Developing A Homeopathic Solution to Minimize Antibiotic Resistance to Four Commonly Used Antibiotics

Word Count: 4164

1. Introduction

1.1 Background

Antibiotics are vital in reducing infectious diseases in humans, livestock, and animals worldwide. However, numerous infections, including tuberculosis, salmonellosis, pneumonia, and gonorrhea, are becoming harder to treat as antibiotic-resistant mutations in microorganisms cause antibiotics to become less effective (World Health, 2020). These mutations cause antibiotics to lose their ability to kill harmful bacteria, increasing infection and disease.

The presence of antibiotic resistance occurs when one bacterium undergoes a specific mutation. This mutation creates a new gene that codes for resistance to a particular antibiotic. This gene can then spread to other bacteria through multiple processes, resulting in a fast transfer of the new gene and fast spread of this new antibiotic-resistant capability.

Since this gene increases the chance of survival against antibiotics, the bacteria with the gene are more likely to survive and reproduce more bacteria with this same resistant gene. Therefore, evolution by natural selection increases the presence of the gene worldwide and decreases the effectiveness of antibiotics. The rise of antibiotic resistance is considered one of the most significant public health issues, according to the World Health Organization. The CDC reports that antibiotic resistance may be the cause of 48,000 deaths worldwide and more than 2.8 million infections (CDC, 2022). Since antibiotics are beginning to lose effectiveness, this study aims to find another solution to prevent antibiotic resistance genes and avoid infection.

1.2 Purpose and Gap

The purpose of this research study is to find or create a solution that keeps plants healthy while reducing the presence of genes coding for antibiotic resistance.

Previous solutions have tested ways to decrease antibiotic resistance in microorganisms; however, most solutions are unsafe for the environment, use costly materials, or have slightly different goals than this study. For example, one study uses bacteriophages (viruses) to prevent antibiotic resistance. The problem is that this poses a risk of infection from the bacteriophage, is unsafe for the environment, and is extremely expensive. (Jassim & Limoges, 2014).

Another study looked at ways to decrease antibiotic resistance in humans instead of plants (Packham et al., 2019). Furthermore, another study investigated new antibiotics to kill bacteria instead of investigating how to reverse the antibiotic-resistance genes that lead to the problem (Al-Kalbani et al., 2022).

This study introduces a new solution that can be sprayed directly onto soil. This novel approach, aimed at reducing antibiotic resistance in sports fields or residential areas, can potentially improve public health and environmental sustainability. Its affordability and ease of use also empower consumers to play a crucial role in this battle.

This research paper addresses a gap by focusing on soil and plant bacteria instead of bacteria in humans. This study also uses affordable and commonly found items, including lavender and cinnamon essential oil, to reverse the antibiotic resistance gene without harming the environment.

1.3 Implications

If research is conducted to find a solution to reduce these harmful genes, then it may help reduce the threat of antibiotic resistance. Therefore, the development of a solution has the potential to guide research in reducing the number of deaths and infections due to antibiotic resistance. If this research study meets its engineering goals, it may help develop a solution that could assist in fighting this problem.

2. Literature Review

This literature review aims to explore and discuss existing research regarding essential oils and antibiotic resistance. This section will also justify using essential oils as a solution and tetracycline as a variable in this experiment. Finally, it will provide the necessary background to understand the methods used.

2.1 Essential Oils

Essential oils were selected as a possible solution to antibiotic resistance for this experiment because previous studies have shown that essential oils can harm bacteria (Swamy, 2016). This ability to harm bacteria is due to their antimicrobial activity, which multiple sources state can also help fight infections (Eden et al., 2020; Kajjari et al., 2022; Oukil & Bedjou, 2013; Rao & Gan, 2014). Studies have also supported that essential oil can improve plant growth (Dyadiuchenko, 2020).

