The Effects of Lavender, Eucalyptus, Lemongrass, and Cinnamon Essential Oils on E. *coli* and *S. aureus*

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19 October 2021

Abstract

Antibiotic resistance is on the rise due to the misuse and overuse of antibiotics, so an alternative is necessary. Essential oils may be that alternative as they have been shown to inhibit the growth of many strains of different bacteria. There have been a wide range of essential oils tested on different strains, including antibiotic resistant ones, of bacteria, however, many are not pure or food safe. Lavender, Eucalyptus, Lemongrass, and Cinnamon essential oils were tested against nonpathogenic strains of *E. coli* and *S. aureus* along with a negative saline control and positive Tetracycline antibiotic control. Blank disks were dipped in different concentrations (undiluted, 1:10, 1:100) of pure, food safe Lavender, Eucalyptus, Lemongrass, and Cinnamon essential oils and placed on inoculated petri dishes. The diluted and 1:10 concentrations of Cinnamon Essential oil inhibited more *S. aureus* than the other essential oils tested and the positive Tetracycline control while the undiluted concentration of Cinnamon Essential oil inhibited more *E. coli* than the other essential oils tested and the positive Tetracycline control. One application of these results would be using Cinnamon Essential Oil in place of the antibiotics given to livestock during growth.

Acknowledgements

I would like to thank Dr. Margarethe Cooper and Ms. Elyse Wexler for mentoring me for the past few months. I would also like to thank the University of Arizona and the STAR Lab for allowing me to conduct our experiment on campus and giving us the chance to earn college credit in the process. Finally, I would like to thank Hannah Lee. Although she was not able to complete the project, she helped with background research and designing the experiment.

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Introduction

Bacteria are in and on everything, whether it is the human body or food. One bacteria in food that can make humans sick is *Listeria monocytogenes*. According to the U.S. Food and Drug Administration, the risk of getting sick from *L. monocytogenes* in pasteurized milk is moderate [1]. This does not necessarily mean that pasteurized milk is risky, as the term refers to the median cases for the total United States population on an annual and per serving basis, which is estimated to be 1.0×10^{-9}. The majority of *L. monocytogenes* in raw milk is killed when it is heated to 71.6°C during pasteurization [2]. The "moderate risk" for pasteurized milk comes from the possibility of it getting contaminated with raw milk which may contain *L. monocytogenes* from being contaminated with feces [3].

A correlation between poor quality of cow feed and *Listeria* spp., the broad term for *Listeria* that includes its 21 species, has been demonstrated by Skovgaard & Morgen (1988) . They found that when a large percentage of feed contained *Listeria* spp. and *L. monocytogenes*, the cow feces also contained a large percentage of *Listeria* spp. and *L. monocytogenes* [4].

Most bacterial infections can be treated with antibiotics, including *L. monocytogenes*. Antibiotics can be effective at killing bacteria, but certain species, like *Escherichia coli*, and *Staphylococcus aureus* can evolve resistance to antibiotics [5]. Gram staining is used to determine if bacteria are gram-positive or gram-negative, which are major categories of bacteria. Gram-positive bacteria stain violet because of a thick layer of peptidoglycan, a polymer made up of amino acids and sugars, in the cell's wall. Gram-negative bacteria stain pink due to their thin layer of peptidoglycan in their cell's wall, which is exposed during the Gram-stain method and must be counterstained using safranin [6]. Multidrug-resistant bacteria, like certain strains of *S. aureus*, and gram-negative bacteria like *Acinetobacter baumannii*, can be a cause of

Postoperative Meningitis (POM). Sari (2019) showed the ineffectiveness of multiple antibiotics on two multidrug-resistant bacteria:, gram-negative *Acinetobacter baumannii* and *Klebsiella pneumoniae*, reporting that 57% of patients who developed POM died. Of the four patients who had *K. pneumoniae*, half died, and of the ten patients who were infected with *A. baumannii*, six died [7]. As more bacterial strains become antibiotic resistant in common illnesses like pneumonia and tuberculosis, more people will likely die from these once easily treatable diseases [8]. However, the antimicrobial properties of certain essential oils combined with antibiotics may help kill antibiotic resistant bacteria.

