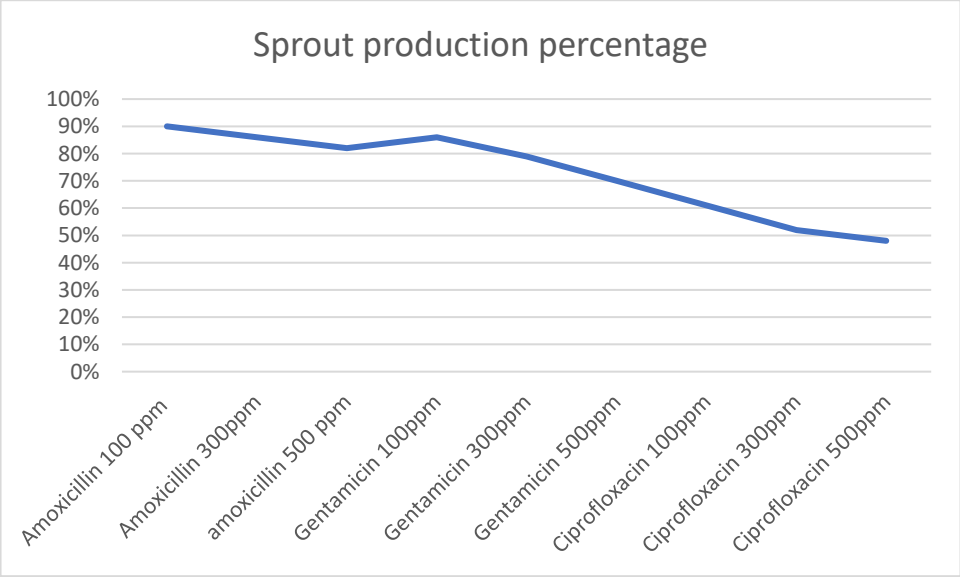
Antibiotic treated mung bean sprouts organism achieved resistance against amoxicillin and gentamicin at every concentration, used to treat the sprouts. *Stenotrophomonas maltophila* earned resistance against ciprofloxacin at 100ppm, and sensitive towards others. *Enterobacter aerogenes* showed sensitivity towards all concentrations of ciprofloxacin used to treat the mung sprouts.

Most of the antibiotic treated samples also showed resistance against commercially available antibiotic discs. *Enterobacter* was sensitive against tetracycline, doxycycline, ampicillin and *Stenotrphpmonas maltophilia* was only sensitive towards ampicillin and trimethoprim-sulphamethoxazole.

3.12. Percentage of sprouts:

List of antibiotics		Germination percentage		
Amoxicillin 100 ppm		90%		
Amoxicillin 300ppm		86%		
amoxicillin 500 ppm		82%		
Gentamicin 100ppm		86%		
Gentamicin 300ppm		79%		
Gentamicin 500ppm		70%		
Ciprofloxacin 100ppm		61%		
Ciprofloxacin 300ppm		52%		
Ciprofloxacin 500ppm		48%		

Germination and sprout formation was gradually decreased by increasing the concentration of antibiotics. Sprout's production in every concentration of amoxicillin was acceptable, highest concentration of gentamicin has less production percentage of sprouts. Sprout's production was critically affected by ciprofloxacin. From Lowest concentration to highest concentration ciprofloxacin inhibited germination and sprout formation.



3.13. Bacterial count from sample seed

Organisms	Bacterial count (Log CFU/ml)
TABC	5.55
E. coli	5.38
Salmonella enterica	A

Initial organism count from raw seed was taken, TABC was lower than the result of TABC after sprouts, no salmonella was detected. However, *E. coli* was found. Throughout the whole process, distilled water was used from washing seeds to prepare antibiotic solution with distilled water for germinating seeds and growing sprouts using it. Antibiotic solution was added to sprouts with pipette, foil paper and glasses where sprouts were grown were sterilized with distilled water. No possible contamination of *Enterobacter aerogenes or other pathogens* can arise during sprouting production. *Enterobacter* and other specified bacteria found later after sprouting must be resided withing the internal particles of mung seed and displayed during sprout production after getting proper nutrients during germination

Chapter 4: Discussion:

The objective of this study was to observe the consequences of applying antibiotics directly on the hydroponically grown salad item mung bean and risk associated with antibiotic-resistance of residing organisms in mung bean.

