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Antibiotic Resistance of Bacteria Isolated from Soils

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ANTIBIOTIC RESISTANCE OF BACTERIA ISOLATED FROM SOILS

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By

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ABSTRACT

After the discovery of antibiotics, antibiotics have been increasingly implemented into human and veterinary medicine. In addition, antibiotics are inserted into animal feed for non-therapeutic purposes, which potentially leads to the development of antibiotic-resistant pathogens. When the livestock excrete waste onto the soil, the antibiotic resistant bacteria are introduced to the environment. With soil collected from different farms throughout the Bowling Green area, the microbial communities were analyzed to determine its bacterial compositions and their resistances to common antibiotics through a modified agar dilution technique. Once resistant colonies were isolated, they underwent more testing to determine if the colonies could be potential pathogens and express multidrug resistance. Through these methods, possible pathogenic enteric bacteria were identified. While the soils did show increased amounts of antibiotic resistant bacteria because of livestock fecal matter, this rise in antibiotic resistant bacteria does not necessitate an immediate public health concern because the soil bacteria would need to be either ingested or introduced into the bloodstream to produce an infection. However, further research is needed to identify the antibiotic resistant strains to determine if the strains had any chance of infecting humans.

Keywords: Antibiotic Resistant Bacteria, Enteric Bacteria, Fecal Coliforms, Soil, Livestock, Antimicrobial Resistance Genes

I dedicate this thesis to my parents, advisors, and friends whose support I relied upon while researching and writing.

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CHAPTER 1

INTRODUCTION

Throughout the United States' history, meat shortages posed a major problem to both citizens and the government. Before World War I, the United States suffered a meat shortage of 18 million heads of livestock, which prompted an increase in animal science research to solve the problem (1). Similarly, during World War II, the United States government appropriated a disproportionate amount of the country's meat to servicemen, and this resulted in a meat shortage in the mainland (2).

When Vitamin B_{12} was identified as a cure for pernicious anemia, it was also thought to offer a solution to the meat shortage (3). When testing this assumption, scientists from Lederle discovered that when chickens ate B_{12} supplements that contained aureomycin residues, the chickens grew faster while experiencing less disease (4). In addition to causing faster growth and less illnesses, antibiotics were not shown to affect meat quality (5). Thus, the administration of antibiotics to the livestock helped solve the frequent meat shortages.

Since the discovery and administration of antibiotics, the practice of supplementing animal feed with antibiotics has greatly increased. In the Federal Drug Association's most recent report on antimicrobials used for food-producing animals, 13.98 million kilograms of antimicrobials were approved for use (6). Furthermore, domestic sales showed that 40% of these approved antimicrobials did not have a

therapeutic purpose (6). Because of this high usage, the FDA is attempting to limit the number of antimicrobials used for livestock due to the possibility of antimicrobial resistant bacteria (ARBs) becoming more prevalent (7).

The reason for concern about rising numbers of ARBs is because the scale for antibiotic resistance depends upon the scale of antibiotic manufacture, so more antibiotic usage selects for ARBs (8). Innately, soil bacteria produce antimicrobial substances to inhibit their competitors' growth, and pristine locations that are isolated from human contact display very small levels of antibiotic resistance (9, 10). Furthermore, the production of some of these substances are only expressed when the bacteria receive signals from the environment or from other microbes (11). Antimicrobial resistance (AMR) genes have been in existence for at least 30,000 years based upon an analysis by d'Costa et. al of Beringian permafrost sediments, so AMR genes predate the use of therapeutic antibiotics (12).

While using antibiotics can work therapeutically, they also select for antimicrobial resistance (AMR) genes that promote resistance to their own action (13). Because bacteria can replicate quickly, this results in a high level of genetic plasticity, so bacteria can adapt to their environments in a short time frame (14). Furthermore, through horizontal gene transfer, bacteria can transfer the AMR genes in plasmids through transformation, transduction, and/or conjugation to different species of bacteria and increase the abundance of ARBs (15).

Through the introduction of antibiotics in livestock feed, enteric bacteria within the livestock can select for AMR genes. Before the high usage of antibiotics, enteric bacteria have been minimally resistant to antibiotics because they lacked the antibiotic-

producing competitors that are present in soil ecology (8). Now, AMR genes are needed to increase the likelihood that the enteric bacteria can survive (8).

Through the fecal matter of livestock, enteric ARBs can be introduced to the environment (16). The presence of these enteric ARBs can be identified if fecal coliforms are found (17). Fecal coliforms are Gram-negative bacilli, oxidase negative, facultative anaerobic, non-sporulating, and lactose fermenting bacterium (17). Common genera of fecal coliforms include *Escherichia, Klebsiella, Enterobacter,* and *Citrobacter* (18).

Despite coliforms indicating the presence of fecal contamination, they are not usually harmful to humans (18). However, if found in the environment, their presence can indicate that other harmful pathogens may also be present (18). Because coliforms are easy and quick to test, testing for coliforms is the simplest way to determine if potentially pathogenic bacteria are in the environment and could raise a public health concern (18).

While no ARB outbreak has been associated with farm soils, these fecal coliforms from livestock have been identified in several locations and have shown multiple resistances to common antibiotics (14). Several studies have shown increased ARBs on vegetables if the soil is fertilized by animal manure (19-21). Also, in a comparison between several soil sites, dairy farm soil, along with soil from hospitals that administer high dosages of antibiotics and from gardens that used pesticides and herbicides, displayed the highest levels of ARB (16). In addition to cattle farms, poultry farms have shown an increase of ARBs in poultry litter (22). Another type of livestock that has shown the presence of ARBs is swine when multidrug resistant *E. coli* was discovered in swine farms in four European countries (23). Thus, ARBs are being introduced to the soil through the fecal matter of several different types of livestock.

To determine the levels of ARBs present in different soil environments, the Prevalence of Antibiotic Resistance in the Environment (PARE) Project has provided resources for students to study ARB in soils throughout the United States. Based upon their database, no ARB levels have been recorded in southcentral Kentucky. Through this lack of ARB identification, this project will focus on discovering the prevalence of ARBs around Bowling Green, Kentucky.