Certain plant essential oils have shown promising results against certain strains of antibiotic resistance bacteria. For example, the antibacterial activity of cinnamon essential oils was compared to that of *streptomycin, ampicillin,* and *chloramphenicol* and was reported to be successful against three strains of bacteria: *E. coli, S. aureus,* and, *P. aeruginosa* [9]. While cinnamon essential oil and *streptomycin* were able to inhibit all the strains tested, *ampicillin* and *chloramphenicol* inhibited only *E. coli*. The antibacterial effect of *ampicillin* was higher than that of *chloramphenicol* against *S. aureus* but *P. aeruginosa* was resistant to *ampicillin* and *chloramphenicol*. Such studies have led to the conclusion that the cinnamon essential oil was found to resist bacteria through the use of anti-quorum sensing effects which allows the inhibition of cell division, ATPase, biofilm formation, membrane porin, and mobility which altered the lipid profile [6] and influenced the acting cell membrane in producing lumps and autoaggregation [9].

Bacteria are everywhere, and most people will take antibiotics at some point in their lives. It is estimated that more than 1.7 million adults in the United States develop sepsis, a complication of infections, every year [5]. The antimicrobial properties of certain essential oils

combined with antibiotics may help kill antibiotic resistant bacteria. Mixing cinnamon essential oil with antibiotics has been shown to reduce the minimum inhibitory concentration (MIC) of antibiotics, which means that it may decrease the needed effective drug dose [9]. This has the possibility of decreasing the use of antibiotics which can help with preventing more bacteria from evolving into antibiotic resistant strains.

Will different concentrations of lavender, Eucalyptus, Lemongrass, and Cinnamon essential oils inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*? It is predicted that the higher the concentration of essential oil, the more the bacterial growth will be inhibited.

Methodology

Procedures:

The concentration of certain essential oils may inhibit the growth of *E. coli* or *S. aureus.* The positive control was tetracycline antibiotic disks (30 μg) for its bacterial inhibition in *E. coli* and *S. aureus*. The negative control was a blank disk for its inability to inhibit bacterial growth in *E. coli* and *S. aureus*.

A serial dilution was made for each of four essential oils (Lavender, Eucalyptus, Lemongrass, and Cinnamon) using saline solution. Four quadrants of each petri dish contained the following: a negative control, undiluted essential oil, 1:10 dilution, and 1:100 dilution. Each bacterium (*E. coli* and *S. aureus*) was mixed with saline solution and inoculated onto a petri dish with Mueller-Hinton agar using a sterile swab. Sterile disks were dipped in the essential oil concentration that corresponded with the quadrant and placed in the middle of each quadrant. Petri dishes were incubated at 37^{o}C for 18 hours. The zone of inhibition was determined in millimeters using a ruler (see Appendix A for a step-by-step protocol). Each treatment was replicated three times.

Data Analysis:

Average zone of inhibition was calculated based on the three trials performed. A bar graph was created using the concentrations of the four essential oils and the positive and negative controls and the mean zone of inhibition in millimeters. Essential oil concentration and controls were plotted on the x-axis while the average zone of inhibition was plotted on the y-axis. Standard deviation for each essential oil was calculated using means. Error bars were added to each graph point using standard error of mean that was calculated with standard deviation. Statistical significance was determined using a t-test, with $p < 0.05$ being significant.

Risk/Safety:

All necessary safety precautions will be taken in order to protect those involved in the project. This includes closed-toed shoes, tied-back hair, gloves, a lab coat, and other proper lab clothing to prevent contamination from any hazardous substances and tools that may be used. All work will be performed in a biosafety cabinet in a BSL 2 lab. COVID-19 precautions will also be taken to ensure the safety of those involved in the project. This includes wearing masks at all times, social distancing when possible, washing hands before and after completing activities, and being tested regularly.