People prefer to eat sprouts which is a germinated form of seeds, ready to eat item either eaten as raw or cooked. Mung bean sprout is a very popular salad item and side dish in many other countries because of their availability throughout the year, rich in enzymes and nutritional value, and easy to grow in home environment and industry. (18) Sprouting needs water to grow, this water must be contamination-free. many industries do not monitor in which environment and which water is used to grown sprouts. sometimes seeds are being contaminated because of soil and the farm where they are cultured and culture. (1) During the seed cultivation process, mung bean may get contaminations. To reduce this contamination many scientists represented many methods in their study how effectively contamination can be eliminated. It is assumed that those methods are followed by industry and commercially grown mung bean sprouts. Although, contamination and disease-causing pathogens have already caused many outbreaks and health hazards related to mung bean sprouts. People who eat sprouts exposed to *E. coli* 0157:H7 or *salmonella* are at a risk to get diseased. (19)

In 2016 May. thirty-two cases were reported by 11 states and federal public health officials, an outbreak of salmonella braenderup linked to consumption of mung bean sprouts served at restaurants. On August 13, 2014, FDA isolated listeria monocyrogenes from mung bean sprouts and sprout irrigation water samples. Around 2000-2002, six *Salmonell*a infection outbreaks associated with mung bean sprouts were identified in Canada (February 2001), Netherlands also in the United States. (20) Five outbreaks linked to Vietnamese and Thai cuisine were occurred due to not following the guidelines of FDA seed disinfection. (20). Two investigations found out; outbreak strains of *salmonella* were also found in environmental specimens like irrigation water. The only prior outbreaks we identified worldwide in which mung bean sprouts were identified as the cause of *Salmonella enterica* outbreaks occurred in England and Sweden in 1988(1). A survey of Thailand revealed contamination of *salmonella enterica* is very common and about 9%. Only

Salmonella enterica is associated with most of the outbreaks but no other serotypes are notable. In most of the cases of the outbreak, the seed was been contaminated and no evidence found contamination from either the sprout growers or industrial employees. Contamination of seeds results from growing them in fields fertilized with untreated manner. (23)

This is why many scientists are giving importance to develop a good disinfectant method that can inhibit the pathogen without damaging seed and germination factors. Wrinkled seed may contain a high concentration of microbial flora with pathogens. Microbial flora and pathogens can be internalized in sprouts during sprouting as a result external cleaning of sprouts will not eliminate the microbial population. (12) Sprouts production requires temperature with high humidity, during sprout formation many nutrients get released and this becomes a suitable environment for microbial growth like Salmonella *and E. coli* can increase their population to 2.0 to 3.0 logs after sprouting. (16)

Many country use antibiotics to eliminate disease causing pathogens from salad item or freshly cut vegetables legally or illegally. There is a report found, Thailand mostly uses antibiotics on hydroponically grown vegetables to reduce organisms load however, there is a big issue relating antibiotic resistance is also noted in commensal organisms within vegetables. (17)

Though antibiotic use on crops is prohibited in many countries, concerning the health issue regarding antibiotic resistance, this study aims to find out how antibiotics effects on mung bean sprouting and leading commensal bacteria resistant to treated antibiotics.

For this study. three different antibiotics were chosen Amoxicillin, Ciprofloxacin, and gentamicin based on their three different modes of action. Antibiotics are classified according to their mode of action, generation and coverage. Aminopenicillin and aminoglycosides are noted before use on crops though, fluoroquinolones are not reported to be used on vegetable crops. (9). Three different concentrations were also taken of each antibiotic because in vegetable farms, growers are not given guidelines to follow any concentration and how to use antibiotics over crops. They are being told that antibiotic inhibit food-borne pathogens and it increases the shelf life of freshly cut vegetable items. The reason for taking their different concentration to see at the performance of antibiotics at minimum 100ppm to maximum 500ppm concentration and at which concentration organisms get resistant to antibiotics.