While the PARE Project only identifies if ARBs are present, this experiment will expand upon these concepts by also testing for enteric bacteria and multidrug resistance. By sampling soil from local cattle farms, the scope of this experiment was limited to cattle for better comparisons between samples rather than comparing multiple types of livestock. With these samples, bacterial resistance to tetracycline was tested because this is the antibiotic that is most in cattle (24). Using MacConkey media to select for enteric bacteria, the resulting colony growth would indicate the number of potential enteric ARBs present in the soil.

For the next part of the experiment, the potential pathogenicity of these bacteria were determined by their ability to grow at human body temperature by growing in an incubator set at 37°C. The colonies that grew and survived under these conditions were tested for multidrug resistance. The results of these steps suggested the possibility of multidrug resistant opportunistic pathogens being introduced to the soil environment through cattle fecal matter. Based upon these results, soil ARBs increase due to high antibiotic levels in fecal matter, but they do not pose an imminent public health concern due to the low probability of ARBs being opportunistic pathogens and due to the undertaking of proper sanitary precautions when handling soil.

CHAPTER 2

MATERIALS AND METHODS

2.1: Soil Collection and Serial Dilutions

For the farm soil collections, four cattle farms located around Bowling Green, Kentucky, gave consent for their soil to be sampled. At each location, two samples were taken several feet apart to account for variations in the soil. The soil's top two inches were discarded because the topsoil does not provide an accurate representation of the soil due to its topical proximity to variable outside environmental factors. The next three inches were collected in plastic bags and were stored at 4°C to prevent bacterial death. For the residential soil collections, soil from two houses and two parks around Bowling Green, Kentucky, were used as controls. The soil collection process as used in the farm soil collection was implemented, but only one sample from each site was taken. Each site's specific location was recorded, but they were not included for confidentiality reasons.

For the serial dilutions, six 1/10 dilutions were performed as demonstrated in Figure 2.1. First, one gram of the soil was measured on an electronic balance, and the soil was placed in labelled 10 mL conical tubes. 9 mL of DI water were then added to each tube for the first 1/10 dilution, which would be represented as the $1/10^1$ tube in Figure 2.1. The conical tubes were centrifuged at 3000 RPM for one minute to mix the soil and DI water to prevent the soil from settling at the bottom of the tube. Then, 150 µL of this

mixture was added with a micropipette to a 1.5 mL microcentrifuge tube that contained 1350 µL of DI water to create the $1/10^2$ dilution. 150 µL of this mixture was used for the next 1/10 dilution, and this technique was repeated until a $1/10^6$ mixture was created. Each microcentrifuge tube was centrifuged at 3000 RPM for five seconds to mix its contents.

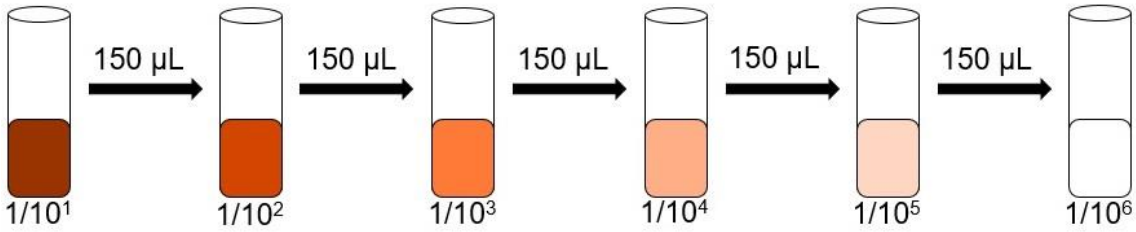


Figure 2.1: Serial Dilutions. 1/10 dilutions were performed with each soil sample. 1 gram of soil and 9 mL of DI water were used to create the $1/10^1$ mixture. 150 µL of this mixture was placed in 1350 µL of DI water, and this ratio was repeated for each successive dilution until a $1/10^6$ dilution was made.

2.2: Differential Plating and Purification

Tryptic soy agar (TSA) and MacConkey plates were used, and tetracycline was the antibiotic used for antibiotic resistance testing. The TSA plates were used as the control because it allowed for the growth of al bacteria. The MacConkey plates selectively grew Gram-negative bacteria, which would include enteric bacteria from cattle.

For each type of medium, five plates contained no tetracycline (no Tet), three plates contained 3 mcg/mL of tetracycline (Tet-3), and three plates contained 30 mcg/mL of tetracycline (Tet-30). 200 µL of one of the dilutions were added by a micropipette in a circular motion, and the plates and their dilutions are shown in Table 2.1. For the no Tet plates, $1/10^2$, $1/10^3$,$1/10^4$, $1/10^5$, and $1/10^6$ dilutions were added. For the Tet-3 and Tet-

30 plates, $1/10^1$, $1/10^2$, and $1/10^3$ dilutions were added. Plastic cell spreaders were used to distribute the mixtures evenly. The plates were incubated at 28°C for 72 hours.

Table 2.1: Dilutions for Plating. Antibiotic levels and dilutions that were used for the TSA and MacConkey plates.

	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Yes	Yes	Yes	Yes	Yes
Tet-3	Yes	Yes	Yes			
Tet-30	Yes	Yes	Yes			

After 72 hours had elapsed, the colonies were counted using a cell counter. Only the plates with less than 500 colonies were counted to ensure accurate measurements. In addition, the different characteristics for the colonies were noted, and unique colonies were identified. To purify each identified colony, the colony was transferred with a heat-sterilized inoculating loop to a new plate that contained the same media and antibiotic level. The colony was streaked with the inoculating loop in a T-streak pattern as demonstrated in Figure 2.2 so that the plate would not be overgrown with bacteria. It would also yield individual colonies that could be isolated easily. In section 1 in Figure 2.2, the initial inoculate was streaked in a zigzag pattern, and the inoculating loop was sterilized by passing it through the flame of a Bunsen burner. With the now-sterilized loop, the loop was placed at the end of section 1, and the loop dragged into section 2. This step was repeated for section 3 to obtain purified colonies, and the plates were incubated at 28°C for 24 hours.