Non-pathogenic strains of *E. coli* and *S. aureus* will be used, meaning they are unlikely to be a potential hazard and will not cause harm to individuals. This means that Biosafety Level 1 is all that is required. Proper lab clothing will be worn, including gloves and a lab coat to prevent the bacteria from touching the skin. Lab equipment contaminated with *E. coli* or S. *aureus* will be sterilized with 70% alcohol or disposed of in a biohazard waste bin.

The University of Arizona Biosafety Plan and Incident Response Plan will be followed in addition to procedures described above [10,11].

Results

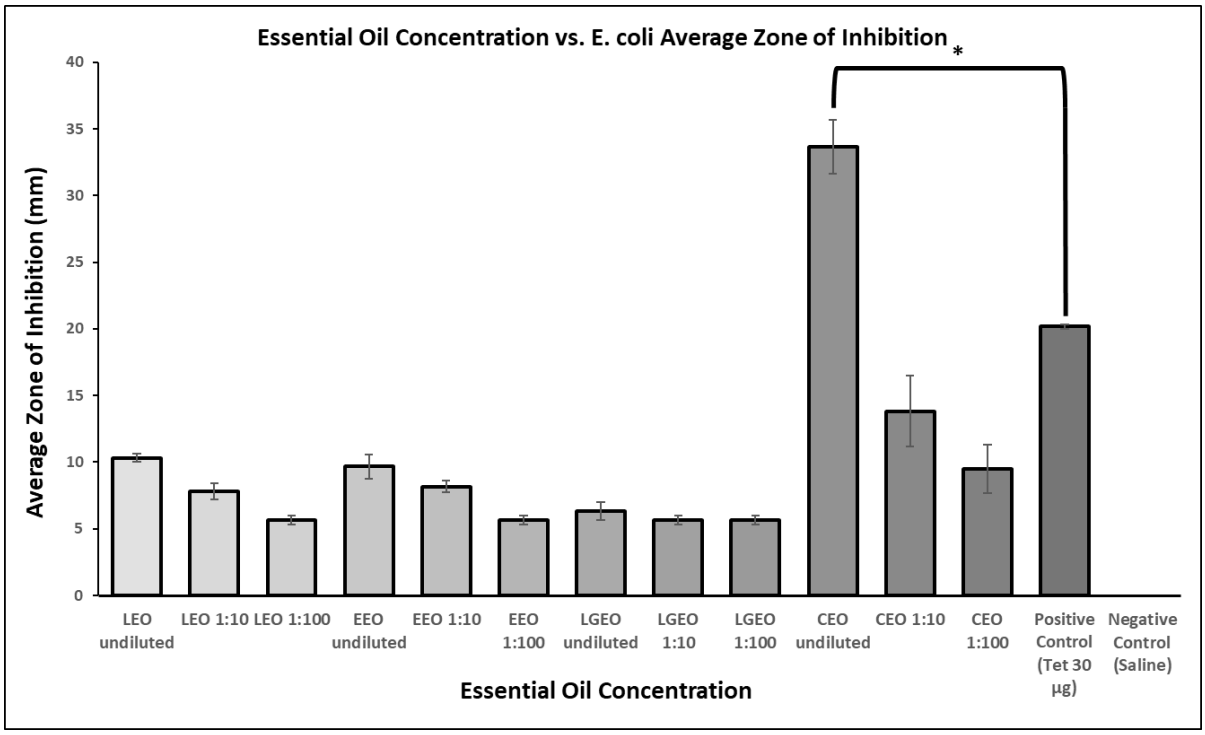

Figure A. Average zone of inhibition from each essential oil concentration for *E. coli*. The error bars represent the standard error of mean for each data set. * indicates a P value < 0.05 with a 2-tailed t-test.

On average, the undiluted Cinnamon Essential Oil (CEO) inhibited the most *E. coli*. This included the positive control of Tetracycline, which had an average zone of inhibition of 20.12 mm, whereas undiluted CEO had an average zone of inhibition of 33.67 mm. 1:100 Lavender (LEO), Eucalyptus (EEO), and 1:10 and 1:100 Lemongrass Essential Oil (LGEO) inhibited the least bacteria, with all average zones of inhibition being 5.67 mm.