Mung bean was collected from the local market. It was washed with distilled water before it was used for sprouting. Sterilized plastic cups were prepared for the germination. There were ten working samples of three-gram mung bean seed, nine of them are prepared for antibiotic treatment and one was a control sample. Seeds were germinated into 100ppm,300 ppm and 500 ppm of amoxicillin, gentamicin and ciprofloxacin antibiotic prepared in distilled water solution. After the growth of sprouts, they are soaked with antibiotic solution slightly up to seven days until they are harvested. Control sample sprouts were grown using distilled water without any antibiotic treatment. All this preparation and treatment were done under biosafety cabinet with full safety management. After harvesting all the samples. they were ready for microbiological preparation and platted over TSA, BSA. Chromocult and Sorbitol MacConkey agar. TSA agar was selected to see the difference of total aerobic count in sample sprouts and antibiotics-treated sprouts. Bismuth sulfite agar and sorbitol MacConkey were selected to see the presence of *E. coli and salmonella enterica*, gram-negative food borne pathogen.

After platting the sprout samples over media, organisms were detected through API 20 E biochemical test and gram staining. In gram staining all organisms was discovers as gram negative rods. Organisms identified were *E. coli*, *Stenotrophomonas maltophila*, *Enterobacter aerogenes. E. coli* was collected from sorbitol MacConkey agar, *Stenotrophomonas maltophilia* was isolated from both BSA and sorbitol MacConkey media and *Enterobacter aerogenes* isolated from BSA plates.

Amoxicillin is a third-generation antibiotic under the penicillin group. Few studies stated using antibiotic of penicillin groups over crops. Under amoxicillin treatment of sprouts, there was no significant decrease was noted in total aerobic count plates in three different concentrations. Even the organisms of selective plates shown a large number of the microbial population. There was no gradual logarithmic decrease noted in the microbial population in different concentrations of amoxicillin-treated sprouts. Amoxicillin failed to inhibit any organisms from both selective and nonselective media. In antibiotic susceptibility test at concentration 100 ppm and 300 ppm, untreated mung bean sprout sample organisms showed resistance, zone of inhibition was shown only by Enterobacter aerogenes at the highest concentration of amoxicillin and intermediate growth shown by *Stenotrophomonas melophilia*. Treated sprouts sample organisms showed resistance against amoxicillin at every concentration. Organisms found in the control sample

exhibited resistance at a lower and moderate concentration of amoxicillin must get their resistance gene before at any point. Many antibiotic-resistant bacteria have also been isolated before from irrigation water, soil amendments, and soil and this resistance came from many environmental factors like antibiotics used for human beings can also reach agricultural soil by wastewaters and surface water. Applying amoxicillin to treat mung bean sprout displayed no elimination of organisms, remained ineffective however, lead antibiotic resistance of the organisms. At the highest concentration 500 ppm, treated sprout organisms got resistant to amoxicillin. Mung bean Sprout's growth was well achieved and unaffected by Amoxicillin treatment.

Gentamicin is a broad-spectrum antibiotic and it is reported used over vegetable crops before. Treatment of gentamicin reduced total aerobic count 1.0 log at the highest concentration 500ppm however, not so significant but a gradual reduction of TABC was seen from 100ppm to 300 ppm. *E. coli* was inhibited at the highest concentration, reduced 1.0 log at moderate concentration and similar growth control sample at 100 ppm. *Enterobacter aerogenes* decreased 1.0 log CFU/ml at 100 ppm to 300 ppm but not inhibited completely, also showed lower growth at 500 ppm. Stenotrophomonas reduction was similar to TABC, 1.0 log CFU/ml decrease was noted at 500 ppm. All the treated sprout organisms showed resistance to every concentration of antibiotic used for treatment 100,300 and 500 ppm. Untreated sample organism Enterobacter showed sensitivity and intermediate growth to gentamicin at minimum to higher concentration 500ppm, *Stenotrophomonas maltophilia* resulted gradual reduction, resistance to lower concentration 100ppm and sensitivity to highest concentration 500ppm. gentamicin was successful in inhibiting *E. coli* at higher concentration, reducing *Enterobacter and E. coli* from moderate concentration, *Enterobacte*r was not fully inhibited however TABC and *Stenotrophomonas maltophila* only seen reduced at the higher concentration. Lower and moderate concentrations have negligible effect to eliminate total aerobic bacterial count. This antibiotic also is not a good alternative for eliminating many pathogens, only can work on one or two, however, making organisms resistant towards antibiotic from minimal concentration 100ppm to maximum 500ppm. Germination remains unaffected and sprouts growth was good at concentration 100 and 300 ppm, however, decreased at 500ppm.