Although previous studies have identified essential oils as method of harming bacteria and improving plant growth, little to none have scientifically tested their ability to rid the bacteria of antibiotic-resistant genes. However, due to their antimicrobial activity and ability to kill bacteria, it can be hypothesized that essential oils may reverse the impact of antibiotic genes, making antibiotics more effective and decreasing infection and disease.

2.2 Tetracycline

There are a multitude of different antibiotics and different genes that can create resistance to those antibiotics. One example of these antibiotics is tetracycline, found in soil bacteria. Tetracycline works by binding to the 16 S RNA, an essential type of ribosomal DNA that aids in making proteins and stops the production of essential proteins in the cell. Stopping the production of vital proteins stops the cell from growing or dividing, causing the cell to die (Daghrir & Drogui, 2013). Although tetracycline can kill bacteria cells, it works differently in human cells, specifically targeting bacteria and leaving the rest of the body unharmed.

Tetracycline is highly effective and inexpensive, becoming a popular antibiotic in humans and animals. Today, most animal feed contains tetracycline, and a recent study stated that out of 353 feed samples, 52.7% had undeclared tetracycline (Przeniosło-Siwczyńska et al., 2020). Because tetracycline is a common additive to animal feed, bacteria have been under constant selection for the resistant gene. As a result, resistant genes to tetracycline are prevalent, with over 40 genes coding for resistance to this antibiotic.

The most popular resistance to this antibiotic occurs in genes that code for efflux pumps (proteins inserted into the cell membrane and force molecules out of the cell before they can enter). The tetB gene is one example of this resistance, which pumps tetracycline out of the cell before it can enter (Marti et al., 2006). However, genes, including the tetM gene, produce resistance differently. This gene produces a protein that binds to the 16S RNA, like tetracycline

(Doherty et al., 2000). Instead of inhibiting protein production, it blocks tetracycline from binding onto the 16S RNA, effectively blocking tetracycline from interfering with protein production.

2.3 Horizontal Gene Transfer

Genes can be transferred to different organisms through a few processes, including vertical gene transfer, where genes are passed down from parent to offspring. In contrast, horizontal or lateral gene transfer is passing genes to other organisms through pathways other than parent to offspring (Burmeister, 2015).

One method of horizontal gene transfer involves bacteria physically attaching in bacterial conjugation, where genes can be spread to entirely different species (Llosa et al., 2002). Additionally, horizontal gene transfer can occur through bacterial transformation, where bacteria pick up genes that leak out of other cells and are in their external environment (Johnston et al., 2014). Although uncommon, bacterial transformation can occur in animals and plants. Finally, the last method of horizontal gene transfer is bacterial transduction, where genes are passed to other organisms by viruses (Schneider, 2021). This type of gene transfer occurs when bacteriophages infect bacteria cells with foreign DNA. Conjugation, transformation, and transduction are examples of how horizontal gene transfer can occur and affect how antibiotic resistance spreads worldwide.

2.4 Analysis of Resistance Genes

In this experiment, sod plugs were used to replicate grassy fields out in the environment. To test the sod plugs (See Figure 1.) for resistant genes, a few processes need to occur prior to the gel electrophoresis. First, eDNA needs to be extracted from the soil.

Figure 1

Sod plugs



eDNA extraction is the procedure's most complicated and time-consuming part. The procedure involves putting the supernatant (the liquid extracted when mixing soil with water) through filtration systems using binding buffers and wash buffers, which help separate the eDNA from the rest of the solution (Rohland & Hofreiter, 2018).

After the eDNA is extracted, it needs to be amplified. A polymerase chain reaction (PCR) replicates the DNA (See Figure 2.) and produces multiple copies (Schochetman, 1988). Replication occurs in three steps: the opening of the DNA double helix, the template strand's priming, and the creation of the new corresponding DNA strand (Chagin et al., 2010). The PCR ends with around 1 billion replicates of the original DNA strand (Sinclair, 2002). This replication is necessary for the final step of analyzing resistant genes, the gel electrophoresis.