The undiluted LEO's zone of inhibition was slightly higher than EEO's which was 9.67 mm as compared to undiluted LEO's 10.33 mm. The opposite was true of the 1:10 concentrations of LEO and EEO, with EEO having a higher zone of inhibition of 8.17 mm whereas LEO's was 7.83 mm. The undiluted LGEO had an average zone of inhibition of 6.33 mm, which was the lowest of any of the undiluted concentrations of essential oil. The 1:10

concentration of CEO had an average zone of inhibition of 13.83 mm whereas the 1:100 concentration had an average zone of inhibition of 9.5 mm.

The results of the t-test showed that the p-value for undiluted CEO and the Tetracycline antibiotic control was 0.0027. The p-value for the 1:10 concentration of CEO and the Tetracycline antibiotic control was 0.0779.

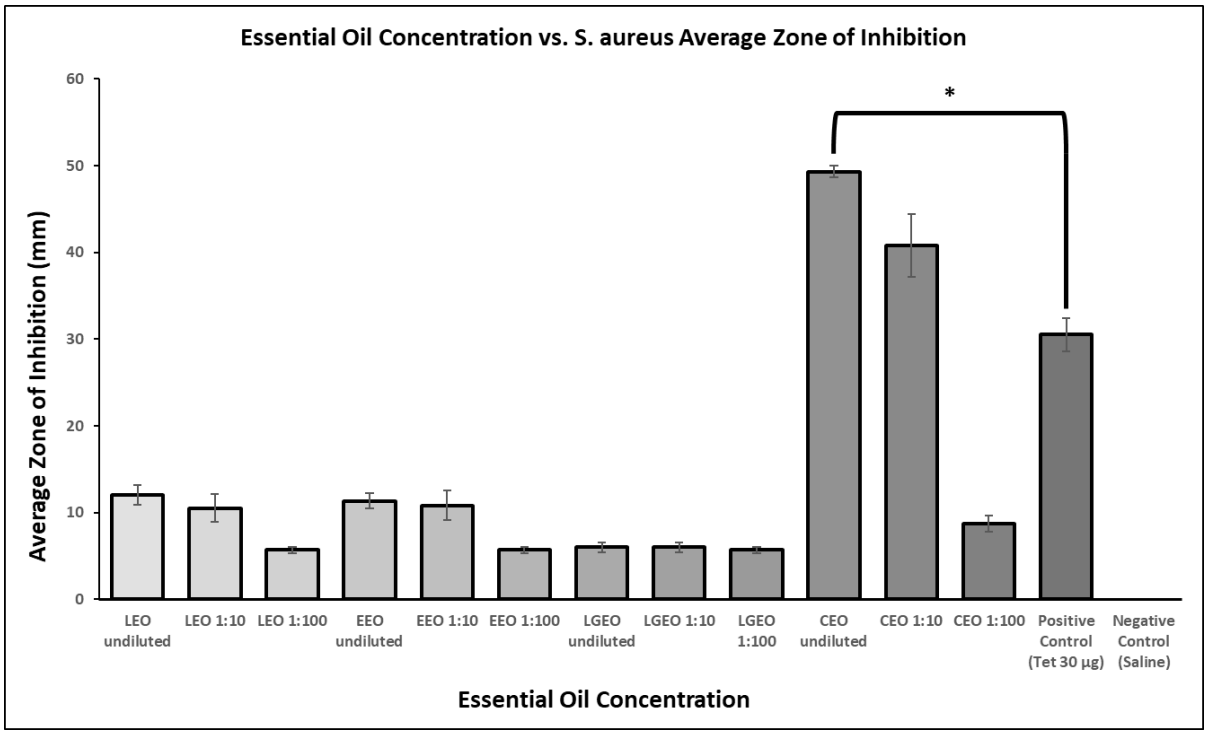

Figure 2. Average zone of inhibition from each essential oil concentration for *S. aureus*. The error bars represent the standard error of mean for each data set. * indicates a P value < 0.05 with a 2-tailed t-test.