Ciprofloxacin is a broad-spectrum second-generation fluoroquinolone antibiotic, used to treat different types of bacterial infections associated with gram-negative bacteria. It is not reported before using on any vegetable or crops. It was chosen for a different and sensitive mode of action which is associated with stopping DNA replication, transcription and repair. Mung bean sprouts treated with Ciprofloxacin eliminated *E. coli and Enterobacter aerogenes* at moderate and higher concentrations. (300,500ppm) however, total aerobic bacterial count and *Stenotrophomonas maltophila* was noted 1.0 log CFU/ml decrease at the highest concentration 500 ppm and a gradual decrease from 100ppm to 300 ppm. Organisms from untreated sample expressed sensitivity towards every concentration of antibiotic treatments. Nevertheless, *Enterobacter* from the treated sample was sensitive to 300 and 500 ppm , intermediate growth at lower concentration 100 ppm, *Stenotrophomonas maltophila* was sensitive at 500 ppm, and intermediate growth is shown at 300 ppm and got resistant at 100 ppm. Ciprofloxacin showed effectiveness at moderate and higher concentrations eliminating pathogenic bacteria however, it did not show a greater decrease of aerobic bacterial count from moderate concentration and also *Stenotrophomonas maltophila*. These organisms of total aerobic bacterial count may be the residing microflora of mung bean sprout or also can be *Stenotrophomonas maltophilia* which were not possible to eliminate properly using ciprofloxacin. Treating with ciprofloxacin also exhibited antibiotic resistance to *S. maltophilia* and intermediate growth on *Enterobacte*r at lower concentration 100ppm can extrapolate that, as a newly administrated antibiotic it already had exhibit resistance at the lower concentration, long term use of this antibiotic may result in greater resistance to higher doses also. Germination rate was poor than other antibiotics in fact it also delayed the germination of sprouts.

As per many findings, *Stenotrophomonas malthophilia* is an emerging multi-drug-resistant global opportunistic organism. It is a gram-negative aerobic bacterium that previously was under the pseudomonas group. They are motile, well grown in MacConkey agar show big flat pigmented colonies. It is an environmental bacterium found in watery habitants, plant rhizospheres, animals, foods, and water sources. It is also commonly found in cruciferous plants and beneficial for plant growth stimulating siderophore, indole acetic acid and inorganic phosphate solubilization. It is also quite dominant in the rhizosphere of cereal crops. It can persist under the tissue of plants and is found as an effective biocontrol in controlling fungal and oomycetes plant pathogens. *S.*

maltophilima may have a synergistic interaction with mung seed, maybe be an opportunistic organism, may get inside the seed due to contamination, or how it came to mung bean is unidentified. Further study is required to identify the relation between *Stenotrophomonas maltophilia* and mung bean sprout, whether it is also a commensal organism and beneficial for mung bean or it is pathogenic. There are several numbers of microflora found from mung bean and many are unidentified, which becomes misleading. Due to the high number of microflorae inside mung bean, pathogenic organisms from mung bean sometimes get laborious to isolate. Thus it is found in this study, *S. maltophila* has been reported to cause many nosocomial diseases though it is not yet broadly consider as a pathogenic organism or human health. It can cause respiratory infection in the patient having cystic fibrosis and chronic lung disease. *S. maltophilia* exhibits resistance to a broad array of antibiotics including, β-lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins, as a result in this study, there was also no gradual reduction found from any antibiotics at different concentrations. Growth of *Stenotrophomonas maltophilia* was higher comparing to other organisms, did not get eliminated rather inherited antibiotic resistance, resulted in antibiotic susceptibility test.