During the gel electrophoresis (See Figure 3.), charged molecules move across a gel depending on size. A DNA ladder, samples, and controls are continuously passed through the gel. The DNA ladder creates different bands in the gel; these bands are compared to the samples; if one sample contains a particular size band, then the researcher knows there is a gene coding for antibiotic resistance (Lee et al., 2012).

Figure 2.

Polymerase Chain Reaction

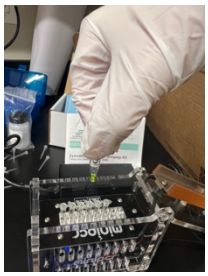
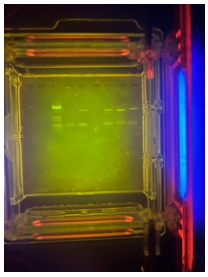


Figure 3.

Gel Electrophoresis



Although this experiment uses a gel electrophoresis to find antibiotic-resistant genes, it can be used to identify a multitude of different items present in DNA. The entire eDNA extraction and analysis was completed with a kit from miniPCR providing all necessary materials and instructions (miniPCR, 2022).

2.5 Zone of Inhibition Test

In addition to the gel electrophoresis, a zone of inhibition test is a much easier and faster way to determine the presence of antibiotic resistance. For this test, a soil sample is mixed with water and put on an agar plate, a circular dish with a gel-like solid on top (Fujikawa & Morozumi, 2005). After putting the soil sample on the plate, one places antibiotic disks on top for different antibiotics. The antibiotics used in this experiment are penicillin, ampicillin, and streptomycin. After 24 hours, bacteria grow on top of the agar plate.

If the bacteria grown is resistant to the antibiotic, it will grow near and around the antibiotic disks. However, if some are resistant, there will be a zone of inhibition around the antibiotic disk. A zone of inhibition means there will be a small circle around the disk, either with no bacteria present or very few present (Barry et al., 1979). Researchers can measure the zone of inhibition using a ruler and conduct a statistical test to see if the data is significant. In this experiment, one can compare the zone of inhibition in the treatment groups versus the control groups to see if the treatment decreased antibiotic-resistant bacteria in the soil samples.

Figure 3.

Zone of Inhibition



2.6 Summary

Although previous studies have identified essential oils as a way to kill bacteria and support plant growth, little to none have analyzed their ability to rid bacteria of antibiotic-resistant genes. The tetB and tetM genes were analyzed because they code for resistance to tetracycline, a very common and accessible antibiotic. Previous solutions were either too expensive or inaccessible to produce commercially. Similarly, previous studies aimed to lower antibiotic resistance in humans instead of plants.

3. Methods

This study aims to create a solution that can minimize antibiotic resistance in plants without harming them. It did so using two main methods: gel electrophoresis and a zone of inhibition test. These two methods were used because they both test for antibiotic resistance but function differently, allowing for a comprehensive understanding of whether the solution is working.

Due to the nature of the proposed research goal, a quantitative and qualitative scientific experimental study was used for the zone of inhibition and gel electrophoresis, respectively. Using quantitative and qualitative data collection methods allows for a more significant collection of data using different strategies. This method aimed to discover a potential correlation between the use of essential oils and the presence of antibiotic resistance. Antibiotic resistance was measured using gel electrophoresis and zone of inhibition tests. The institutional review board fully approved this research.

3.1 Experiment Method

An experiment is a research method that involves adding an independent variable and examining its impact on a separate group or dependent variable. A hypothesis is commonly made before collecting data, and data can be presented as either quantitative or qualitative, both of which were collected and analyzed for this current study.

An experimental study was most appropriate for this research because the proposed question requires the examination of two variables (The use of essential oils and the presence of antibiotic resistance). Similarly, an experimental study allows a more controlled environment to examine their relationship (Knight, 2010). Although a scientific experiment has its benefits, it has many limitations, including a lack of materials due to their high costs. Because of this, a smaller number of data points were collected than would have been ideal.