On average, the undiluted and 1:10 Cinnamon Essential Oil (CEO) inhibited the most bacteria. This included the positive control of Tetracycline, which had an average zone of inhibition of 30.5 mm, whereas undiluted CEO had an average zone of inhibition of 49.33 mm, and 1:10 CEO had an average zone of inhibition of 40.83 mm. 1:100 Lavender (LEO),

Eucalyptus (EEO), and Lemongrass Essential Oil (LGEO) inhibited the least bacteria, with all average zones of inhibition being 5.67 mm.

The undiluted LEO had an average zone of inhibition of 12 mm, while the 1:10 concentration had an average zone of inhibition of 10.5 mm. The average zone of inhibition for undiluted EEO was slightly more than for the 1:10 concentration of EEO, with it being 11.33 mm as opposed to the 1:10 concentration's 10.83 mm. The undiluted and 1:10 concentrations of LGEO had the same zones of inhibition, with both being 6 mm. The 1:100 concentration of CEO had the highest zone of inhibition of any 1:100 concentrations of the other essential oils, with it being 8.75 mm.

The results of the t-test showed that the p-value for undiluted CEO and the Tetracycline antibiotic control was 0.0007. The p-value for the 1:10 concentration of CEO and the Tetracycline antibiotic control was 0.0652.

See Appendix B for the data tables and data analysis results for both bacteria.

Discussion

The hypothesis was generally supported, as the 1:10 concentrations inhibited more bacteria than the 1:100 concentrations and the undiluted ocncentrations inhibited more bacteria than the 1:10 and 1:100 concentrations. This was only not the case with *E. coli'*s trial 1 results for Lemongrass essential oil and *S. aureus*' trial 3 results for Lemongrass essential oil, where the zone of inhibition was the same for all three concentrations. These results were expected because of similar results seen in previous studies, many of which used the same type of essential oils. The unexpected result of Cinnamon essential oil inhibiting more bacteria than the Tetracycline antibiotic control was seen in another study, where Cinnamon essential oil inhibited more bacteria than the three antibiotic controls for all three bacterial strains tested [9].

For both *E. coli* and *S. aureus*, the p-values for the two t-tests performed show that the results for undiluted CEO and the Tetracycline control are significant, while the 1:10 concentration of CEO and Tetracycline control are not significant.

The gram-negative nature of *E. coli* versus the gram-positive nature of *S. aureus* may explain why only one concentration of Cinnamon essential oil inhibited more E. *coli b*acteria than the antibiotic rather than two for *S. aureus.* While the strains tested were nonpathogenic and testing was on a controlled medium, this still gives insight into the antibacterial properties of essential oils. The components of Cinnamon essential oil may explain why it performed better than the other essential oils tested, as it contains trans-cinnamaldehyde, which is used for sterilization [12]. While this compound's mechanisms are not fully understood, it may work like many antibiotics and prevent peptidoglycan from being synthesized by the bacteria [13, 14].

There were some difficulties during the trials that may have impacted the results. This included possible poor mixing of the essential oils and saline when creating the 1:10 and 1:100 concentrations. Most of the essential oils were the same color as the saline, which was clear, so it was difficult to determine if they were well mixed or if the essential oils were sitting on top. This was not as much of a problem with the Cinnamon essential oil as it was a brown color. The disks used for the essential oils were pre-cut and were not all the same thickness. This may have also impacted the results because thicker disks could have absorbed more essential oil and inhibited more bacteria than the thinner disks.

Further studies would need to be done to determine how these results apply to outside of the lab setting. This includes testing essential oils on bacterial growth in and on food that often get contaminated with bacteria, like produce, meat, dairy products, and other perishables. If similar results are seen where essential oils perform better than antibiotics, essential oils could be

used to wash produce and added to dairy products to prevent bacterial growth from spoilage and antibiotic resistant infections. One possible study that would be a direct continuation of this study would be to test the same essential oils when used alongside antibiotics. While essential oils and antibiotics may be effective alone, there may be compounds in each that would decrease their effectiveness when used together, so future research is needed to determine if this is the case.