The intention of this study was to perform antibiotic treatment in the production of mung bean sprouts to determine how antibiotics work on eliminating pathogens or commensal bacteria, majorly if it leads to persist commensal organisms to antibiotic resistance. After performing the antibiotic treatment, it is anticipated that the beta-lactamase penicillin group antibiotics amoxicillin and aminoglycoside group failed to reduce the number of pathogenic and commensal bacteria from the total aerobic bacterial count in a satisfying number. There was a gradual decrease of organisms we can see from the result but that is not significant enough to kill pathogenic organisms, rather they result in organisms getting resistant at minimal concentrations to maximum concentrations. Gentamicin only could inhibit *E. coli,* and no other bacterium. If one antibiotic inhibits a few among many that cannot be a suitable alternative to be used. Rather killing other organism, they may persist in the genes of existing organisms. Ciprofloxacin is introduced newly over the crops; it can only decrease the number of multi-drug-resistant *S. maltophila* at its higher concentration but cannot inhibit it and it was successful to kill *E. coli* and Enterobacter from medium

concentration to higher concentration. However, being a newly presented antibiotic to plants organisms shows resistance at its minimal concentration of 100 ppm. It is understood that if it is being used for long-term organisms may get resistance to other concentrations within a few years. Moreover, it has shown an adverse effect on germination. it lowers the germinating percentage to 50%. Sprouts with seed started blackening from day four and started to die off. On day seven it seems ciprofloxacin-treated sprouts were not edible, not in a state of harvesting. Among many, very few sprouts were left were edible. It may inhibit pathogen but has a serious effect associated with seed's biochemical formation. blackening and mortification may be a result of reduced porosity, evaporation and overall hydrological mixing. Fluoroquinolones inhibit DNA synthesis of organisms, gradually they may also interact with the plant DNA and stop the growth. A method that affects the germination process of a plant is not suitable anyway to use.

Conclusion:

Antibiotic use does not effectively suppress microbial populations, and a high percentage of microorganisms develop antibiotic resistance. Since, antibiotics are not an effective method that can be used to inhibit pathogenic microbes in mung bean sprouts and hence, the use of antibiotics in freshly eaten vegetable leads to persistent of MDR commensal bacteria on hydroponically grown mung bean sprouts. This investigation urges the necessity for effective antibiotic management in order to prevent the development of multi-antibiotic resistance bacteria through the consumption of fresh vegetables.

Recommendations:

Since antibiotic use is banned over crops in many countries, whereas, middle-income countries and many other well-known countries are distinguished using antibiotics over crops illegally without following any guidelines and they also are unaware of proper concentration to use. There are very little data can be found about the usage and effectiveness of antibiotics over crops to reduce pathogenic organisms. This study can be an eye-opening to those whoever is aiming to use antibiotic over mung bean sprouts to prevent foodborne illness in near future, or using antibiotics over any other vegetables that are eaten freshly with or without little cooking. Salad items are not

cooked and freshly eaten by humans, antibiotic-resistant genes of any organisms may interpret with the human genome and modify. Cooking may inactivate the antibiotics on foods, but for salad items cooking is not a way to eat.

Antibiotic residues and resistant bacteria are found from many environmental factors, such as swage water, soil, surface water, drinking water, vegetable, and many more objects. These antibiotics are exposed to the environment willingly or unwillingly without proper monitoring. Many antibiotics are used over human disease, veterinary medicine, and agriculture resulting in resistance of antibiotics at the environmental stage. The constant rise of antibiotic resistance has a negative ecological effect and resistance genes can be transferred again to humans; serious illness will be hard to treat. Introducing antibiotics over crops may cause serious environmental hazards.

Scientists should find effective alternatives, other than using antibiotics to kill pathogens from freshly producing vegetables.

Limitations:

Other examination is required to confirm the persistence of antibiotic-resistant developed gene in organisms via PCR testing, bacterial identification by 16s rDNA sequencing, and identifying antibiotic residue needs sample extraction or high-performance liquid chromatography analysis. Other methods have to perform to give more clarification about this study, due to the lack of technology in the laboratory and shorter time, it was not possible to perform these experiments. Only three different concentrations and three different groups of antibiotics were taken initially, in further work, taking diversified antibiotics and concentrations will help more to know about their effect on pathogens and commensal organisms. This study focused on finding two disease-causing organisms *E. coli 0157;h7 and salmonella* , previously responsible for mung bean outbreaks. Salmonella was not found besides other organisms were detected. Future studies may focus on isolating more organisms by using different media and biochemical tests, and also their activity against different antibiotics. Due to lack of time and during the 2nd lock down was announced, this is why antibiogram of *E. coli* was not possible to perform.

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