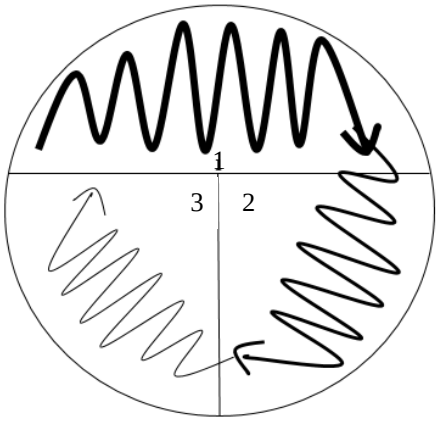


Figure 2.2: T-streak Method. The T-streak pattern was performed by streaking the bacteria in a zigzag pattern with a sterilized inoculating loop.

2.3: Gram-Staining

To prepare for the Gram-staining, an individual microscope slide was used for each unique colony. In addition to the singular colonies, *Escherichia coli* was used at the Gram-negative control, and *Micrococcus luteus* was used as the Gram-positive control. On the back of the slide, a black circle was drawn, and the slide was labelled with the correct soil sample abbreviation, media, and dilution factor. Then, a small drop of DI water was added to each circle. Using a sterilized inoculating loop, a drop of each pure culture was added to its respective circle, and the loop was sterilized by heating before and after each transfer. After the slide had dried, the cells were heat-fixed by passing the slide through the flame three times.

For the Gram staining, the slide was flooded with crystal violet for one minute and rinsed with DI water to stain the peptidoglycan in the cell walls. For another minute, the slide was flooded with iodine, a mordant, and the slide was rinsed again. Then, for fifteen seconds, the slide was flooded with 95% ethanol, and it was rinsed. Next, safranin was used as a counterstain, and the slide was flooded for thirty seconds to flood the smear and was rinsed afterwards. After the slides dried, they were viewed under a bright field microscope under a 1000X oil immersion lens, and the results were recorded.

2.4: Multidrug Resistance Testing

Before beginning the multidrug resistance testing, the purified colonies were inoculated with a sterile inoculating loop into plastic culture tubes containing 2 mL of tryptic soy broth (TSB). The tubes were incubated at 37°C for 24 hours to produce liquid cultures to see which cultures grew at human body temperature and had the potential to be pathogenic. Only the tubes with visible growth were saved.

To test for multidrug resistance, Mueller-Hinton plates were used for Kirby-Bauer antibiotic resistance testing, and the plates were sectioned into four quadrants. 200 µL of the cultures that grew at 37°C were transferred to Mueller-Hinton plates with a micropipette, and a plastic cell spreader distributed the cultures evenly. In each quadrant, one antibiotic wafer was placed in the center as shown in Figure 2.3. Common antibiotics in animal feed were tested, and the antibiotics used were 10 mcg of streptomycin, 30 mcg of tetracycline, 15 mcg of erythromycin, and 10 mcg of penicillin. Then, the plates were incubated at 37°C for 24 hours, and the lack of bacterial growth around the wafer was measured to the nearest millimeter. These diameters were called the zones of inhibition, and the strains' levels of resistance were classified as either resistant, intermediate, or susceptible based upon their zones of inhibition. The standardized criteria for each antibiotic's resistance level was listed in Table 2.2.

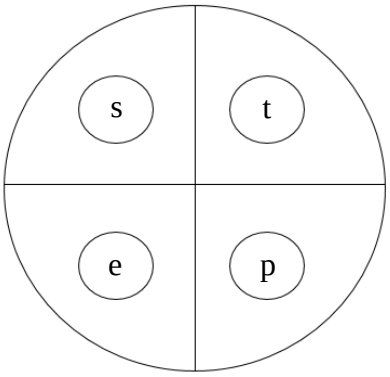


Figure 2.3: Kirby-Bauer Test. Wafers of streptomycin (s), tetracycline (t), erythromycin (e), and penicillin (p) were placed in each quadrant.

Table 2.2: Zones of Inhibition. The bacterial resistance levels for streptomycin, tetracycline, erythromycin, and penicillin were based upon the measured zones of inhibition. Each level was also assigned a color.

Antibiotic	Dosage (mcg)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Streptomycin	10	≤11	12-14	≥15
Tetracycline	30	≤14	15-18	≥19
Erythromycin	15	≤13	14-22	≥23
Penicillin	10	≤14	-	≥15

CHAPTER 3

RESULTS

3.1: Soil Collections and Serial Dilutions

As demonstrated in Table 3.1, the different locations were given an abbreviation along with defining characteristics. At each farm, the owner allowed access to specific fields. This provided different timeframes for when the cattle were last in contact with the soil.

Table 3.1: Soil Samples. Each soil sample was given an abbreviation, and the type of farm and general characteristics were recorded for further comparisons.

Soil Sample	Cattle Farm/Residential	General Characteristics
A	Cattle Farm 1	Cattle were settled on this field.
B		
C	Cattle Farm 2	Cattle recently switched to another field.
D		
E	Cattle Farm 3	Cattle were settled on this field.
F		
G	Cattle Farm 4	Cattle moved off this field four months prior.
H		
RA	House 1	Taken from garden soil.
RB	House 2	Taken from the backyard.
RC	Park 1	Taken near the road.
RD	Park 2	Taken off the path.

3.2: Differential Plating and Purification

After the incubation period, the TSA plates and the MacConkey plates were compared, and a sample comparison is shown in Figure 3.1 using the Sample B plates. As demonstrated in Figure 3.1, bacterial lawns were grown on the plates with the first dilutions, and individual colonies grew on the plates with later dilutions. Visually, the

TSA plates had significantly more growth than the MacConkey plates, which was expected because the MacConkey plates selectively grew enteric Gram-negative bacteria.