3.2 Materials

This research study aims to find a solution that reduces antibiotic resistance in soil. The solution is meant to be sprayed in sports fields, parks, playgrounds, or anywhere where humans become susceptible to being exposed to this antibiotic resistance. To best represent these settings, sod plugs were used (See figure 1.).

All materials for the gel electrophoresis were purchased from miniPCR. Some of these materials included primer mixes, positive control plasmids, DNA ladders, and nuclease-free water, all necessary for the gel electrophoresis portion of the procedure (miniPCR, 2022). Carolina Biology provided the materials for the concentration gradient and zone of inhibition test to test for the presence of antibiotic resistance.

3.3 Concentration Gradient

Before beginning data collection, the possible solutions needed to be tested on plants to ensure they do not harm them. Any signs of plant death or stunted growth suggest that the solution would not be a good fit to test in this experiment. To ensure that the solutions were safe, they were sprayed onto sod plugs (See Figure 1.) in varying concentrations, and then their growth was measured over the following two weeks. Lavender essential oil, cinnamon essential oil, and a 1:1 mix of both essential oils were tested at 100%, 75%, 50%, 25%, and 10% concentrations. A control group was also sprayed with water instead of essential oils.

The growth and health of the control group were compared to the experimental groups to ensure the solutions did not stunt plant growth. Grass spades were randomly sampled from each sod plug and measured using a ruler. To measure if the solutions were stunting plant growth, the length of random grass spades was compared between the control and experimental groups. If there

was a statistical difference between the two, it was deemed unsafe for plants. Additionally, visual data was observed to see if the plant was losing color. Testing plant length and color allows for a better-rounded estimate of plant health.

3.4 Testing for Antibiotic Resistance

Once safe solutions were established, another set of sod plugs was sprayed with the solutions and then tested for antibiotic resistance a few days later. The two methods used to test for antibiotic resistance include testing for tetB and tetM genes through a gel electrophoresis and conducting a zone of inhibition test. The gel electrophoresis provides qualitative or visual results on whether these specific genes that code for antibiotic resistance are present (see Figure 3.). Alternatively, the zone of inhibition test provides quantitative data. It gives a numerical value that one can correlate with the presence of antibiotic resistance (the larger the size of the zone of inhibition, the greater the presence of antibiotic resistance).

3.5 Gel Electrophoresis

As previously mentioned, a gel electrophoresis is the first method to test for antibiotic resistance. A few steps are necessary to produce a gel electrophoresis. First, eDNA needed to be extracted from each of the sod plugs. A kit from miniPCR came with all the necessary materials to extract this eDNA. Once eDNA has been extracted, it needs to go through a PCR. PCR stands for a polymerase chain reaction, a scientific procedure that allows for copying strands of DNA. Therefore, the PCR starts with one strand of extracted DNA and copies it multiple times until it has one billion copies of the original DNA (which is necessary to run the next step). After these two steps, the gel electrophoresis can occur. The gel electrophoresis results show if tetB or tetM genes are present in the sample by displaying a neon line if the genes are present. Both the tetB and tetM genes code for resistance to the antibiotic tetracycline. Therefore, if these genes are present, so is antibiotic resistance. Comparing the presence of these genes before and after treatment (of essential oils) can help determine whether the two are correlated.

3.6 Zone of Inhibition

The second method to test for antibiotic resistance is a zone of inhibition test. To begin this procedure, the researcher must prepare agar plates (see Figure 5.) with a solid agar layer. Then, the supernatant was extracted from the soil samples. One can acquire a supernatant by adding water to a soil sample, mixing the solution, and then separating the liquid from the soil sample. Separating the liquid from the soil allows for liquid that contains the bacteria and eDNA found in the soil sample but in a liquid form. This liquid (the supernatant) can now be placed on the agar plate. After adding the supernatant, small discs of designated antibiotics were placed on top.