Conclusion

Cinnamon Essential Oil showed to inhibit more *E. coli* and *S. aureus* than the other essential oils tested as well as the negative saline control and positive Tetracycline antibiotic control.

There are many applications to this research. This includes using essential oils in replace of or in tandem with antibiotics in a number of settings. Disinfectant sprays that are not food safe are often used on kitchen and other food preparation surfaces, so using food safe essential oils could be an alternative. The overuse of antibiotics is often attributed to them being used on livestock. Essential oils could be placed in the feed and water of livestock rather than antibiotics to still prevent infections that affect livestock growth while limiting antibiotic usage. Different concentrations of essential oils would have to be researched to determine what is safe for livestock.

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Appendix A

Lavender essential oil (LEO) was added to a test tube to avoid contamination with the original bottle. Two plastic microcentrifuge tubes were filled with 900 µl (microliters) saline. 1000 µl LEO was pipetted into an empty microcentrifuge tube. 100 µl LEO was pipetted from the undiluted solution into one 900 µl saline microcentrifuge tube for a 1:10 ratio. 100 µl 1:10 ratio solution was pipetted into a 900 µl saline microcentrifuge tube for a 1:100 ratio. Solutions were vortexed for 10 seconds. Procedure described above was repeated for Eucalyptus Essential Oil, Lemongrass Essential Oil, and Cinnamon Essential Oil. 900 µl saline was pipetted into a microcentrifuge tube. Some *E. coli* was transferred into the 900 µl saline microcentrifuge tube via an inoculation loop. Solution was vortexed for 10 seconds.

Petri dishes were labeled with initials, the date, the type of bacteria, and four quadrants were drawn and labeled with I, II, III, and IV. Quadrant I was labeled control (blank disk), quadrant II was labeled undiluted, quadrant III was labeled 1:10, and quadrant IV was labeled 1:100. Sterile swab was dipped in *E. coli* solution and evenly spread on the entire surface of a Mueller-Hinton agar plate. Lab tweezers were dipped in 100% ethanol and placed over the Bunsen burner for one second and then moved away from the bunsen burner and allowed to sterile for 15 seconds before and after each disk was placed. Sterile, blank disk was placed in the middle of quadrant one. Sterile, blank disk was dipped in undiluted LEO and placed in the middle of quadrant two. Sterile, blank disk was dipped in 1:10 LEO solution and placed in the middle of quadrant three. Sterile, blank disk was dipped in 1:100 LEO solution and placed in the middle of quadrant four.

Petri dishes were labeled with initials, the date, the type of bacteria, and two sections. Section 1 was labeled negative control and section 2 was labeled positive control. Sterile swab

was dipped in *E. coli* solution and evenly spread on the entire surface of a Mueller-Hinton agar plate. Sterile, blak disk was placed in section 1 of the petri dish. Tetracycline antibiotic disk was placed in section 2 of the petri dish. Procedure described in paragraphs two and three were repeated for *S. aureus*.

Petri dishes were covered and allowed to incubate at 37^{o}C for 18 hours. Ruler was used to measure the diameter of the zone of inhibition in millimeters.

Appendix B

E. coli				
Essential Oil Concentration	Trial 1 Zone of Inhibition (mm)	Trial 2 ZI (mm)	Trial 3 ZI (mm)	Average ZI (mm)
LEO undiluted	10	10	11	10.33333333
LEO 1:10	7.5	9	7	7.833333333
LEO 1:100	5	6	6	5.666666667
EEO undiluted	10	8	11	9.666666667
EEO 1:10	7.5	8	9	8.166666667
EEO 1:100	5	6	6	5.666666667
LGEO undiluted	5	7	7	6.333333333
LGEO 1:10	5	6	6	5.666666667
LGEO 1:100	5	6	6	5.666666667
CEO undiluted	30	34	37	33.66666667
CEO 1:10	12.5	10	19	13.83333333
CEO 1:100	10.5	6	12	9.5
Positive Control (Tet 30 µg)	20	20.5	20	20.16666667
Negative Control (Saline)	0	0	0	0

Figure 3. Data table for *E. coli*. Includes zone of inhibition results for each of the three trials and the average of the three trials.