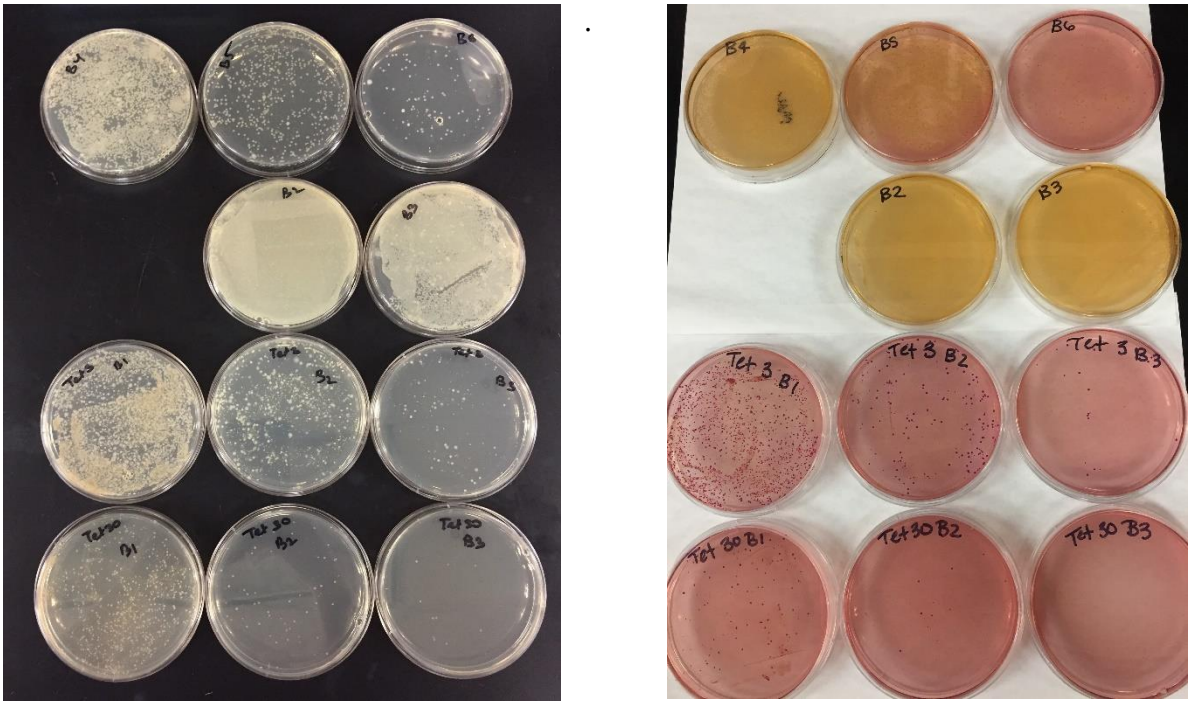


Figure 3.1: Plating. Sample B grown on the TSA plates (left) and the MacConkey plates (right) after 72 hours.

The distinguishable colonies on the TSA and MacConkey plates were counted, and the results were recorded in their respective tables (Tables 3.2 – 3.13). To keep the data consistent, only the plates that had less than 500 colonies were recorded. If a dilution was not used, the respective space in the tables were grey. Like with the visual comparisons, the MacConkey plates had less bacterial growth than the TSA plates, so the MacConkey plates successfully allowed the growth of Gram-negative enteric bacteria.

Table 3.2: Sample A. Sample A's colony count for the TSA and MacConkey plates after 72 hours.

Sample A with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	27
Tet 3	Too High	Too High	Too High			
Tet 30	Too High	340	40			
Sample A with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	184	62	4	0
Tet 3	232	27	11			
Tet 30	8	3	0			

Table 3.3: Sample B. Sample B's colony count for the TSA and MacConkey plates after 72 hours.

Sample B with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	273
Tet 3	Too High	Too High	Too High			
Tet 30	Too High	Too High	79			
Sample B with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	126
Tet 3	Too High	482	63			
Tet 30	199	15	0			

Table 3.4: Sample C. Sample C's colony count for the TSA and MacConkey plates after 72 hours.

Sample C with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	19
Tet 3	Too High	Too High	Too High			
Tet 30	Too High	Too High	27			
Sample C with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	66	6	0	0
Tet 3	58	9	2			
Tet 30	65	3	1			

Table 3.5: Sample D. Sample D's colony count for the TSA and MacConkey plates after 72 hours.

Sample D with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	21
Tet 3	Too High	Too High	Too High			
Tet 30	Too High	372	25			
Sample D with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	91	10	4
Tet 3	Too High	147	2			
Tet 30	48	10	4			

Table 3.6: Sample E. Sample E's colony count for the TSA and MacConkey plates after 72 hours.

Sample E with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	4
Tet 3	Too High	Too High	342			
Tet 30	Too High	272	25			
Sample E with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		205	25	2	0	0
Tet 3	126	3	1			
Tet 30	7	0	0			

Table 3.7: Sample F. Sample F's colony count for the TSA and MacConkey plates after 72 hours.

Sample F with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	10
Tet 3	Too High	Too High	Too High			
Tet 30	Too High	289	46			
Sample F with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	299	36	3	0
Tet 3	78	7	1			
Tet 30	90	10	1			

Table 3.8: Sample G. Sample G's colony count for the TSA and MacConkey plates after 72 hours.

Sample G with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	7
Tet 3	Too High	Too High	213			
Tet 30	Too High	238	15			
Sample G with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	41	2	0	0
Tet 3	98	2	0			
Tet 30	9	1	0			

Table 3.9: Sample H. Sample H's colony count for the TSA and MacConkey plates after 72 hours.

Sample H with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	2
Tet 3	Too High	Too High	244			
Tet 30	Too High	157	9			
Sample H with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		187	20	0	0	0
Tet 3	37	6	0			
Tet 30	10	0	0			

Table 3.10: Sample RA. Sample RA's colony count for the TSA and MacConkey plates after 72 hours.

Sample RA with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	18
Tet 3	Too High	Too High	324			
Tet 30	Too High	170	12			
Sample RA with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	194	15	4	0
Tet 3	127	12	1			
Tet 30	86	6	0			

Table 3.11: Sample RB. Sample RB's colony count for the TSA and MacConkey plates after 72 hours.