Figure 5.

Agar Plates



After 24 hours, bacteria from the soil sample will grow on the agar plate. If bacteria grow right next to the antibiotic discs, one can infer that the bacteria in the sample are resistant to that antibiotic. If there is a “zone of inhibition” or a small circle around the antibiotic, one can infer that the bacteria is not resistant (see Figure 6.)

Figure 6.

Zone of Inhibition



Furthermore, if a zone of inhibition is present, it can be measured using a ruler. The ruler was placed against the dish to measure the circumference of the "zone of inhibition" or the circle around the antibiotic disc where bacteria were absent. This portion of the experiment allowed testing for penicillin, ampicillin, and streptomycin resistance, which were chosen as they are some of the most common and inexpensive antibiotics. Testing multiple antibiotics ensures the solution can prevent antibiotic resistance to multiple antibiotics instead of just one.

3.7 Data Analysis

To analyze the zone of inhibition data, a 2-way ANOVA test was run. ANOVA stands for analysis of variance, and this test is used to test how two independent variables impact a dependent variable. Using this test, one can compare the difference in antibiotic resistance between the control group and each of the experimental groups. Contrastingly, because the gel electrophoresis obtained qualitative data, it was analyzed using percentages instead of a statistical test. After attempting to rid the eDNA of the tetB and tetM genes, the percentage of experimental groups that had these genes removed after treatment was measured.

4. Results

This experiment aimed to find a solution to minimize antibiotic resistance safely and healthily. The following sections first state the results of each research portion: the concentration gradient, zone of inhibition test, and gel electrophoresis. Then, the section explains the statistical significance of each finding by using tests and finding connections throughout the data.

4.1 Data Cleanup

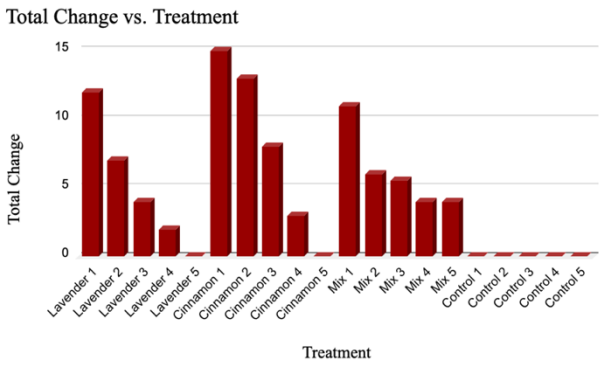
Before analysis, data was rearranged and organized into multiple Excel spreadsheets. Data was cleaned by rearranging groups and variables into the correct format to run statistical tests.

4.2 Concentration Gradient

The concentration gradient was meant to test if the solutions impede plant growth. The "total change" represents the total number of grass spades per each sod that visually appeared to have stunted growth after a week had passed. Different concentrations of 100%, 75%, 50%, 25%, and 10% are represented by 1,2,3,4, and 5 respectively.

The results show that around 10% concentration, both cinnamon and lavender essential oil begin to have no noticeable effect on plant growth and health when compared to the control groups. The plants were monitored for a few days; however, experienced no noticeable change after 7 days. The changes in plant health were reported after 7 days as follows (See Table 1).

Table 1.
The impact of solutions at different concentrations on plant health



4.3 Zone of Inhibition

The results of the zone of inhibition test using a 10% concentration of lavender, cinnamon, and a mix of both essential oils supported the idea that cinnamon could enlarge the zone of inhibition. Creating a bigger zone of inhibition means more bacteria are being killed by the antibiotic, which is the goal. Cinnamon significantly increased the zone of inhibition for both penicillin and ampicillin compared to the control group. Similarly, a mix of both essential oils significantly increased the zone of inhibition for streptomycin.