S. aureus				
Essential Oil Concentration	Trial 1 Zone of Inhibition (mm)	Trial 2 ZI (mm)	Trial 3 ZI (mm)	Average ZI (mm)
LEO undiluted	10	12	14	12
LEO 1:10	7.5	13	11	10.5
LEO 1:100	5	6	6	5.666666667
EEO undiluted	10	11	13	11.33333333
EEO 1:10	7.5	13	12	10.83333333
EEO 1:100	5	6	6	5.666666667
LGEO undiluted	5	7	6	6
LGEO 1:10	5	7	6	6
LGEO 1:100	5	6	6	5.666666667
CEO undiluted	50	48	50	49.33333333
CEO 1:10	47.5	40	35	40.83333333
CEO 1:100	10.25	7	9	8.75
Positive Control (Tet 30 µg)	30	27.5	34	30.5
Negative Control (Saline)	0	0	0	0

Figure 4. Data table for *S. aureus.* Includes zone of inhibition results for each of the three trials and the average of the three trials.

E. coli												
LEO undiluted	**LEO 1:10**	**LEO 1:100**	**EEO undiluted**	**EEO 1:10**	**EEO 1:100**	**LGEO undiluted**	**LGEO 1:10**	**LEGO 1:100**	**CEO undiluted**	**CEO 1:10**	**CEO 1:100**	**Positive control (Tet 30 µg)**
10.33333333	7.833333333	5.666666667	9.666666667	8.166666667	5.666666667	6.333333333	5.666666667	5.666666667	33.66666667	13.83333333	9.5	20.16666667
STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV
0.577350269	1.040833	0.577350269	1.527525232	0.763762616	0.577350269	1.154700538	0.577350269	0.577350269	3.511884584	4.645786622	3.122498999	0.288675135
n	n	n	n	n	n	n	n	n	n	n	n	n
3	3	3	3	3	3	3	3	3	3	3	3	3
SEM (standard error or mean)	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM
0.333333333	0.600925213	0.333333333	0.881917104	0.440958552	0.333333333	0.666666667	0.333333333	0.333333333	2.02758751	2.682246157	1.802775638	0.166666667
Undiluted CEO and Tet t-test	**1:10 CEO and Tet t-test**											
0.002676297	0.077946464											
S. aureus												
LEO undiluted	**LEO 1:10**	**LEO 1:100**	**EEO undiluted**	**EEO 1:10**	**EEO 1:100**	**LGEO undiluted**	**LGEO 1:10**	**LEGO 1:100**	**CEO undiluted**	**CEO 1:10**	**CEO 1:100**	**Positive control (Tet 30 µg)**
12	10.5	5.666666667	11.33333333	10.83333333	5.666666667	6	6	5.666666667	49.33333333	40.83333333	8.75	30.5
STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV	STDEV
2	2.783882181	0.577350269	1.527525232	2.929732639	0.577350269	1	1	0.577350269	1.154700538	6.291528696	1.639359631	3.278719262
n	n	n	n	n	n	n	n	n	n	n	n	n
3	3	3	3	3	3	3	3	3	3	3	3	3
SEM (standard error or mean)	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM	SEM
1.154700538	1.607275127	0.333333333	0.881917104	1.691481928	0.333333333	0.577350269	0.577350269	0.333333333	0.666666667	3.632415786	0.946484724	1.892969449
Undiluted CEO and Tet t-test	**1:10 CEO and Tet t-test**											
0.00071844	0.065166001											

Figure 5. Data analysis results for *E. coli* and *S. aureus*. Includes the average zone of inhibition, standard deviation, standard error of mean, and t-test results.