Sample RB with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	2
Tet 3	Too High	Too High	302			
Tet 30	Too High	185	7			
Sample RB with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		210	45	5	0	0
Tet 3	21	0	0			
Tet 30	42	0	0			

Table 3.12: Sample RC. Sample RC's colony count for the TSA and MacConkey plates after 72 hours.

Sample RC with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	Too High	6
Tet 3	Too High	Too High	318			
Tet 30	Too High	Too High	115			
Sample RC with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		312	65	4	0	0
Tet 3	91	12	3			
Tet 30	91	7	1			

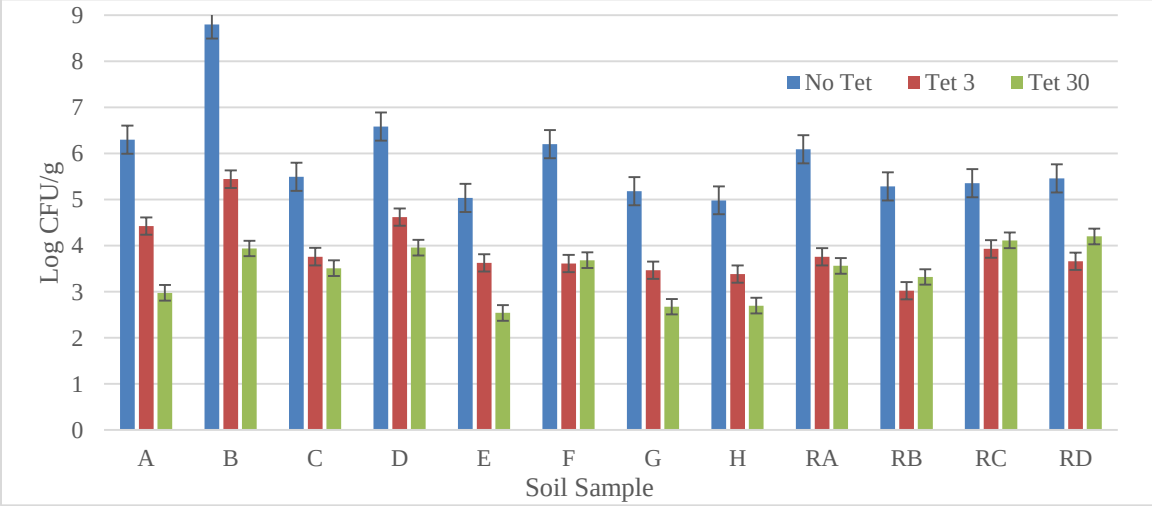
Table 3.13: Sample RD. Sample RD's colony count for the TSA and MacConkey plates after 72 hours.

Sample RD with TSA						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		Too High	Too High	Too High	36	1
Tet 3	Too High	Too High	Too High			
Tet 30	Too High	Too High	340			
Sample RD with MacConkey						
	$1/10^1$	$1/10^2$	$1/10^3$	$1/10^4$	$1/10^5$	$1/10^6$
No Tet		367	58	8	0	0
Tet 3	136	4	1			
Tet 30	28	19	1			

The counted colonies in Tables 3.2 – 3.13 were divided by their dilution factors to obtain the colony forming units (CFUs), and a sample calculation can be found in Appendix A with Equation A1. For each type of MacConkey plate, the CFUs for the dilutions were averaged together with Equation A2 in Appendix A to obtain the CFUs in the original soil sample. The values were inputted into Table A1 in Appendix A. The log values of these amounts were found and inputted into Table A2 in Appendix A. Because many TSA plates had too high of bacteria count to produce accurate results, they could not be easily compared.

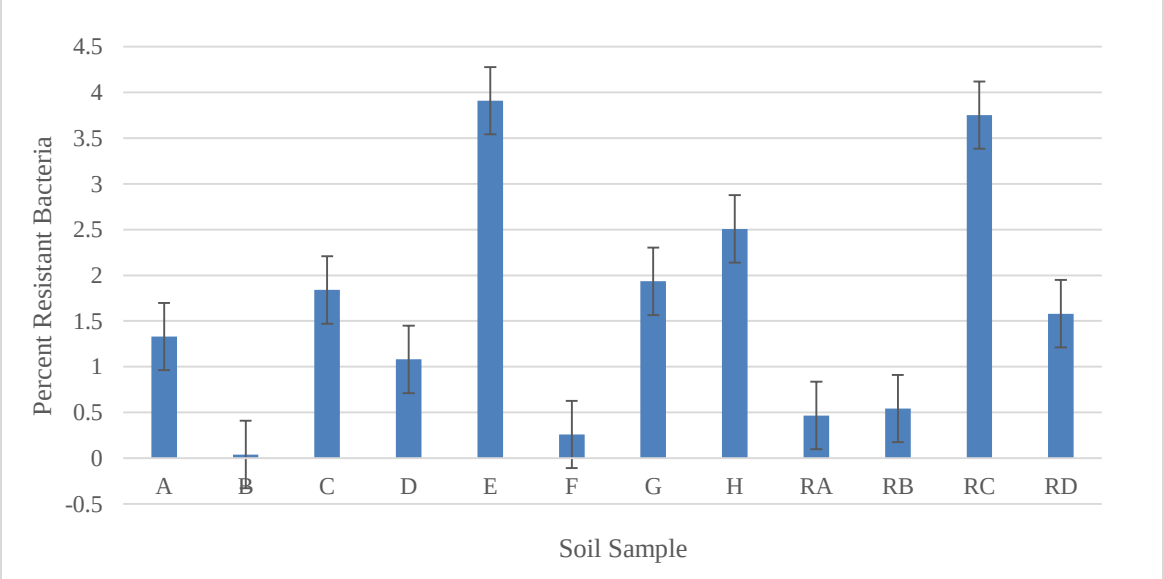
With the log values from Table A2, Figure 3.2 was created to compare both the differences between the soil samples and the differences between the three antibiotic levels. In Figure 3.2, variation occurred between all the samples, but many of their error ranges overlapped. Notable differences are shown in Samples B, E, F, and RC. With Samples B and F, the difference in magnitude between the No Tet CFUs and the Tet 3/Tet 30 CFUs was more than the other samples. Conversely, with Samples E and RC, the difference in magnitude was less than the other samples.