Table 2.
Zone of inhibition results after treatment

	Penicillin	Ampicillin	Streptomycin
Control	0	0.6	2.167
Lavender	0.583	1.02	2.15
Cinnamon	0.733	1.317	2.2
Mix	0.643	1.267	2.7

4.4 Gel Electrophoresis

The gel electrophoresis additionally supported the idea that cinnamon essential oil at 10% would make an effective solution. For the cinnamon group, 100% of the samples had a reversed impact of the tetB and tetM genes coding for antibiotic resistance. The lavender and mix groups worked one-third and two-thirds of the time. However, since cinnamon had a more significant impact, it is highlighted as the best possible solution.

4.5 ANOVA Test for Connections

Statistical tests were run to analyze the relationship between essential oils' treatment and antibiotic resistance to multiple antibiotics for the zone of inhibition test. Two-way ANOVA tests were used to calculate these connections. Since the zone of inhibition provided strictly quantitative and not qualitative data like gel electrophoresis, it can be analyzed to determine its statistical significance. A 2-way ANOVA (analysis of variance) test was conducted to examine the data. This type of test can be used when comparing groups on two categorical variables. It is commonly used for analyzing scientific experiments involving different groups, making it appropriate for this study (National University, 2024).

The results obtained are of significant importance, displaying p-values of 0.02 and 0.04 for cinnamon essential oil, effectively increasing the zone of inhibition of penicillin and ampicillin, respectively. These results are below the alpha value of 0.05, demonstrating a clear statistical significance between the cinnamon essential oil and the control groups. Similarly, the mix had a p-value of <0.01 for increasing the zone of inhibition to streptomycin, showing a vital statistical significance.

5. Conclusion

This study explored using essential oils as a novel solution to combat the spread of antibiotic resistance in the environment. A scientific experiment explored the connection between different essential oils and the change in antibiotic resistance, which was measured through a zone of inhibition test and gel electrophoresis. Cinnamon essential oil at 10% concentration was deemed safe for the environment and beneficial in reducing antibiotic resistance to ampicillin, penicillin, and streptomycin.

This research is essential because antibiotic resistance is the cause of countless deaths and infections worldwide. Previous research had not yet established a clear connection between essential oils and the presence of antibiotic resistance and, therefore, only hinted at links. This scientific experiment established a stronger connection between the variables, agreeing with previous research.

5.1 Limitations and Challenges

One of the main limitations of this study was the amount of data points collected. A lack of data points inhibits the confidence in the findings. Many data points were collected for the zone of inhibition tests (100); however, the gel electrophoresis had fewer. The reason for this was the length of the procedure and the need for more funding for resources, with each kit costing around $300.

Another limitation is the chance of cross-contamination between the soil samples since they were placed near each other. Furthermore, other people in the lab could have contacted the soil and cross-contaminated it since it was placed openly in a classroom. Additionally, this study only looked at two specific genes and only a few different antibiotics, so they cannot yet be generalized to fight antibiotic resistance to all antibiotics.

5.2 Implications & Future Directions

This research has implications in natural medicine since antibiotic resistance is a problem that requires an urgent solution before bacteria grow resistant to all antibiotics. It is crucial to find a solution to reverse the impact of the genes instead of simply creating new antibiotics that bacteria may eventually grow resistant to. This study suggests that cinnamon oil at a 10% concentration may be a good solution for all aspects of the research goal.

These results also may have implications for agriculture workers. If a solution containing cinnamon essential oil can be incorporated into the upkeep of various sports fields, playgrounds, and other outdoor environments, it may have the potential to help slow the spread of antibiotic resistance, creating a safer environment.

Future research should explore different essential oils to combat antibiotic resistance. The solutions should also be tested at more extensive concentrations and on many different antibiotics to ensure they are universally applicable. Future research should also conduct more trials to gain statistical significance and confidence in the findings. Furthermore, more research should be conducted on the possible impacts of putting essential oils into the environment to ensure they are safe before anything is put into the environment. Future research would contribute to filling the gap and further expand the research on solutions to antibiotic resistance.

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