Figure 3.2: MacConkey Log CFU/g Graph. The MacConkey plates' log values were graphed with error bars to show the differences between the No Tet, Tet 3, and Tet 30 antibiotic levels.



To determine the percent resistant bacteria in the soil samples, the ratio between the Tet 3 CFUs and the No Tet CFUs was found using Equation A3 in Appendix A. All the percentages are listed in Table A3 in Appendix A, and the percentages are also shown in Figure 3.3. Majority of the percentages' error bars overlapped, but the notable outliers are Samples B, E, F, RA, RB, and RC. As shown in Table 3.1, RA and RB were both from residential house areas, so these two samples would have less exposure to antibiotics. For Samples B and F, the most probable reason was their high levels of CFUs on the No Tet plates. Because the No Tet plates were in the denominator in Equation A3, this would result in lower percent resistant bacteria in Figure 3.3. Likewise, Samples E and RA had a smaller difference between the No Tet and Tet 3 plates, so the smaller difference would inflate the percent resistant bacteria in Figure 3.3. The fluctuations were also noted in Figure 3.2, and these observations remained consistent between the two graphs.

Figure 3.3: Percent Resistant Bacteria Graph. The percent resistant bacteria were calculated by dividing the No Tet CFUs by the Tet 3 CFUs, and these percentages are shown with error bars.



For colony purification, colonies were chosen based upon their differing morphologies. The different colonies in each soil sample were given a new label, and the purified colonies are listed in Table 3.14.

Table 3.14: Purified Colonies. The purified colonies are listed with their original soil sample, antibiotic level, and dilution factor.

Abbreviation	Sample	Antibiotic	Dilution
A1	A	Tet 3	$1/10^3$
A2			
A3			
A4			$1/10^2$
A5			
B1	B	Tet 30	$1/10^2$
B2			
B3			
C1	C	Tet 3	$1/10^1$
C2			
C3			
C4			
D1	D	Tet 30	$1/10^1$
D2			$1/10^2$
D3		Tet 3	$1/10^3$
E1	E	Tet 3	$1/10^1$
E2			
E3			
F1	F	Tet 3	$1/10^1$
F2			
F3			
G1	G	Tet 3	$1/10^1$
H1	H	Tet 3	$1/10^1$
H2			$1/10^2$
H3			
RA1	RA	Tet 30	$1/10^1$
RA2			
RA3		Tet 3	$1/10^2$
RA4			
RA5			
RA6			
RA7			$1/10^3$
RB1	RB	Tet 30	$1/10^1$
RB2			
RC1	RC	Tet 3	$1/10^2$

3.3: Gram Staining

To confirm that the MacConkey plates grew Gram-negative bacteria, several of the colonies from the MacConkey plates were Gram-stained. All the plates stained Gram-negative, and a sample microscopic picture from B2 in Table 3.14 is shown as Figure 3.4. Also, the bacteria were rod-shaped, which suggests that the bacteria were enteric bacilli. Therefore, antibiotic resistant bacteria from cattle's intestines were present in the soil.

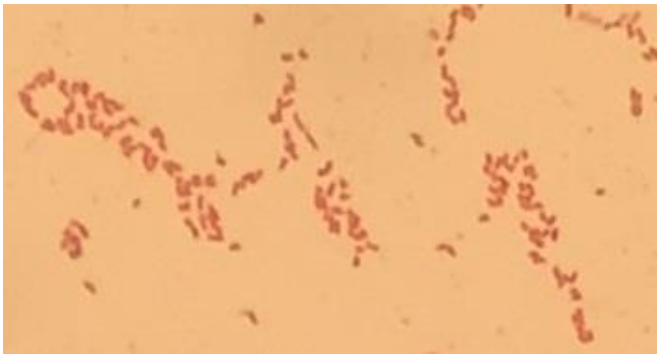


Figure 3.4: Gram-Staining. Colony B2 was viewed as Gram-negative enteric bacilli under a light microscope.

3.4: Multidrug Resistance Testing

The strains that grew at 37°C are listed in Table 3.15, and these strains carry the potential to be pathogenic to humans. After these cultures were inoculated on Mueller-Hinton plates and incubated for 24 hours, the zones of inhibition for streptomycin, tetracycline, erythromycin, and penicillin wafers were marked, and a sample plate for F2 is shown as Figure 3.5. The measured distances were recorded in Table 3.15, and these measurements were compared against the criteria listed in Table 2.2. Resistant strains were red, intermediate resistant strains were yellow, and susceptible strains were green.

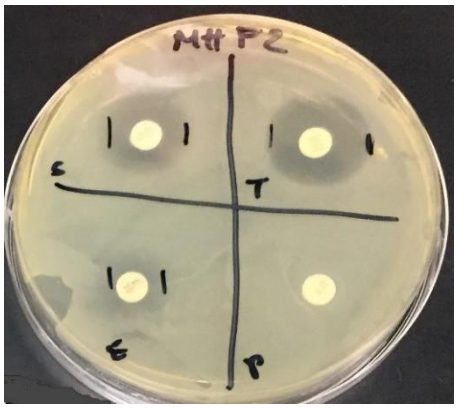


Figure 3.5: Kirby-Bauer Results. The F2 colony was grown on a Mueller-Hinton plate was underwent Kirby-Bauer testing.

Except for RA2, all the samples were resistant to penicillin. Also, all the samples showed some resistance to erythromycin, and majority were susceptible to streptomycin. While the original plates contained tetracycline, not all the samples in Table 3.15 were resistant to tetracycline. However, majority of the cultures were grown on Tet 3, and the tetracycline wafers contained 30 mcg of the antibiotic. This demonstrates that the bacteria were likely to be resistant to tetracycline at lower dosages. The levels of resistance could indicate which antibiotics were likely incorporated into the animal feed and how common the antibiotic resistant gene is present in the environment.

Table 3.15: Measured Zones of Inhibitions for Purified Colonies. The zones of inhibitions for the potentially pathogenic soil samples were recorded and color-coded based upon if they were resistant, intermediate, or susceptible to the antibiotics.

Sample	Streptomycin (mm)	Tetracycline (mm)	Erythromycin (mm)	Penicillin (mm)
A3	15	22	12	6
A5	6	10	11	6
B1	19	6	17	9
C3	16	12	10	6
D3	18	16	12	6
E1	19	21	14	6
E2	25	22	10	6
E3	26	7	16	6
F2	16	20	11	6
H1	15	17	19	6
RA1	20	6	6	6
RA2	22	33	20	37
RA5	6	10	15	6
RA6	21	20	19	8
RA7	16	11	13	6

CHAPTER 4

DISCUSSION

While antimicrobial resistance (AMR) genes have been in the bacterial genomes for thousands of years, antimicrobial resistant bacteria (ARB) typically remain in low numbers in the environment. When antibiotics are introduced for therapeutic or nontherapeutic reasons, they inadvertently select for ARB, which increases the amount of ARB present. Through the comparisons of cattle farm soils and residential areas, the goal of this experiment was to determine the ARB differences in these environments while also testing for pathogenicity and multidrug resistance. Bacterial colonies were counted on tryptic soy agar (TSA) plates and MacConkey plates that had three levels of antibiotics used. These bacteria counts allowed for comparisons between soil samples. After incubating at human temperature of 37°C, the bacteria that grew could potentially infect humans, and these bacteria were also tested for multidrug resistance through Kirby-Bauer testing. Because of the high usage of antibiotics in animal feed, the increased numbers of ARBs in the farm soils suggest that horizontal gene transfer of AMR genes could occur. In addition, the ARBs could then infect humans under specific conditions, but no breakout of pathogenic ARBs have been correlated with the ARBs in soils.

The three types of soils studied were from cattle farms (Samples A-H), residential areas (RA and RB), and parks (RC and RD). The ARB levels between the three areas can

be compared to determine where AMR genes are expressed at a higher rate. AMR genes are more likely to be expressed as a regulatory tool for microbial growth (11). In the residential areas, the lots were developed from farms within the past decade, so the local environments likely contained less competition. This is represented in Figure 3.3 because they have the lowest percentages of ARBs. For the cattle farm soils and park soils, they displayed similar levels of antibiotic resistance in Figure 3.3 despite the parks not being exposed to antibiotics. However, as the park soils were taken from the woods in overgrown areas, the parks likely had a higher level of microbial competition than the farms, which would explain the increased the prevalence of AMR genes being expressed. The high levels of ARBs in parks have been recorded in several areas throughout the United States, so these findings are consistent with current data (25-27). In addition, the ARBs present in cattle farms likely originated from cattle intestines, but the ARBs in parks occur naturally in the environment. So, while the levels were similar, their origins were different and cannot be compared simply.

Comparing the cattle farms, the farm soils displayed roughly the same number of resistant bacteria in the soil. Samples B and F had significantly lower percent resistant bacteria as observed by their high levels of normal microbial counts as observed on the plates containing no tetracycline (no Tet). Samples E displayed the opposite result than Samples B and F by having larger percent resistant bacteria present than the other farms.

In Table 3.1, the four cattle farms each had two samples tested. However, as demonstrated in Figures 3.2 and 3.3, the variation in bacteria levels were not consistent within the same farm. Even though the soil samples came from different areas of the farms, the differences show that bacteria levels are not consistent throughout the pastures.

This was likely due to the area's proximity to manure, and the differing bacteria levels throughout the farms likely caused the variations in Samples B, F, and E. More samples from each farm should be taken to determine the average ARB level for future comparisons.

More samples from each farm would also help in temporal comparisons. In a past study that analyzed ARB change over time in farm soil, it showed that more exposure to manure led to higher levels of ARB, but these levels decreased over time when the manure was removed (28). In Figure 3.3, Samples C, D, G, and H did not have any cattle present on the pasture, but their ARB levels resembled the other farm soil samples rather than the residential area samples. Therefore, the bacterial counts in Samples C, D, G, and H may not be a true representation of the actual ARBs present in the farms, and this result could be supplemented with AMR analysis. Conversely, the AMR gene levels take longer to decrease, so they would be present in the soil environments longer. Thus, more samples would be needed to deduce the reason why the ARB numbers in Samples C, D, G, and H were higher than expected despite not having recent cattle manure.

Also, the multidrug resistance for the soil samples should be expanded. As shown in Table 3.15, all the samples were either resistant to penicillin or erythromycin. For streptomycin, majority were susceptible, while while Samples A5, RA5, and RA7 were resistant. For tetracycline, there was a mixture of resistances between the samples. The differences in the four antibiotics could represent if the antibiotics were used in animal feed and the amount of antibiotics used. However, the areas from where the soils were sampled did not affect the resistances because the residential areas had multidrug resistance without being exposed to antibiotics. This could also suggest how AMR genes

are naturally present in bacteria (9, 10). While all the colonies initially grew on tetracycline plates as shown in Table 3.14, about half of the colonies were susceptible to tetracycline in Table 3.15. The Kirby-Bauer test should be repeated for consistency as external factors could have caused this discrepancy.

As displayed in Table 3.15, all the samples had penicillin resistance. One of the main concerns for ARBs is the prevalence of extended-spectrum β-lactamase (ESBL) resistance mechanism. Currently, beta-lactam antimicrobial agents, such as penicillin, are the most common treatment method of bacterial infections (29). These antibiotics disrupt the enzymes that create the peptidoglycan cell wall, but ESBLs render this activity mechanism ineffective (29). With the number of ESBL-producing bacteria rising, the appropriate antibiotic needs to be selected based upon the the characterization of bacteria. Because of the amount of non-therapeutic antibiotics used in livestock feed, ESBL-producing bacteria could rise due to horizontal gene transfer, which could yield more multidrug resistant bacteria (6, 29). While ESBLs were not studied in this experiment, ESBLs could explain the high prevalence of penicillin resistant bacteria in Table 3.15, but further studies would need to confirm the presence of ESBL-producing bacteria.

Even though this experiment did not conclusively establish a relationship between fecal matter and ARBs, it does display similar results as other experiments that tested for this relationship (19-24). However, this relationship with soil does not necessitate a public health concern. For enteric bacteria to infect humans, they must be ingested. Therefore, the enteric bacteria present in the soil have a very low chance of infecting humans through topical interactions. However, if the cattle that contained pathogenic

enteric ARBs were butchered and eaten raw, then these bacteria have a higher likelihood of causing disease.

Despite this low probability of causing disease, many people encounter the ARBs. Where antibiotic usage and ARB exposure is high, the density of ARBs on people's skin is also high (8). Even with a higher ARB density, the ARBs are still not likely to cause an infection (8). If farmers and others follow proper sanitary procedures, their health should be unaffected.

Thus, enteric ARBs on soil are currently not a concern. While superbugs are a rising topic in healthcare, enteric ARBs introduced from livestock to soil have not been the cause of any outbreak (14). As shown in this experiment, ARB numbers throughout different sites vary widely, which adds inconsistency to their true prevalence. A similar finding has been reported in a review by Pepper et. al that studied the relationship between soil ARBs and healthcare (14). However, the research behind soil ARBs is lacking, so further studies are required to determine the actual relationship enteric ARBs have on the environment and healthcare. Different areas that need to be studied are horizontal gene transfer between soil enteric ARBs and common pathogens, long-term exposure to ARBs, and more sampling of different sites that are exposed to antibiotics. Regardless of these unknowns, this study supports the consensus that antibiotics should only be used for therapeutic reasons, so antibiotic usage in animal feed should be limited.

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APPENDIX A

COLONY FORMING UNIT CALCULATIONS

To determine the CFUs of each soil sample, the countable colonies from Tables 3.2-3.13 were divided by their dilution factors. Using the colonies from the $1/10^3$ dilution from the no Tet MacConkey data in Table 3.2, Equation A1 shows an example calculation,

$$CFU = \frac{number\ of colonies}{dilution\ factor} \tag{A1}.$$

$$CFU = \frac{184\ colonies}{10^{-3}}$$

$$CFU = 1.84 * 10^5 CFU$$

With the CFUs for each dilution, the CFUs (x_{CFU}) were averaged together to obtain the number of CFUs present in the original soil sample. As a fifth of the dilution volume was pipetted onto the plates, the averages were multiplied by 5 to represent this ratio. The results represented the average CFUs per gram of soil. Equation A2 demonstrates a calculation using the no Tet MacConkey data from Table 3.2,

$$Average\ CFU/g = \frac{\sum x_{CFU}}{n_{x_{CFU}}} * 5 \tag{A2}.$$

$$Average\ CFU/g = \frac{(1.84 + 6.2 + 4) * 10^5 CFU}{3} * 5$$

$$Average\ CFU/g = 2.01 * 10^6 CFU/g$$

The average CFU/g were found for all the soil samples and for each of the antibiotic levels. For recording purposes, the results were listed in Table A1. With these values, their logarithms were taken, and the log values are recorded in Table A2. The results in Table A2 were then used to create Figure 3.2, which provided a better visualization of the results.

Table A1: Calculated CFU/g. The CFUs per gram of soil were found using Equations A1 and A2 for all the soil samples and for the three antibiotic levels.

Sample	Total (CFU/g)	Tet 3 (CFU/g)	Tet 30 (CFU/g)
A	2006667	26700	950
B	630000000	278000	8725
C	315000	5800	3250
D	3850000	41750	9133
E	109167	4267	350
F	1598333	4133	4833
G	152500	2950	475
H	96750	2425	500
RA	1240000	5783	3650
RB	193333	1050	2100
RC	227000	8517	13050
RD	291167	4600	15900

Table A2: Log CFU/g. The log values from Table A1 were recorded for Figure 3.2.

Sample	Log Total	Log Tet 3	Log Tet 30
A	6.30	4.43	2.98
B	8.80	5.44	3.94
C	5.50	3.76	3.51
D	6.59	4.62	3.96
E	5.04	3.63	2.54
F	6.20	3.62	3.68
G	5.18	3.47	2.68
H	4.99	3.38	2.70
RA	6.09	3.76	3.56
RB	5.29	3.02	3.32
RC	5.36	3.93	4.12
RD	5.46	3.66	4.20

Using the no Tet and Tet 3 values from Sample A in Table A1, the percent resistant bacteria were found through Equation A3,

$$\boldsymbol{Percent\ Resistant\ Bacteria} = \frac{\boldsymbol{Tet\ 3}\frac{\boldsymbol{CFU}}{\boldsymbol{g}}}{\boldsymbol{No\ Tet}\frac{\boldsymbol{CFU}}{\boldsymbol{g}}} * \mathbf{100}\% \qquad \text{(A3).}$$

$$\boldsymbol{Percent\ Resistant\ Bacteria} = \frac{\mathbf{26700}\frac{\boldsymbol{CFU}}{\boldsymbol{g}}}{\mathbf{2006667}\frac{\boldsymbol{CFU}}{\boldsymbol{g}}} * \mathbf{100}\%$$

$$\boldsymbol{Percent\ Resistant\ Bacteria} = \mathbf{1.33}\%$$

Each of the percentages for each soil sample were calculated and inputted into Table A3,

Table A3: Percent Resistant Bacteria. The percent resistant bacteria for the soil samples were calculated with Equation A3 and recorded.

Soil Sample	Percent Resistant Bacteria (%)
A	1.33
B	0.04
C	1.84
D	1.08
E	3.91
F	0.26
G	1.93
H	2.51
RA	0.47
RB	0.54
RC	3.75
RD	1.58